UNIVERSITAT DE BARCELONA

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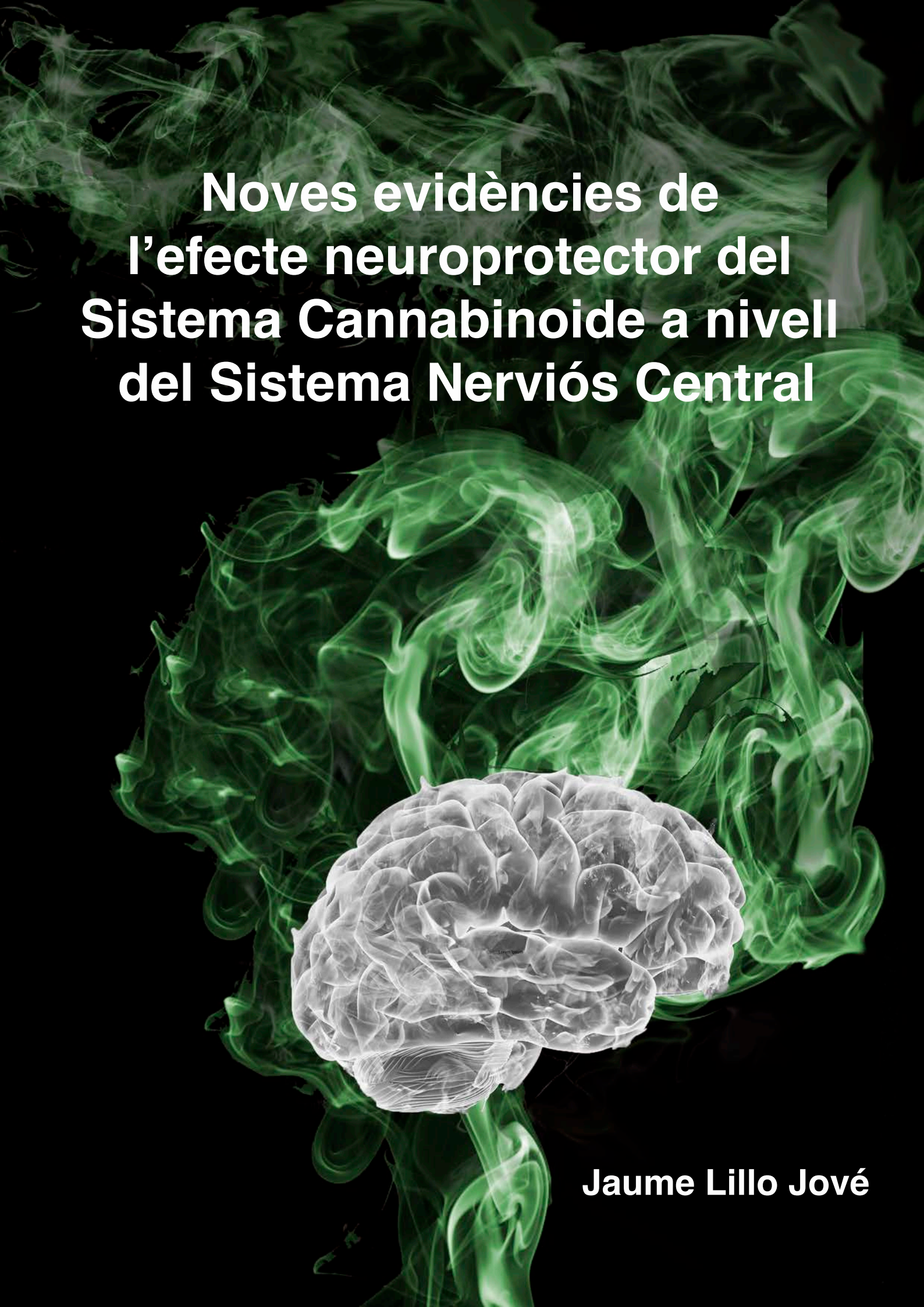

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UNIVERSITAT DE BARCELONA
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Noves evidències de l'efecte neuroprotector del Sistema Cannabinoide a nivell del Sistema Nerviós Central

Memòria presentada pel Graduat en Biotecnologia
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per a optar al grau de Doctor per la Universitat de Barcelona

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Resum

El Sistema Cannabinoide, que inclou els dos receptors de cannabinoides CB_1 i CB_2, els seus lligands endògens i els enzims de síntesi i degradació d'aquests, és un sistema molt complex que controla un gran nombre de processos biològics. Aquest sistema té un paper important tant a nivell del Sistema Nerviós Central com Perifèric, tenint una implicació directa en la plasticitat neuronal i la neuroprotecció entre d'altres.

Els resultats presentats en aquesta Tesi Doctoral aporten noves evidències del rol neuroprotector del Sistema Cannabinoide vers diverses patologies del Sistema Nerviós Central. El fet que el receptor CB_2 es trobi sobreexpressat en episodis de neuroinflamació posa de manifest la seva importància a l'hora d'oferir una resposta neuroprotectora. Aquest efecte l'hem observat en els resultats que es presenten en aquesta Tesi Doctoral en diferents models animals i en diferents patologies.

En el model animal de ratolí d'ictus isquèmic, els individus afectats presentaven uns nivells elevats d'expressió de l'heteròmer CB_2R-$5HT_{1A}R$. És més, la progènie de ratolines alimentades amb dieta alta en greixos també presentaven una sobreexpressió de CB_2R, aquest cop interaccionant amb el receptor GHS-R1a. En la patologia del Parkinson, s'ha demostrat una elevada expressió de l'heteròmer CB_2R-GPR55 en macacos Parkinsonians. Tot i que el CB_2R és el receptor cannabinoide associat més directament amb la neuroprotecció, també hem observat que el CB_1R pot tenir un paper important. És cert que la sobreexpressió de CB_1R no és tan evident com la de CB_2R en la patologia de l'Alzheimer, on els assaigs de qPCR indiquen que en ratolins 5xFAD l'expressió de CB_2R pot incrementar-se fins a gairebé 10 vegades, mentre que el CB_1R es duplica. En aquest sentit hem detectat que l'heteròmer CB_1R-GHR1a es troba sobreexpressat en un model d'obesitat, mentre que el CB_1R-GPR55 ho fa en la patologia de Parkinson.

El CBD, el segon component més abundant de la planta del cànnabis també juga un paper important en el tractament de les patologies amb un component inflamatori. Els resultats presentats en aquesta Tesi Doctoral demostren que el CBD pot modular el fenotip de la micròglia activada, a favor d'un fenotip neuroprotector en detriment d'un fenotip proinflamatori. Aquest efecte sobre la micròglia l'observem en seccions de ratolins isquèmics i en seccions de cervell de ratolins model de la patologia d'Alzheimer, on el tractament amb CBD incrementa la immunoreactivitat de marcadors neuroprotectors com l'Arginasa I i disminueix els de marcadors inflamatoris com iNOS. Potser un dels efectes més atractius del CBD és la seva capacitat per inhibir el transport axonal de proteïnes associades a la patologia de l'Alzheimer i el Parkinson entre neurones, limitant l'afectació a neurones veïnes i alentint el progrés de la malaltia. Els dipòsits d'Aβ i pTau són tòxics per les neurones i mitjançant la tècnica d'immunocitoquímica, hem observat que afecten també a la formació de neurites. Sorprenentment, el tractament amb CBD és capaç de recuperar gairebé els nivells basals de neurites que presenta una neurona sana, revertint la pèrdua patològica d'aquestes neurites.

Abstract

The Cannabinoid System, which includes the two cannabinoid receptors CB_1 and CB_2, their endogenous ligands and the enzymes for their synthesis and degradation, is a very complex system that controls a large number of biological processes. This system plays an important role both at the level of the Central and Peripheral Nervous System, having a direct involvement in neuronal plasticity and neuroprotection among others.

The results presented in this Doctoral Thesis provide new evidence of the neuroprotective role of the Cannabinoid System towards various pathologies of the Central Nervous System. The fact that the CB_2 receptor is overexpressed in episodes of neuroinflammation highlights its importance in offering a neuroprotective response. We have observed this effect in the results presented in this Doctoral Thesis in different animal models and in different pathologies.

In the mouse animal model of ischemic stroke, affected individuals had high levels of expression of the CB_2R-$5HT_{1A}R$ heteromer. What's more, the offspring of mice fed with a high-fat diet also had an overexpression of CB_2R, this time interacting with the GHS-R1a receptor. In Parkinson's disease, high expression of the CB_2R-GPR55 heteromer has been demonstrated in Parkinsonian macaques. Although the CB_2R is the cannabinoid receptor most directly associated with neuroprotection, we have also observed that the CB_1R may play an important role. It is true that the overexpression of CB_1R is not as evident as that of CB_2R in Alzheimer's pathology, where qPCR assays indicate that in 5xFAD mice CB_2R expression can be increased up to almost 10-fold, while the CB_1R is doubled. In this regard, we have detected that the CB_1R-GHR1a heteromer is overexpressed in an obesity model, while CB_1R-GPR55 is overexpressed in Parkinson's disease.

CBD, the second most abundant component of the cannabis plant also plays an important role in the treatment of pathologies with an inflammatory component. The results presented in this Doctoral Thesis demonstrate that CBD can modulate the phenotype of activated microglia, in favor of a neuroprotective phenotype to the detriment of a proinflammatory phenotype. We observe this effect on microglia in sections of ischemic mice and in brain sections of mice model of Alzheimer's disease, where treatment with CBD increases the immunoreactivity of neuroprotective markers such as Arginase I and decreases those of inflammatory markers like iNOS. Perhaps one of the most attractive effects of CBD is its ability to inhibit the axonal transport of proteins associated with the pathology of Alzheimer's and Parkinson's between neurons, limiting the effect on neighboring neurons and slowing the progress of the disease. Aβ and pTau deposits are toxic to neurons and using the immunocytochemistry technique, we have observed that they also affect the formation of neurites. Surprisingly, CBD treatment is able to recover almost the basal levels of neurites that a healthy neuron exhibits, reversing the pathological loss of these neurites.

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Una de les moltes coses que he après a NBM durant aquests anys és la importància d'explicar bones històries. La que us explicaré ara no és només la que he viscut durant el meu doctorat, sinó que és també la de totes les persones, família i amics, que m'heu acompanyat al llarg d'aquest viatge i que heu fet possible que ara estigui escrivint aquestes línies. La ciència, igual que la vida, només pot viure's plenament si ho fas acompanyat de persones que et fan créixer i ser millor, és per això que sense vosaltres ni seria la persona que soc, ni hauria arribat a viure aquesta apassionant aventura. Aquesta història, la vostra, he tingut la gran sort de compartir-la amb moltíssima gent, així que em disculpo per avançat si la llista d'agraïments és llarga, però no se m'acut millor moment per dir-vos coses que potser no expressem tant com caldria.

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Jaume Lillo Jové

Índex

Abreviatures

A

AA	Àcid araquidònic
Aβ	Pèptid β-amiloide
AC	Adenilat ciclasa
AD	*Alzheimer's Disease*
ADP	Difosfat d'adenosina
AEA	Anandamida
AMPA	Àcid α-amino-3-hidroxi-5-metil-4-isoazolepropionic
AMPc	Monofosfat d'adenosina cíclic
APP	Proteïna precursora amiloide
Arg	Arginasa
ATP	Trifosfat d'adenosina

B

BiFC	*Bimolecular Fluorescence Complementation*
BRET	*Bioluminiscence Resonance Energy Transfer*

C

Ca^{2+}	Calci
CaM	Calmodulina
CB_1R	Receptor de cannabinoides tipus 1
CB_2R	Receptor de cannabinoides tipus 2
CBD	Cannabidiol
CBG	Cannabigerol
CBN	Cannabinol
CoH	Coelenterazine-H
COX-2	Ciclooxigenasa-2

D

DAG	Diacilglicerol
DAGL	Diacilglicerol Lipasa
DMR	*Dynamic mass redistribution*
DNA	Àcid desoxiribonucleic
DSI	*Depolarization-induced suppression of inhibition*
DSE	*Depolarization-induced suppression of excitation*

E

EC_{50}	Concentració efectiva mitja màxima
ERK	*Extracellular signal-regulated kinase*

F

FAAH	*Fatty acid amide hydrolase*
FDA	*Food and Drug Administration*
FRET	*Förster Resonance Energy Transfer*

G

GABA	Àcid γ-aminobutíric
GDP	Difosfat de guanosina
GEF	*Guanine nucleotide exchange factor*
GHSR	Receptor secretagog de l'hormona de creixement
GFP^2	*Green Fluorescent Protein* 2
GFAP	*Glial fibrillary acidic protein*
GluR	Receptors metabotròpics de glutamat
GOD	*Glucose and oxygen deprivement*
GPCR	Receptor acoblat a proteïna G
GRKs	*G protein-coupled receptor kinases*
GTP	Trifosfat de guanosina

H

HEK-293T	Cèl·lules embrionàries de ronyó humà
HTRF	*Homogeneous Time Resolved Fluorescence*

I

IC_{50}	Concentració inhibidora mitja màxima
IFN-γ	Interferó gamma
IL	Interleucina
iNOS	Òxid nítric sintasa inductible
IMC	Índex de massa corporal
IP_3	Inositol trifosfat

K

K^+	Potassi
KO	*Knockout*

L

L-DOPA	Levodopa
LTD	*Long term depression*
LPI	Lisofosfatidilinositol
LPS	Lipopolisacàrid

M

M	Molar
MAGL	*Monoacylglycerol lipase*
MAPK	*Mitogen-Activated Protein Kinases*
Mg^{2+}	Magnesi
mM	Milimolar
MPTP	1-metil-4-fenil-1,2,3,6-tetrahidropiridina

N

NAAA	*N-acylethanolamine-hydrolyzing acid amidase*
Na^+	Sodi
NAPE	*N-araquidonil fosfatidiletanolamina*
NAPE-PLD	*NAPE preferring phospholipase D*
NFAT	*Nuclear factor of activated T cells*

NFκß	Nuclear factor κß
NMDA	N-metil-D-Aspartat
nM	Nanomolar
NO	Òxid nítric

P

P	Fosfat
PIP_2	Fosfatidilinositol bisfosfat
PD	*Parkinson's Disease*
PI3K	Fosfoinositol 3-cinasa
PKA	Proteïna cinasa A
PKC	Proteïna cinasa C
PLA	*Proximity Ligation Assay*
PLC	Fosfolipasa C
PP	Proteïna fosfatasa
PPAR	*Peroxisome proliferator-activated receptor*
pTau	Proteïna Tau fosforilada

R

R	Receptor
RLuc	*Renilla luciferasa*
RMN	Ressonància magnètica nuclear
RNA	Àcid ribonucleic
ROS	Espècie reactiva d'oxígen

S

SC	Sistema Cannabinoide
SNC	Sistema Nerviós Central
SNP	Sistema Nerviós Perifèric
STD	*Short term depression*

T

TM	Transmembrana
TRPV1	*Transient receptor potential vaniloid channel*

W

WT	*Wild-type*

Y

YFP	*Yellow Fluorescence Protein*

Altres

2-AG	2-araquidonilglicerol
5-HTR	Receptor de serotonina
6-OHDA	6-hidroxidopamina
α-syn	α-sinucleïna
Δ^9-THC	Δ^9-tetrahidrocannabinol
Δ^9-THCV	Δ^9-tetrahidrocannabivarina
µM	Micromolar

INTRODUCCIÓ

1. Introducció

1.1 GPCR

Els receptors acoblats a proteïna G (GPCR) són la família de receptors de membrana més gran entre eucariotes[1]. La seva funció principal és la transducció de senyal. En humans, els GPCR codifiquen per més de 800 proteïnes diferents, essent la família de receptors més gran codificada en el genoma humà[2].

Des de fa temps, els GPCR han adquirit un gran interès en la indústria farmacèutica degut a la seva implicació en un elevat nombre de funcions fisiopatològiques. Aquest fet es veu reflectit amb aproximadament un 30% dels fàrmacs del mercat global, que tenen com a diana terapèutica un GPCR[3]. Tot i això, únicament el 15% del total dels GPCR són actualment diana terapèutica d'algun fàrmac, restant per descobrir un gran ventall de noves possibilitats i posant de manifest la rellevància d'aquests receptors a nivell farmacològic[1].

Quan els GPCR s'uneixen al seu lligand específic, inicien la transducció de senyal en el nostre organisme. Aquest lligand pot ser endogen, si és sintetitzat pel propi organisme, o exogen si prové de l'exterior. La seva naturalesa és molt diversa, trobem hormones, neurotransmissors, pèptids o inclús fotons o odorants[4]. Un cop activat el receptor, el senyal es transdueix a través de diferents missatgers secundaris, amb la finalitat de produir diferents respostes fisiològiques; entre elles, induir la diferenciació cel·lular, la modulació de la resposta immune o fins i tot la capacitat de percebre el gust i les olors[5].

Els GPCR adquireixen un rol molt important tant en humans com entre tots els animals, insectes i altres organismes. Es caracteritzen per presentar una estructura de 7 dominis transmembrana en forma d'hèlix alfa. Aquestes hèlixs alfa són altament hidrofòbiques i estan separades entre si per 3 *loops* intracel·lulars i 3 *loops* extracel·lulars de manera alternada[6]. L'extrem N-terminal de la proteïna queda expressada a l'exterior cel·lular, mentre que l'extrem C-terminal queda a l'interior[5].

La rodopsina va ser el primer GPCR en ser cristal·litzat, proveint, per primera vegada, l'estructura tridimensional d'un receptor acoblat a proteïna G[7]. Això va significar un abans i un després en l'estudi d'aquesta família de receptors, que gràcies a la criomicroscòpia electrònica (cryo-EM), va obrir un gran ventall de possibilitats per estudiar tant l'orientació com la localització d'aquests receptors en la membrana plasmàtica[8]. Recentment, utilitzant aquesta mateixa tècnica, s'ha resolt l'estructura tridimensional de la rodopsina fusionada a la proteïna G[9]. Arran d'aquest descobriment, han aparegut un elevat nombre d'estudis analitzant altres GPCR, tant en el seu estat conformacional actiu com en la seva forma inactiva[10].

1.1.2 Classes

Tots els GPCR comparteixen una estructura terciària de 7 dominis transmembrana, amb importants diferències a nivell de seqüència i en la llargada dels seus bucles intra o extracel·lulars i les seves cues N- i C-terminals[11]. Aquestes diferències ens permeten classificar els diferents GPCR. Actualment, la classificació més acceptada és la de Kolakowski[12], que separa els GPCR en sis famílies diferents, identificades de la A a la F[13]. D'aquestes 6 famílies, únicament 4 s'expressen en humans[14].

La Família A, també anomenada "*rhodopsin-like family*", és la més coneguda i nombrosa, ja que en formen part el 80% dels GPCR[12]. L'homologia a nivell d'estructura primària es baixa, de fet únicament hi ha un residu conservat, que correspon a la Arginina del motiu Asp-Arg-Tyr (DYR) en el tercer segment transmembrana[15]. Les principals característiques estructurals són un pont disulfur que connecta el primer *loop* extracel·lular amb el segon i una cisteïna palmitoïlada a la cua C-terminal que serveix d'ancoratge a la membrana plasmàtica (Figura 1). El receptor model d'aquesta família és el de rodopsina, però també en formen part els receptors de cannabinoides CB_1 i CB_2 i el receptor de grelina GHS-R1a. En aquesta família, la unió del lligand al receptor es produeix majoritàriament en una cavitat formada pels dominis transmembrana, tot i que en el cas que el lligand sigui un petit pèptid (com el GHS-R1a) la unió a lligand es troba a nivell dels bucles extracel·lulars i el domini N-terminal[16].

La Família B inclou més de 50 receptors que uneixen hormones peptídiques o neuropèptids com la calcitonina o el glucagó. Es caracteritza per un llarg extrem N-terminal ric en cisteïnes, formant un entramat de ponts disulfur[17]. En comparació amb la família A, no presenten el motiu DYR i tot i que la unió del primer *loop* extracel·lular amb el segon per mitjà d'un pont disulfur es manté, la cisteïna palmitoïlada que ancorava els receptors a la membrana plasmàtica també desapareix[18].

La família C es caracteritza per presentar uns llargs extrems N i C-terminals, aproximadament d'uns 600 aminoàcids. A més, presenten un tercer *loop* intracel·lular més curt que la resta de receptors i altament conservat. Exceptuant les dues cisteïnes que formen un pont disulfur entre el primer *loop* extracel·lular i el segon, aquesta família de receptors no tenen gairebé cap més similitud amb els receptors de les Famílies A i B[19]. En aquesta família trobem els receptors de glutamat i els receptors d'àcid γ-aminobutíric $(GABA)_B$[20].

Les famílies D i E són menys nombroses i estan formades per receptors de feromones de llevats. D'altra banda, la família F està constituïda per 4 receptors d'AMPc d'arqueobacteries, sent la més petita de les 6[13].

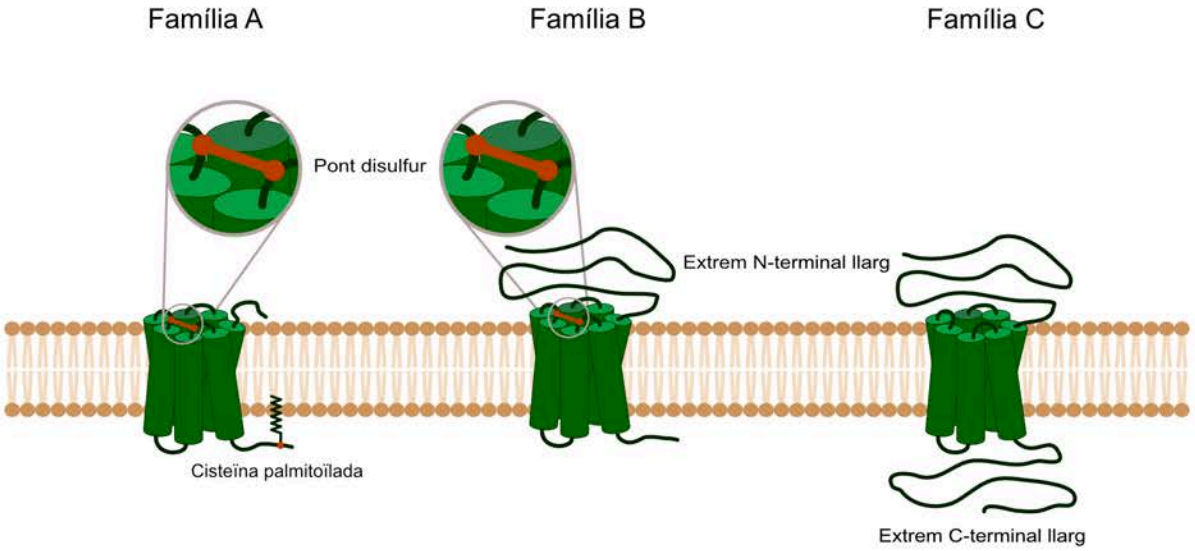

Figura 1: Esquema de les tres principals famílies de GPCR.

La transducció de senyal dels receptors acoblats a proteïna G es basa en la presència de tres components: un receptor GPCR, la proteïna G heterotrimèrica i un sistema efector format principalment per enzims i missatgers secundaris[5].

La proteïna G heterotrimèrica està formada per les subunitats α (39-46 kDa), β (37 kDa) i γ (8 kDa). Els GPCR actuen com a *guanosine exchanging factor* (GEF). Quan un GPCR s'activa per unió a lligand, pateix un canvi conformacional que acoblarà la proteïna G, activant-la. Conseqüentment, s'intercanvia el GDP unit a la subunitat α per un GTP[21]. Aquesta conversió de GDP a GTP comporta un canvi estructural en la proteïna G que es reagrupa en dues subunitats funcionals: Gα i Gβγ[22], que activaran diferents vies de senyalització. La regeneració de la proteïna G es produeix per l'acció GTPasa de la subunitat α que hidrolitza el GTP a GDP[23].

Les proteïnes G es classifiquen en funció de la subunitat Gα en quatre grans famílies: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, i $G\alpha_{12/13}$[21] (Figura 2). La proteïna $G\alpha_s$ o activadora, transdueix el senyal mitjançant l'activació de l'Adenilat Ciclasa (AC), un enzim que converteix ATP en AMPc, incrementant els nivells d'aquest segon missatger a l'interior de la cèl·lula. Elevats nivells d'AMPc poden activar proteïnes regulades per AMPc, com per exemple la proteïna cinasa A (PKA)[24]. Aquesta cinasa fosforilarà diverses proteïnes (receptors, enzims, canals iònics o factors de transcripció) regulant el funcionament cel·lular. D'altra banda, les proteïnes $G\alpha_i$ o inhibidores, inhibeixen a l'AC, produint una reducció intracel·lular dels nivells d'AMPc[24].

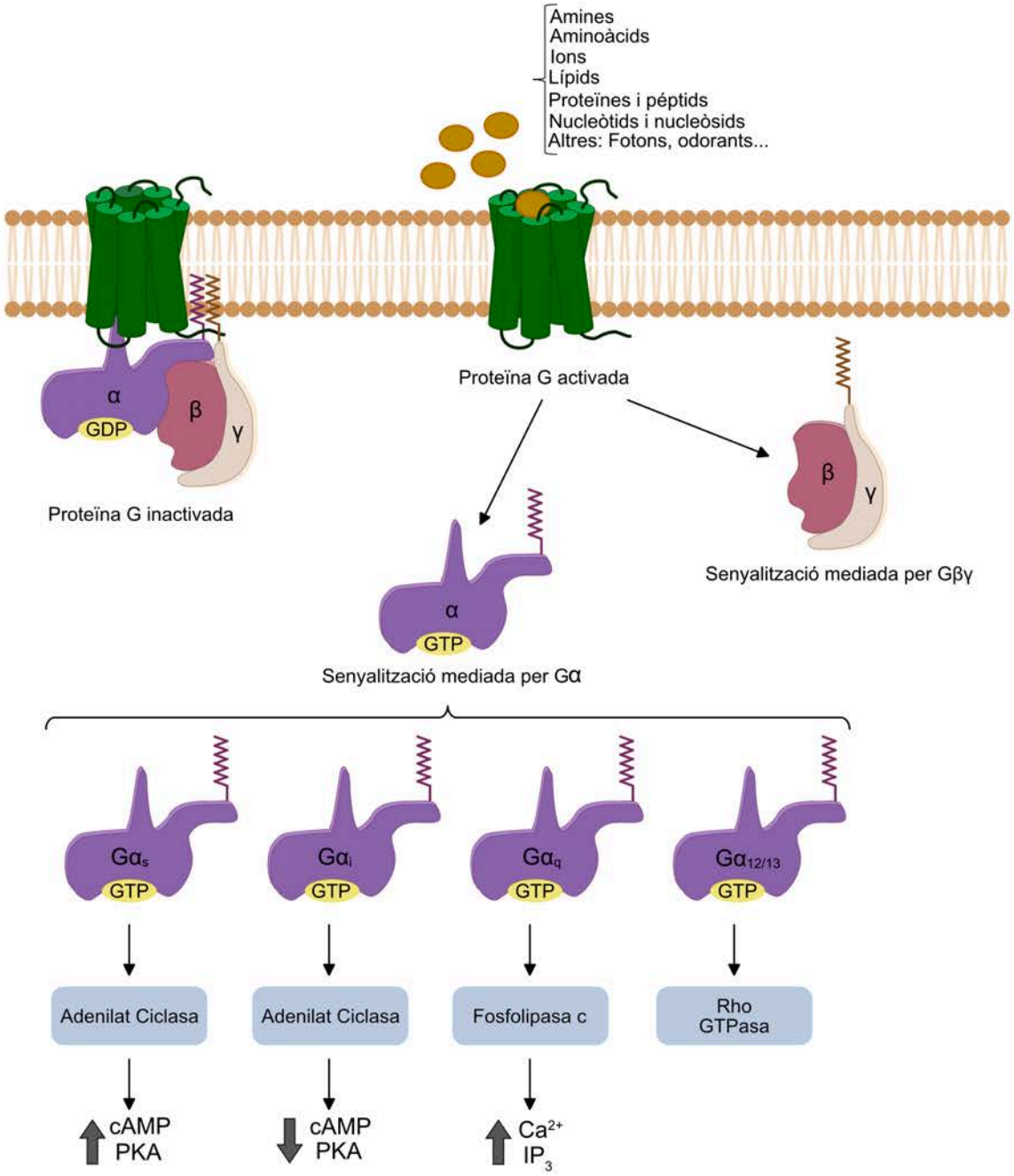

Figura 2: Esquema de la transducció de senyal d'un GPCR.

Les proteïnes $G\alpha_q$ activen l'enzim Fosfolipasa C (PLC), que hidrolitza el fosfatidilinositol-4,5-bisfosfat (PIP_2) produint diacilglicerol (DAG) i Inositol-1,4,5-trifosfat (IP_3)[25]. Per un costat, l'IP_3 actua sobre els canals de Ca^{2+} del reticle endoplasmàtic, provocant l'alliberació de ions Ca^{2+} al citosol, mentre que el DAG activa la proteïna cinasa C (PKC), que fosforila un gran nombre de proteïnes, regulant la funció cel·lular.

Finalment, les proteïnes $G\alpha_{12/13}$ activen petites proteïnes amb activitat GTPasa com per exemple la Rhoa i regulen la reorganització del citoesquelet. La resposta biològica final dependrà del nivell de segons missatgers i el grau d'activació de les GTPases[26].

Hi ha vies de senyalització que poden activar-se de manera independent a la proteïna Gα. Entre les més rellevants destaca la via de les MAPK (*mitogen activated protein kinase)* també coneguda com a via de les ERKs (*extracelular signal-regulated kinases*), essent una peça clau en la transmissió del senyal d'un gran nombre d'agents extracel·lulars per regular processos cel·lulars com proliferació, diferenciació i progressió del cicle cel·lular. Però n'hi ha d'altres que poden funcionar en paral·lel com la via de les JNK, de la p38 MAPK o de les ERK5[27]. Aquestes rutes independents de proteïna Gα solen anar associades a l'activació de les subunitats Gβγ[28].

La via de les MAPK inclou l'activació de la proteïna Ras, que té activitat GTPasa[29] (Figura 3). Segons aquest model, la subunitat Gβγ, interacciona amb la PLC, que per mitjà de l'IP_3 incrementarà els nivells de Ca^{2+} intracel·lulars. Conseqüentment es produirà l'activació de la cinasa Pyk2[30]. Pyk2 activarà l'adaptador Shc, que unirà Src i podrà activar la proteïna Ras mitjançant el reclutament del factor intercanviador de nucleòtids de guanina mSOS a la membrana plasmàtica[29,30]. La proteïna Ras és un missatger secundari que s'uneix i activa a Raf, una serina/treonina que a la vegada fosforila i activa a MEK, la cinasa que finalment fosforilarà les MAPK[31].

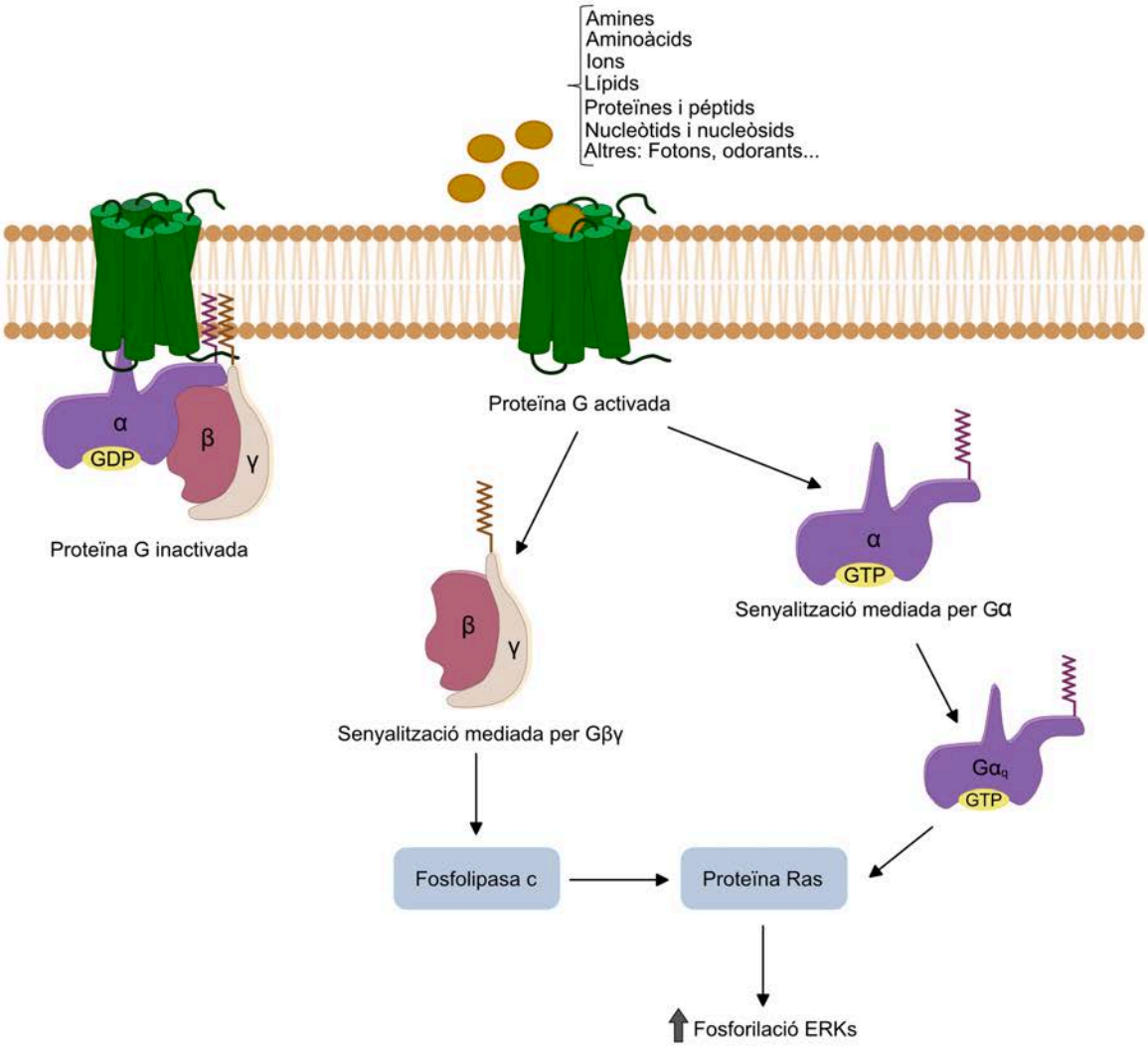

Figura 3: Esquema de l'activació de les MAPK a través de les subunitats Gβγ.

Per evitar els possibles efectes adversos de la sobreestimulació dels GPCR, aquests receptors pateixen un procés de dessensibilització i internalització[32]. La dessensibilització és un fenomen de regulació per *feedback* que es produeix en forma de diferents processos com el desacoblament del receptor a la seva proteïna G a conseqüència de la seva fosforilació, la internalització del receptor dins la membrana plasmàtica cap a compartiments intracel·lulars i la degradació dels receptors existents[33]. Aquest procés pot involucrar proteïnes com les cinases de GPCR (GRK), que s'encarreguen de fosforilar al receptor un cop ha estat activat per unió a lligand. No obstant això, aquesta fosforilació també es pot fer de manera independent a unió a lligand, iniciada per les cinases PKA i PKC[34].

Una vegada el receptor és fosforilat, s'inicia el reclutament de β-arrestina[35]. Aquestes proteïnes seran les encarregades de trencar la unió de la proteïna G amb el GPCR i finalitzar el senyal. Seguidament, les β-arrestines també intervenen en el procés d'internalització del receptor. Un domini fosforilat de la β-arrestina és reconegut per la proteïna clatrina, que s'ancorarà a la membrana afavorint l'endocitosi del receptor en forma de vesícules[36]. Un cop el receptor ha estat internalitzat, pot ser ubiquitinitzat i degradat en els lisosomes o bé ser

desfosforilat i reciclat a la membrana plasmàtica per poder tornar a ser activat[37] (Figura 4).

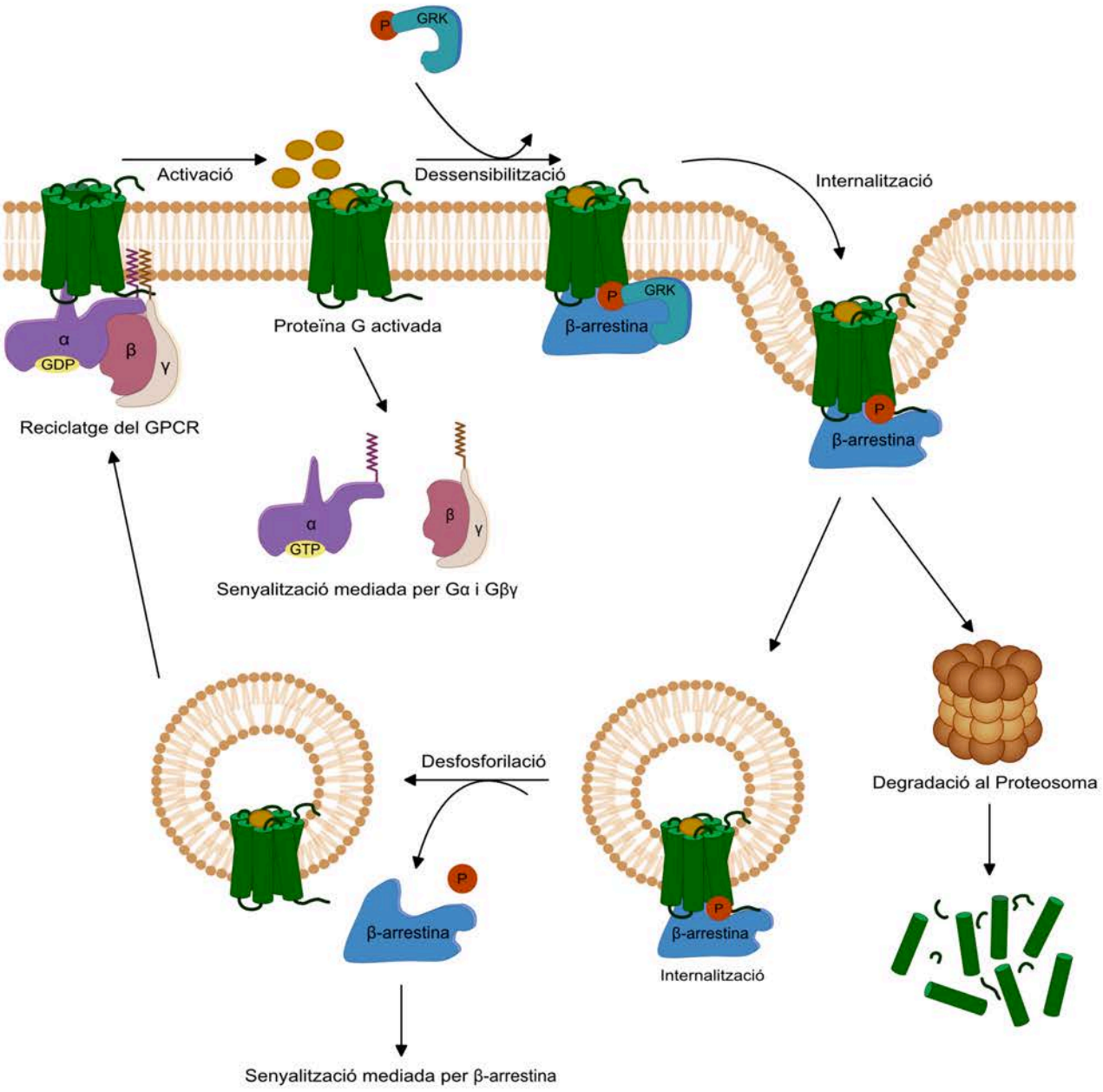

Figura 4: Esquema del procés de dessensibilització, internalització i reciclatge dels GPCR per mitjà de les GRKs i les β-arrestines.

Cal remarcar que les β-arrestines no únicament actuen en aquest fenomen de dessensibilització i internalització dels GPCR, sinó que també poden participar en diferents cascades de senyalització mitjançant la interacció amb proteïnes com c-Src o les MAPK[38,39,40]. De fet, estudis recents demostren que alguns GPCR poden unir simultàniament la proteïna G i les β-arrestines[41].

1.1.3 Lligands dels GPCR

L'activació d'un GPCR depèn de la unió a lligand. Considerem que un lligand és un agonista quan produeix un canvi de conformació en el receptor, passant d'un estat inactiu a un estat actiu[42]. Els dos estats sempre es troben en equilibri. Quan es produeix un canvi d'estat de manera espontània, sense presència d'agonista, parlem d'expressió constitutiva dels GPCR[43]. Segons la intensitat de l'activació, els agonistes poden classificar-se en agonistes totals, quan produeixen l'efecte màxim en el receptor o en agonistes parcials, quan l'activen parcialment (Figura 5).

Els GPCR poden trobar-se en diferents conformacions, aquestes conformacions es veuen estabilitzades per la unió del lligand al receptor, essent la unió a l'agonista total la que desplaçant l'equilibri cap a la conformació activa, afavorint la senyalització[44]. En el cas dels agonistes parcials, no poden activar el 100% de la resposta, ja que tenen menor eficiència per estabilitzar la forma activa del receptor. Els agonistes inversos redueixen l'activitat constitutiva del receptor, estabilitzant la conformació inactiva en menor o major mesura, depenent de si són parcials o totals[45]. Finalment, els antagonistes no tenen preferència d'afinitat entre els estats actiu o inactiu, s'uneixen al receptor i bloquegen l'activitat induïda per l'agonista[46]. Tots aquests moduladors d'activitat interaccionen directament amb el centre ortostèric del receptor, i conseqüentment s'anomenen lligands ortostèrics.

Els lligands al·lostèrics són aquells que s'uneixen als receptors en un centre d'unió diferent al centre ortostèric. Són capaços de modular l'activitat del receptor ja sigui potenciant (activadors al·lostèrics) o inhibint (inhibidors al·lostèrics) l'acció del lligand ortostèric[46]. Els lligands al·lostèrics endògens poden ser ions, lípids, pèptids o aminoàcids i aporten un grau més de complexitat a la regulació de la senyalització dels GPCR. L'ús de lligands al·lostèrics exògens obre les portes al disseny de nous fàrmacs, ja que els centres al·lostèrics són una diana amb moltes possibilitats i no impedeixen la unió dels lligands ortostèrics endògens[47].

No obstant això, la visió clàssica dels dos estats, actiu i inactiu, desplaçats per la presència d'agonistes o antagonistes sembla ser més complexa del que a priori podria semblar. Cada vegada hi ha més evidències de què diferents lligands actuant en el mateix receptor i en el mateix entorn biològic, poden estabilitzar diferents conformacions, de manera que s'activaran unes vies de senyalització en detriment d'altres[47,48]. A aquest fenomen se'l coneix com a "*biased agonism*", i és un indicador de què els GPCR poden adoptar més d'un estat actiu.

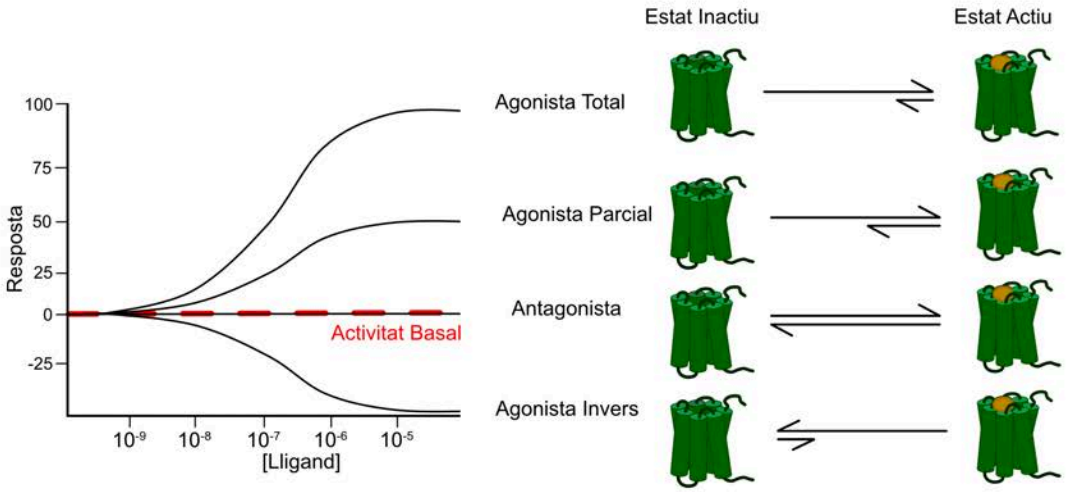

Figura 5: Representació dels diferents tipus de lligands d'unió a GPCR.

1.1.4 Implicació dels GPCR en patologies del Sistema Nerviós Central

Els GPCR participen en una àmplia varietat de processos fisiològics en l'ésser humà[49]. És per aquest motiu que aquestes proteïnes es troben implicades en un gran nombre de disfuncions i malalties. La primera mutació natural d'un GPCR associada a una patologia descoberta, estava relacionada amb la *retinitis pigmentosa*. Des d'aleshores, la llista de malalties provocades per mutacions en GPCR no ha deixat de créixer[50]. L'hipotiroïdisme, l'enanisme o inclús la sensibilitat a la infecció per VIH són condicions que poden ser provocades per mutacions en un determinat GPCR[49].

Els GPCR no només es troben implicats en malalties per culpa de mutacions, diferents patologies cursen amb nivells d'expressió anòmals d'aquestes proteïnes. Un exemple recau en els receptors de dopamina D_1 que es troben sobreexpresats en l'esquizofrènia, o els receptors de dopamina D_2 que augmenten la seva expressió en malalts de Parkinson no tractats amb L-DOPA[51]. Per un altre costat, els receptors de serotonina tenen un rol molt important en la depressió, en episodis d'isquèmia/hipòxia, en la malaltia d'Alzheimer i en l'ansietat[52] i els receptors d'adenosina i dopamina són de gran importància en l'addicció a la cocaïna, la malaltia de Huntington o el Parkinson[53]. A part d'aquests exemples, els GPCR es troben implicats en moltes altres que cursen en altres teixits, com la diabetis tipus 2, l'obesitat o el càncer[2].

Els GPCR orfes són aquells receptors que no tenen un lligand endogen conegut, però que tenen una estructura semblant a altres receptors i es troben implicats en funcions fisiològiques i patològiques[54]. Un exemple és el GPRC5A, que es troba directament relacionat amb el càncer de pàncrees i la seva supressió resulta en una reducció de la proliferació tumoral[55]. El GPR22 té un rol important en malalties cardíaques, com la hipertrofia[56]. També s'ha descrit que en la malaltia d'Alzheimer, la pèrdua del receptor GPR3 afavoreix la reducció de les

plaques amiloides i millora la memòria en ratolins model de la malaltia[57]. Tots aquests receptors són una potencial àrea d'estudi molt prometedora per poder detectar noves dianes terapèutiques i descobrir nous compostos capaços de tractar aquestes patologies que sovint no tenen cura.

1.1.5 Els heteròmers de GPCR

Tradicionalment, els mecanismes de transducció de senyal i la senyalització dels GPCR es basaven en la suposició que aquests receptors actuaven com a una unitat funcional independent, amb una estequiometria 1:1 amb la proteïna trimèrica G. A mitjans dels anys noranta, cada cop més evidencies indicaven que els GPCR podien oligomeritzar[17,58]. Així doncs, la imatge clàssica de l'estequiometria 1:1 quedava desplaçada per la 2:1, és a dir, un receptor necessitava formar part d'un dímer per acoblar una proteïna G.

L'heteromerització entre GPCR es defineix com la interacció directa entre un mínim de dos receptors amb propietats tant bioquímiques com funcionals específiques, diferents de les que presenten els seus components individualment[59]. Definim així com homodímer el complex format per dos receptors iguals i com a heterodímer el complex format per dos receptors diferents (Figura 6). Tot i que els GPCR monomèrics també poden activar les proteïnes G, el complex format per un homodímer i la seva proteïna G sembla ser la unitat funcional principal. També s'han descrit complexes trimèrics[60] o inclús tetramèrics amb estequiometria 4:2, amb dos homodímers de GPCR acoblats a dues proteïnes G[61].

La majoria d'evidències indiquen que tant la família de GPCR A com la C, formen homodímers i heterodímers, sent els homodímers l'espècie dominant, amb potencial per formar oligòmers de major ordre, sobretot tetràmers[62]. L'any 1999 va ser publicat el descobriment del primer heteròmer format per dos receptors de la mateixa família, en aquest cas, els receptors delta i kappa opioides[63].

La formació de complexos heteromèrics entre dos GPCR no únicament està limitada a la interacció de receptors dins la mateixa família, com hem comentat. L'any 2000, es va publicar l'heteròmer format pel receptor d'adenosina A_1 i el receptor de dopamina D_1[64]. Actualment, la formació de complexos oligomèrics de GPCR és una realitat que està acceptada en el camp de la biologia[65].

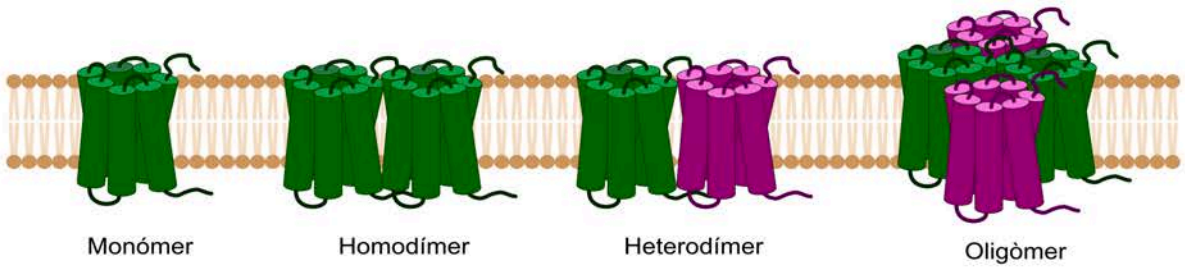

Figura 6: Oligomerització dels GPCR.

1.1.6 Implicacions funcionals de l'oligomerització

L'oligomerització dels GPCR té un paper important en gairebé totes les etapes del cicle de vida d'aquests receptors, des de la maduració de la proteïna al reticle endoplasmàtic. La sortida dels GPCR del reticle endoplasmàtic és una etapa crucial en el control de l'expressió del receptor a nivell de membrana plasmàtica[66]. Únicament els receptors ben plegats seran traslladats, mentre que els defectuosos o incomplets seran retinguts i degradats en compartiments intracel·lulars[67].

Per algunes proteïnes dimèriques, el seu correcte plegament i migració passa per un solapament de les seves senyals de retenció al reticle, evitant així la seva retenció. Un exemple és el receptor $GABA_B$, que requereix la interacció entre dos GPCR anomenats $GABA_{B1}$ i $GABA_{B2}$ per ser funcional[68]. Per un costat, el receptor $GABA_{B2}$ expressat de forma individual, pot viatjar fins a la membrana plasmàtica, però no es comportarà com una unitat funcional. D'altra banda, $GABA_{B1}$ presenta un senyal de retenció al reticle endoplasmàtic que impedeix la seva migració cap a la membrana plasmàtica. Quan ambdós receptors heteromeritzen, el senyal de retenció es veu emmascarada per $GABA_{B2}$ i es genera un dímer funcional capaç de ser expressat a membrana plasmàtica i senyalitzar[69].

S'ha observat també que l'oligomerització confereix diversitat farmacològica, ja que la unió d'un lligand a un receptor de l'oligòmer pot influir a la unió o l'activitat d'un segon lligand a un altre receptor de l'heteròmer a causa de modulacions al·lostèriques entre els receptors. Com hem descrit anteriorment l'al·losterisme és el procés pel qual la interacció d'un lligand en un lloc específic del receptor o de l'heteròmer (el lloc al·lostèric) influeix a la unió o funció d'un altre lligand a un lloc topogràficament diferent (lloc ortostèric). Actualment, s'han descrit diversos casos de modulació al·lostèrica, principalment negativa. Recentment s'ha reportat el cas de l'heteròmer CB_1R-CB_2R, en què el cotractament amb agonistes dels dos receptors donava lloc a una disminució de la seva senyalització. Un altre fenomen que pot ocórrer quant dos GPCR interaccionen és el del crosantagonisme, on l'antagonista d'un receptor és capaç de bloquejar l'efecte de l'agonista de l'altre receptor[70].

L'oligomerització també pot modificar les propietats de la senyalització d'un lligand afectant la interacció entre un receptor i la seva proteïna G. Per exemple, els receptors CB_1 i D_2 interaccionen formant complexos heteromèrics, i ambdós receptors s'acoblen a la proteïna Gi. Quan els dos receptors són coactivats, l'heteròmer CB_1R-D_2R s'acobla a una proteïna Gs en lloc d'una Gi[71].

S'ha observat, que un cop un GPCR ha arribat a la membrana plasmàtica, el seu estat dimèric pot veure's regulat de manera dinàmica per la presència de lligands, fluctuant entre el seu estat dimèric i monomèric. Aquest fenomen de regulació del grau d'oligomerització per lligands s'ha descrit en un gran nombre de publicacions. Encara no hi ha un consens sobre la seva rellevància fisiològica, ja que hi ha literatura que demostra que la unió al lligand pot promoure i inhibir l'oligomerització, però també hi ha estudis que defensen que l'oligomerització és un procés constitutiu que funciona independentment de la unió a lligand[72,73]. Tot

i això, el que sí que està ben acceptat és que els heteròmers tenen una naturalesa dinàmica a nivell de membrana plasmàtica[74].

L'oligomerització també pot afectar l'endocitosi i al tràfic intracel·lular dels GPCR, que en última instància decidiran el destí de reciclatge o degradació d'aquests receptors, sent internalitzats des de la membrana plasmàtica cap a compartiments intracel·lulars[75].

1.2 Cànnabis

1.2.1 La planta del Cànnabis

El cànnabis és una planta dioica, alguns éssers tenen flors masculines mentre que d'altres femenines, i aquestes, floreixen anualment[76]. Les fulles tenen folíols que creixen de manera radial des de la base de la fulla[76] i es caracteritza per una espècie de pèls que recobreixen la planta anomenats tricomes. La funció dels tricomes no està del tot clara, però sembla ser en part, per protegir la planta de petits insectes. Hi ha diferents tipus de tricomes que en les plantes femenines tenen una gran concentració de Δ^9-tetrahidrocannabinol[77].

La planta del cànnabis es classifica en 3 espècies: *Cannabis sativa*, *Cannabis indica* i *Cannabis ruderalis.* A més, se'n coneixen diferents varietats, com el *Cannabis indica var. kafiristanica* o el *Cannabis sativa var. spontanea*[78]. Aquestes tres espècies poden reproduir-se entre elles formant híbrids. Precisament per aquest motiu i basant-se en la definició d'espècie (que indica que diferents espècies no poden donar descendència fèrtil), certs autors consideren que únicament existeix una espècie de cànnabis, el *Cannabis L (Linnaeus)*, considerant com a subespècies el *C. sativa, el C. indica* i *el C. ruderalis*[79].

Estudis paleobotànics asseguren que fa més de 11.700 anys el *Cannabis sativa* ja s'utilitzava a les muntanyes Altai a l'Àsia Central[80,81]. Les llavors de la planta del cànnabis van estendre's per la resta del món a través d'Euràsia durant l'últim període glacial[80]. Els primers usos del *Cannabis sativa* no van ser recreatius, la planta es cultivava per produir cordes, roba i paper. Precisament, a la Xina hi ha restes d'aquests materials que daten de l'any 400 a.C[82].

El primer ús medicinal documentat del cànnabis data entre els anys 300 a.C i 200 d.C, en la farmacopea de Shen Nung Pen Ts'ao Ching, un emperador xinès que es creu va ser el primer en usar el cànnabis amb finalitats curatives. En aquests escrits, també es parlava d'altres plantes medicinals com el ginseng, la canyella o la taronja[83]. Des de llavors, durant segles, la planta del cànnabis s'ha utilitzat tant amb finalitats recreatives com medicinals, encara que el mecanisme d'acció d'aquests compostos han restat desconeguts fins fa unes dècades.

Actualment, aproximadament 200 milions de persones consumeixen cànnabis anualment, sent el mètode de consum més habitual la crema i inhalació de la flor[82]. El cànnabis és la tercera substància d'abús més consumida al món, per darrere de l'alcohol i el tabac[84]. L'any 2018, les nacions unides van presentar un

informe que estimava que el 3,9% de la població mundial adulta havia consumit cànnabis el darrer any[85].

Els països en vies de desenvolupament realitzen un menor consum de cànnabis comparat amb els països desenvolupats tot i que es veu una tendència a l'alça[86]. Des de l'any 2000 fins al 2020, el consum de cànnabis s'ha incrementat en un 16%, mentre que la percepció del risc en el consum ocasional ha disminuït un 39%, i en el cas del consum regular, en un 42%[87]. A més, aproximadament el 9,9% dels individus que afirmaven haver consumit cànnabis en l'últim any ho feien a diàriament[85]. Tots aquests factors han afavorit que el trastorn per consum de cànnabis (CUD) afecti gairebé al 10% dels consumidors de cànnabis arreu del món[85]. El trastorn per consum de cànnabis es defineix com la inhabilitat de parar de consumir cànnabis encara que estigui causant un perjudici físic o psicològic[88]. Una estimació a nivell global del 2016 xifrava els casos que complien els criteris de diagnòstic de CUD en 22,1 milions de persones (289,7 casos per cada 100.000 habitants)[85].

Tot i l'elevat potencial mèdic dels cannabinoides i malgrat que la seva dependència tant física com psicològica és relativament baixa, el cànnabis és considerat una droga de classe C, la categoria que engloba les substancies controlades menys nocives, en diversos països de la Unió Europea i té diferents graus de legalitat als Estats Units[82]. Tot i això, el consum de cànnabis està marginalitzat i considerat un vici a tot el món[89]. Entre els anys 1980 i 1990 es va iniciar un canvi en la percepció que es tenia sobre el cànnabis i es va començar a prescriure com a antiemètic i com a fàrmac per incrementar la gana en pacients amb càncer i HIV[90].

1.3 Sistema Cannabinoide

El Sistema Cannabinoide és un sistema neuromodulador que juga un paper important en el desenvolupament del Sistema Nerviós Central, la plasticitat sinàptica i en la resposta a *inputs* tan endògens com exògens[91]. En mamífers, aquest sistema està format pels receptors de cannabinoides CB_1 i CB_2; els lligands endògens d'aquests receptors, anomenats endocannabinoides, sent els més importants l'anandamida i el 2-araquidonilglicerol; i els enzims de síntesi i degradació[92].

El Sistema Cannabinoide controla un gran nombre de processos biològics. La seva activitat, tant a nivell del Sistema Nerviós Central com Perifèric, està estretament relacionada amb la plasticitat neuronal, la regulació del dolor, la resposta a l'estrès, la reproducció, la regulació del comportament i l'estat d'ànim i en el control de la ingesta calòrica. El Sistema Cannabinoide té un rol modulador en el Sistema Nerviós Central, controlant processos cognitius com l'aprenentatge i la memòria[91]. Canvis en la funcionalitat del Sistema Cannabinoide causen alteracions en altres sistemes neuromoduladors com el de les monoamines i produeixen desequilibris en el control del sistema GABA/glutamat[93]. Com a conseqüència, la disfunció del Sistema Cannabinoide comporta l'aparició de

condicions psiquiàtriques com la depressió, ansietat, psicosi o esquizofrènia. El bloqueig del receptor CB_1R produeix un estat en rates equiparable a la depressió, causant ansietat, estrès i reducció de la ingesta[94]. La reducció de l'activitat dels endocannabinoides produeix dolor crònic i una disminució de l'activitat serotoninèrgica, que molts cops acompanya a la depressió.

Una de les particularitats del Sistema Cannabinoide és la seva senyalització retrògrada. En la sinapsi clàssica (també anomenada anterògrada) les dendrites de la neurona presinàptica alliberen neurotransmissor que interaccionen amb els receptors de la neurona postsinàptica (Figura 7). Contràriament, en el cas de la neurotransmissió retrògrada, els receptors es troben expressats a la neurona presinàptica, els lligands són sintetitzats a la neurona postsinàptica i alliberats a l'espai sinàptic, així que el transport axonal es dona des de l'axó de la neurona postsinàptica cap a la presinàptica[95]. Els endocannabinoides actuen principalment com a inhibidors de l'alliberació de neurotransmissors. Així, l'activació dels receptors de cannabinoides té com a conseqüència la inhibició de l'alliberació de senyals neuronals, tan activadores, com seria el cas de glutamat[96], com inhibidores en el cas del GABA[97].

Els endocannabinoides poden funcionar com a missatgers retrògrads a través de tres mecanismes: la supressió de la inhibició/excitació induïda per despolarització (DSI i DSE respectivament), la supressió de la inhibició/excitació induïda per un senyal metabotròpic (MSI i MSE respectivament) i la depressió a llarg termini mitjançada per cannabinoides (LTD)[91]. En el cas de la despolarització, ocorre quan la neurona postsinàptica pateix una forta estimulació de varies dècimes de segon de durada causada per repetits potencials d'acció[98]. El senyal metabotròpic apareix quan a la membrana postsinàptica un receptor metabotròpic acoblat a una proteïna Gq, és activat pel seu lligand, elevant els nivells intracel·lulars de Ca^{2+} i activant la PLCβ que sintetitzarà 2-araquinodilglicerol a partir de fosfatidilinositols de la membrana plasmàtica[99]. En darrer lloc, la depressió a llarg termini es produeix després d'una estimulació continuada de la neurona postsinàptica que pot ser homosinàptica (sobre la mateixa sinapsi) o heterosinàptica (sobre una sinapsi adjacent)[91].

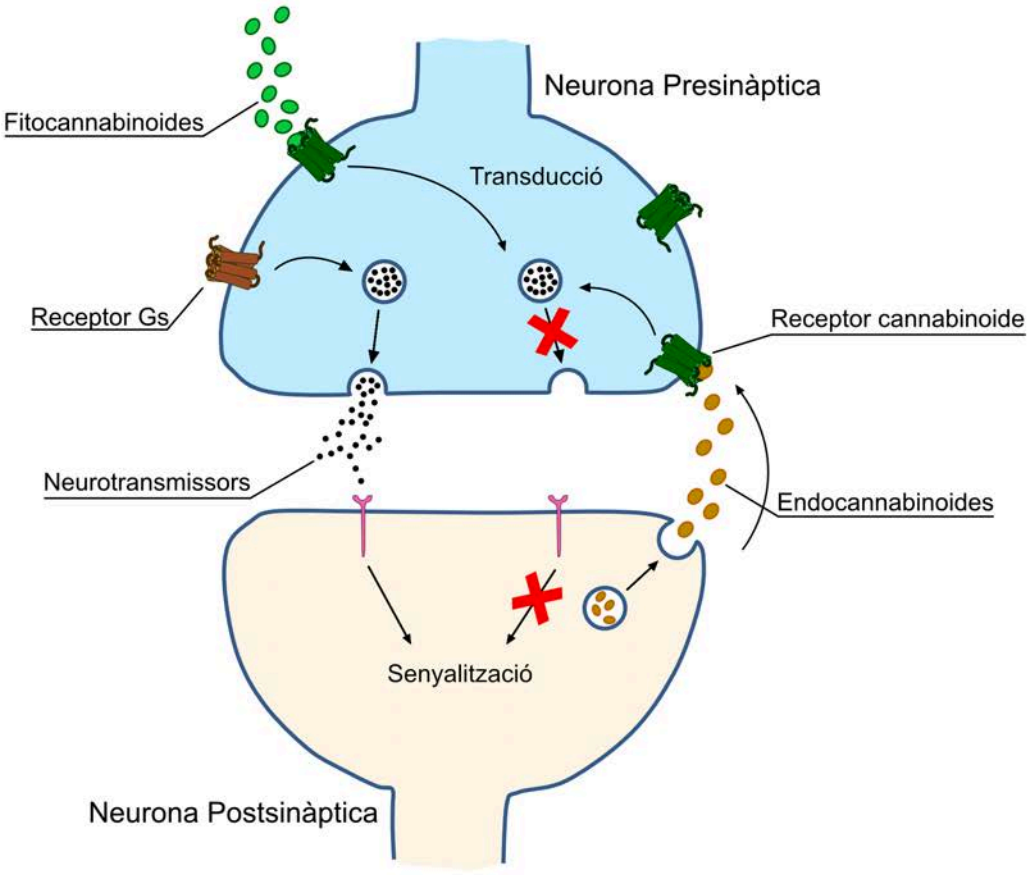

Figura 7: Esquema de la senyalització retrògrada del Sistema Cannabinoide.

1.3.1 Lligands del Sistema Cannabinoide

Els lligands cannabinoides es divideixen en: fitocannabinoides, compostos que s'extrauen de la planta *Cannabis sativa,* com per exemple el Δ^9-tetrahidrocannabinol (Δ^9-THC) o el cannabidiol (CBD); cannabinoides sintètics, que han estat sintetitzats en un laboratori, com el JWH-133; i endocannabinoides, compostos sintetitzats pel nostre organisme, sent els més importants i els primers en ser descoberts l'anandamida (N-araquidonil-etanolamida, AEA) i el 2-araquidonilglicerol (2-AG) (Figura 8).

El Δ^9-THC és el principal compost psicoactiu i més abundant en la planta *Cannabis sativa*[100]. Va ser descobert el 1964 i és un terpenoide de 21 carbonis altament lipofílics, de tacte resinós i color lleugerament groc[82]. Es troba de manera abundant en forma de cera al voltant de les fulles de la planta del cànnabis i les seves flors[101].

El Δ^9-THC s'uneix tant el receptor CB_1 com al receptor CB_2 i és, de fet, el més potent de tots els fitocannabinoides[102]. No obstant això, actua com a agonista parcial, ja que no indueix l'activació total ni de CB_1R ni de CB_2R[103]. Així doncs,

Δ^9-THC pot actuar també com a antagonista segons la presència d'endocannabinoides o altres agonistes totals[103].

El Δ^9-THC produeix els seus efectes psicoactius a través de l'activació del receptor CB_1. Principalment, produeixen eufòria, augment de la percepció sensorial, taquicàrdia, dificultat de la concentració i alteracions de la memòria[104]. Algunes d'aquestes deficiències cognitives poden persistir en el temps després del consum. Cal destacar que el Δ^9-THC conté un grup fenol amb efecte antioxidant que protegeix les neurones en situacions d'estrès oxidatiu produït per excitotoxicitat induïda per glutamat[105,106]. Un efecte àmpliament estudiat del consum de cànnabis és l'augment de la gana i la intensificació de les propietats hedòniques del menjar[107]. Aquest efecte és un reflex del rol fisiològic dels endocannabinoides en la regulació de la gana[107].

El potencial terapèutic del Δ^9-THC com a cànnabis medicinal està limitat pel seu efecte psicoactiu. En aquest sentit, la comunitat científica ha centrat els seus esforços en la recerca de nous compostos cannabinoides que posseeixin les mateixes propietats beneficioses sense aquest efecte psicoactiu. Un clar exemple és el precursor del Δ^9-THC, l'àcid tetrahidrocanabinolic (Δ^9-THCA) que dona lloc a Δ^9-THC per mitjà de la seva descarboxilació. Té un perfil biològic interessant essent un potent activador del receptor PPARγ (*peroxisome proliferator-activated receptor-γ*), el qual la seva activació està relacionada amb l'activitat neuroprotectora[108] i amb el tractament de malalties metabòliques, però sense l'efecte psicoactiu[109].

Un altre compost que està essent estudiat amb resultats prometedors a causa de la seva absència d'efectes psicoactius és el Δ^9-THCV, un fitocannabinoide amb una estructura molt semblant químicament al Δ^9-THC, però amb una ruta de síntesi completament diferent a partir de l'àcid cannabigerovarínic (CBGVA)[110]. Els efectes d'aquest compost inclouen la supressió de la sensació de gana, el control glicèmic i, com el Δ^9-THCA, un paper neuroprotector molt important[111]. El Δ^9-THCV ha estat utilitzat clínicament per tractar la pèrdua de pes i la diabetis tipus 2[112].

Tant el Δ^9-THC com el Δ^9-THCV tenen una major afinitat pel CB_1R que pel CB_2R. Contràriament, el Δ^9-THCA mostra, en general, menys afinitat que els altres dos compostos tant pel CB_1R com pel CB_2R[113]. Tant el Δ^9-THCA com el Δ^9-THC es comporten com agonistes parcials, i conseqüentment poden actuar com antagonistes en presència d'un agonista total. Per un altre costat, el Δ^9-THCV actua com a antagonista de CB_1R, de manera molt semblant al rimonabant, però sense mostrar els seus efectes adversos, com són la depressió entre altres afectacions psiquiàtriques[114]. Així doncs, tant el Δ^9-THCA com el Δ^9-THCV podrien ser una bona alternativa per tractar diferents malalties sense induir un efecte psicoactiu.

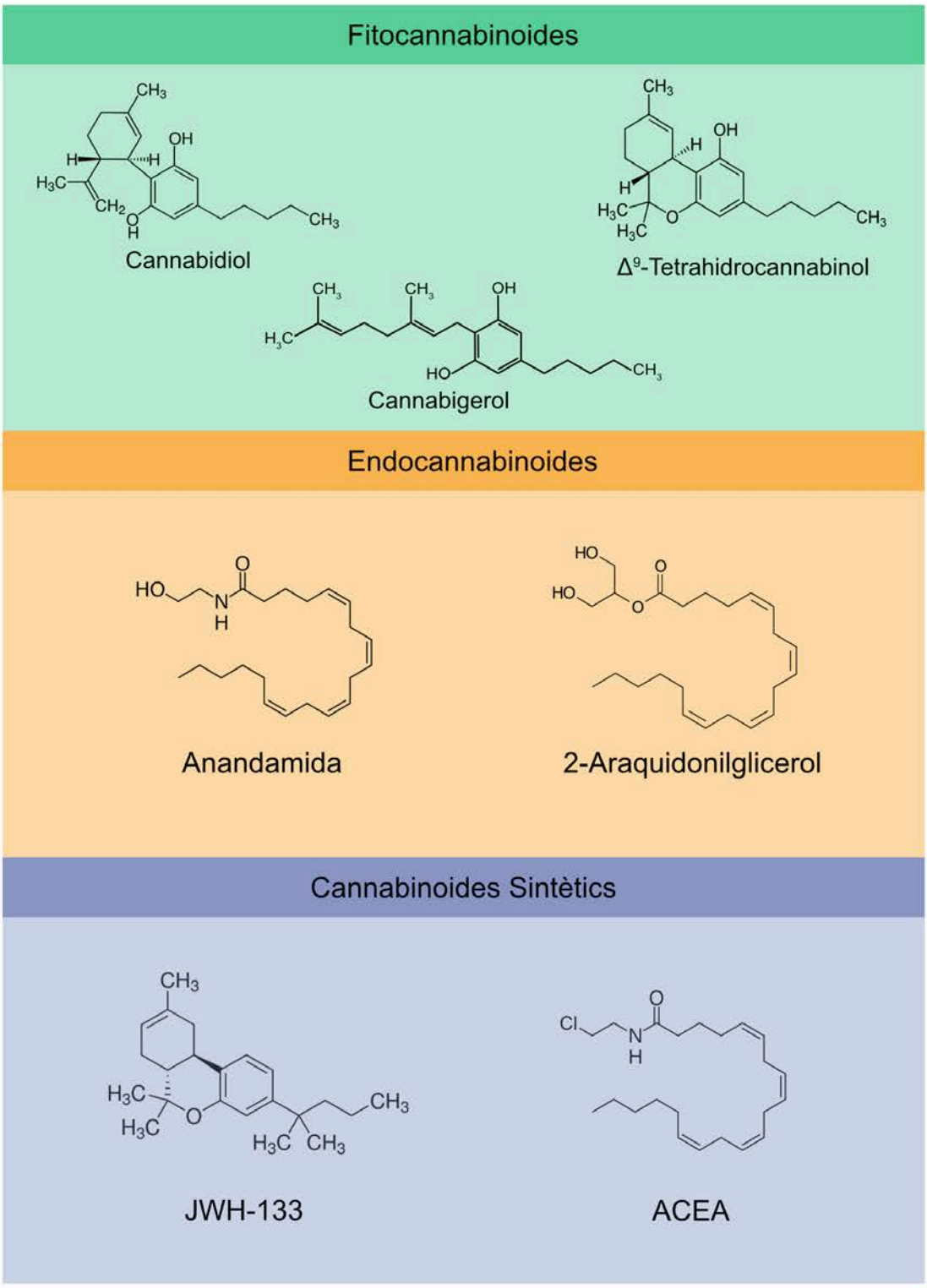

Figura 8: Classificació dels compostos cannabinoides més importants.

El segon component més estudiat i abundant de la planta del cànnabis és el cannabidiol (CBD)[82]. El CBD va ser descobert el 1963 i cada cop apareixen més evidencies del seu potencial com a agent terapèutic en nombroses patologies, ja que a diferència del Δ^9-THC, no indueix efectes psicoactius[115,116]. Hi ha hagut molta controvèrsia sobre l'afinitat i el centre d'unió del CBD per ambdós receptors

cannabinoides[117,118]. Actualment, les dades obtingudes apunten que a baixes concentracions actua com a modulador al·lostèric, mentre que a altes concentracions, pot actuar com a agonista parcial unint-se al centre ortostèric[119]. Recentment, s'ha publicat un article que conclou que el CBD també pot afectar indirectament a la senyalització del CB_1R, ja que inhibeix els enzims de degradació d'endocannabinoides com l'anandamida, evitant que s'eliminin i promovent una activació prolongada en el temps[120,121]. Com a curiositat, tant el CBG com el CBD s'uneixen al receptor 5-HT_{1A}, però tenen efectes contraris. Mentre que el CBG actua com a antagonista del receptor de serotonina, el CBD es comporta com agonista i/o modulador al·lostèric positiu[122].

El CBD és un dels cannabinoides més interessants i amb un potencial terapèutic més elevat. S'ha observat un efecte farmacològic beneficiós en patologies neurodegeneratives. El seu efecte antiinflamatori recau en la seva capacitat d'inhibir la producció de citocines proinflamatòries com la IL-1β, la IL-6, i l'interferó-β (IFN-β)[123,124]. L'efecte neuroprotector i antiinflamatori del CBD en casos d'ictus també es dona per l'activació dels receptors PPARγ i $5HT_{1A}R$[125]. L'activació dels receptors PPARγ resulta en la inducció de la micròglia cap al fenotip antiinflamatori M2, promovent la síntesi de citocines antiinflamatòries i activant el seu potencial antioxidant i neuroprotector[126]. El cannabidiol també s'està estudiant en l'epilèpsia, l'esquizofrènia, el càncer i algunes malalties autoimmunes[116].

Més enllà del CBD, el Δ^9-THC i els seus derivats, la planta del Cànnabis produeix més de 400 compostos diferents, dels quals més de 100 són cannabinoides. Entre ells, el cannabigerol (CBG) és el precursor de la majoria dels fitocannabinoides[127]. El CBG presenta una afinitat pels receptors de cannabinoides CB_1 i CB_2 molt semblant al Δ^9-THC[128]. Sorprenentment, també s'ha descrit la seva capacitat d'activar el receptor de serotonina (5-$HT_{1A}R$)[129]. A nivell funcional, el CBG té activitat neuroprotectora, ja que a través de l'activació de PPARγ, disminueix la producció de citocines proinflamatòries en astròcits[130]. En la mateixa línia, diferents estudis indiquen que el CBG té un important potencial terapèutic en malalties neurològiques com el Huntington, el Parkinson o l'esclerosi múltiple[129]. També presenta activitat antibacteriana i sembla ser una diana prometedora en malalties inflamatòries com la malaltia de Crohn[131]. Finalment, mitjançant la inhibició de l'aldosa reductasa ALR2, un enzim implicat en la degradació de la glucosa, també podria tenir un rol important en trastorns alimentaris i diabetis, ja que s'ha vist que indueix hiperfàgia en rates[132,133].

De la mateixa manera que amb el Δ^9-THC, també s'han estudiat compostos derivats del CBG, essent els més investigats el CBGA i el CBGV[134,135]. El seu interès no és recent, de fet hi ha estudis sobre aquests compostos des de l'any 1990, on s'estudiava el metabolisme del CBG en mamífers[136].

Des que es va descobrir el Δ^9-THC i es van començar a observar els efectes beneficiosos d'aquests i d'altres fitocannabinoides com el CBD i el CBG, la indústria farmacèutica va començar a interessar-se per aquests compostos i va centrar els seus esforços a sintetitzar noves molècules que mantinguessin l'activitat biològica natural dels cannabinoides, però no produïssin els efectes psicoactius indesitjats[137]. Aquestes noves molècules podien ser similars

estructuralment a altres fitocannabinoides o compostos nous amb noves estructures químiques completament diferents[138]. Aquests nous compostos es van anomenar Cannabinoides Sintètics (SCs).

Els primers cannabinoides sintètics es van produir en laboratoris de la indústria farmacèutica. L'objectiu principal era sintetitzar molècules que poguessin unir-se als receptors CB_1 i CB_2 per ser utilitzades com a analgèsics amb propietats semblants a les del Δ^9-THC[139]. Entre els pioners trobem Pfizer, que l'any 1974 sintetitzava el cyclohexylphenol (CP 55,940)[140]. A continuació, laboratoris de la Universitat d'Hebrew i de Carolina del Sud van començar a sintetitzar els agonistes selectius HU-210 i JWH-018[141].

El 2016 experiments *in vitro* van demostrar que els agonistes selectius de CB_2R, JWH-015, JWH-133 i HU-308; i els agonistes tant de CB_1R com CB_2R, WIN 55,212-2 i HU-210 disminuïen l'alliberació de citocines proinflamatòries en cultius de micròglia exposats a diferents espècies del pèptid β-amiloide (Aβ) i prevenien deficiències cognitives i la pèrdua de memòria en la malaltia d'Alzheimer[142].

A principis dels anys noranta es va identificar i clonar el primer receptor cannabinoide, el CB_1; no va caldre esperar gaire fins que es va descobrir el seu agonista endogen, l'anandamida (AEA)[143]. Com que l'AEA no podia reproduir exactament tots els efectes del Δ^9-THC es va suposar que havia d'existir un altre endocannabinoide per tal de poder explicar aquests efectes. Així va ser com l'any 1995 es va proposar al 2-AG com el segon lligand endogen dels receptors de cannabinoides[144]. Aquests dos endocannabinoides estan molt ben documentats i posseeixen característiques i propietats farmacològiques diferents.

L'anandamida actua de manera molt semblant al Δ^9-THC, es comporta com un agonista parcial amb alta afinitat pel receptor CB_1 i menor pel receptor CB_2[145]. L'AEA també pot interaccionar amb altres receptors com per exemple el receptor vanilloide 1 (TRPV1), un canal iònic que té un rol molt important en la transmissió sinàptica i la regulació del dolor[92]. A causa de la seva capacitat per unir cannabinoides, hi ha diferents estudis que consideren també a TRPV1 com un receptor cannabinoide[146].

D'altra banda, el 2-araquidonilglicerol (2-AG) actua com a agonista d'ambdós receptors cannabinoides, CB_1 i CB_2[147], amb una afinitat lleugerament superior per a CB_1R i més activitat envers CB_1R i CB_2R que l'anandamida[148]. El 2-AG també s'uneix al receptor TRPV1, que es troba coexpressat amb CB_1R i CB_2R tant en neurones com en glia, i en episodis de neuroinflamació incrementa la seva expressió[149]. Cada cop hi ha més estudis de la seva interacció amb altres receptors no cannabinoides, un exemple són els receptors PPAR, que des de 2002, amb l'ajut d'animals KO i siRNA pels receptors cannabinoides han anat ampliant la llista de cannabinoides que s'uneixen a ells[150].

1.3.2 Fàrmacs al mercat

El fort increment en la investigació durant els darrers anys ha comportat que el rol del cànnabis en medicina estigui evolucionant molt ràpidament. Els progressos en la química i en la farmacologia del cànnabis han aportat noves propostes terapèutiques i funcionals, com per exemple analgèsiques o antidepressives[151]. Tot i això, la gran majoria d'efectes d'aquests compostos encara no han estat provats en assajos clínics o no es tenen suficients dades per ser aprovats. Aquests estudis es veuen agreujats per la complicada situació legal en què es troba el cànnabis en l'actualitat, i comporta que l'ús adequat d'aquestes substàncies romangui lleugerament incert[151].

Efectes secundaris dels compostos derivats del cànnabis, com ara fatiga, mareig, mal de cap o nàusees han limitat el seu ús en gran mesura[152]. Mentre evoluciona tant l'estatus legal com mèdic d'aquests derivats, els científics treballen per potenciar al màxim els seus efectes beneficiosos i reduir els efectes adversos i conseqüentment, les seves limitacions.

A dia d'avui, la FDA ha aprovat dos fàrmacs derivats del cànnabis: Epidiolex® (CBD), i Sativex® (Δ^9-THC i CBD) i tres fàrmacs sintètics derivats del cànnabis: Marinol® (dronabinol), Syndros® (dronabinol) i Cesamet® (nabilone)[152].

El Sativex® és un esprai oromucós que conté els dos cannabinoides més abundants de la planta del cànnabis, el Δ^9-THC i el CBD, en una relació molecular 1:1[153]. Aquest fàrmac funciona com un modulador del Sistema Cannabinoide i ha demostrat eficàcia en més de 1500 pacients amb esclerosi múltiple[154].

L'Epidiolex® conté una forma purificada d'un fitocannabinoide, el CBD, com a principi actiu. Va ser acceptat per la FDA el 2018, convertint-se en el primer fàrmac derivat del cànnabis en ser aprovat[155]. Ha resultat ser efectiu en certs casos d'epilèpsia i malalties com el síndrome de Lennox-Gastaut o síndrome de Dravet[156].

El Marinol® i el Syndros® tenen com a principi actiu el Dronabinol, un derivat sintètic del Δ^9-THC que no té efectes psicoactius[157]. Aquest compost s'utilitza per tractar les nàusees associades amb el tractament de quimioteràpia i per tractar l'anorèxia associada amb la pèrdua de pes dels malalts de VIH[158].

Finalment, el Cesamet® conté el principi actiu Nabilone, que és un altre derivat sintètic del Δ^9-THC sense efectes psicoactius[159]. De la mateixa manera que el Dronabinol, s'utilitza, sobretot, per tractar les nàusees produïdes per la quimioteràpia[160].

1.3.3 Enzims de síntesi i degradació

A diferència de la majoria de neurotransmissors clàssics, que se sintetitzen i s'emmagatzemen en vesícules sinàptiques, els endocannabinoides se sintetitzen en funció de la demanda fisiològica, en el moment i lloc necessari, a partir de precursors lipídics de la membrana plasmàtica[91]. Els dos endocannabinoides més coneguts i més importants són l'anandamida i el 2-araquidonilglicerol. Ambdós contenen àcid araquidònic conjugat amb diferents molècules com l'etanolamina o el glicerol, però les seves rutes de síntesi i degradació són i estan regulades per enzims diferents[161] (Figura 9).

El 2-AG se sintetitza a partir de la hidròlisi del fosfatidilinositol bisfosfat (PIP_2) mitjançant la PLC, produint així diacilglicerol, que serà hidrolitzat per l'enzim diacilglicerol lipasa (DAGL)[162]. El 2-AG a més de ser un lligand endogen dels receptors cannabinoides, també és un important metabòlit intermediari en la síntesi de lípids i una de les fonts principals d'àcid araquidònic per la síntesi de prostaglandines[163]. És per aquest motiu que l'alteració de la síntesi i degradació de 2-AG pot tenir efectes independents al Sistema Cannabinoide.

La degradació del 2-AG es du a terme, principalment, per tres enzims: la monoacilglicerol lipasa (MAGL) i les hidrolases ABHD6 i ABHD12[164]. Els tres enzims tenen localitzacions diferents, fet que permet la síntesi del 2-AG en diferents compartiments cel·lulars. Per un costat, el MAGL es troba, principalment, en els terminals sinàptics de les neurones del SNC adult. La seva inhibició ha estat estudiada com a estratègia terapèutica per disminuir els nivells d'àcid araquidònic, necessari per a la síntesi de prostaglandines que es troben involucrades en processos d'inflamació[165]. L'ABHD6 es troba, majoritàriament, en les dendrites i espines dendrítiques de les neurones excitadores del còrtex cerebral[166]. Finalment, tot i que el ABHD12 té un rol important en la degradació de lisofosfatidilserines de cadena llarga, el seu paper *in vivo* no està totalment caracteritzat[167]. No obstant això, el 2-AG també pot ser oxidat per mitjà de la ciclooxigenasa-2 (COX-2) o l'enzim hidrolasa d'amines d'àcids grassos (FAAH)[164].

La síntesi de l'anandamida és més complexa que la del 2-AG i pot dur-se a terme per diferents mecanismes. L'AEA és un producte del metabolisme dels fosfolípids de membrana, sintetitzada per mitjà de la hidròlisi de la N-araquidonil fosfatidiletanolamida (NAPE); la principal via d'hidròlisi de la qual està regulada per la fosfolipasa D (NAPE-PLD)[168,169]. Una altra via de síntesi de l'anandamida, que sobretot té lloc en les cèl·lules immunitàries, és per mitjà d'una fosfolipasa C selectiva (PLC) que trenca els enllaços fosfodièster de la NAPE i seguidament desfosforila la fosfo-AEA resultant[170]. Es coneixen altres mecanismes, però encara no està clar el seu paper en el SNC[171].

La degradació de l'anandamida es dona principalment per l'enzim FAAH[172]. La inhibició dels enzims de degradació té com a conseqüència nivells més elevats d'endocannabinoides, potenciant els seus efectes beneficials. Com que la FAAH també degrada altres amines com per exemple l'oleoil etanolamida, la seva inhibició pot tenir com a conseqüència un increment d'aquestes etanolamides[173]. Una segona via de degradació és per mitjà de la COX-2. La diferència estructural

entre l'anandamida i l'àcid araquidònic, permet la síntesi d'inhibidors d'oxidació d'AEA sense veure's afectada la síntesi de prostaglandines[174]. Finalment una tercera via de degradació de l'AEA és per mitjà de l'amidasa NAAA[175].

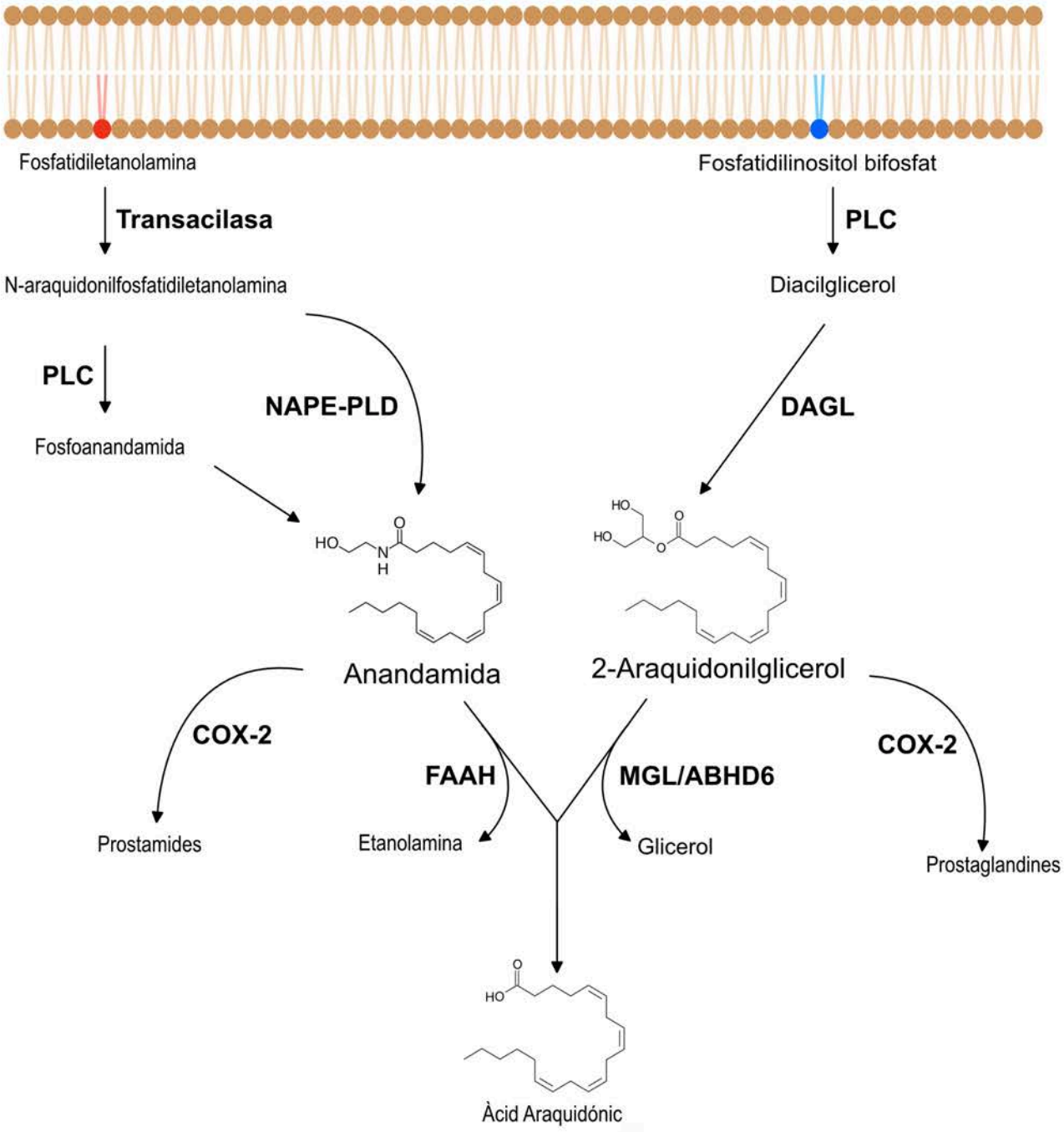

Figura 9: Rutes de síntesi i degradació dels endocannabinoides.

1.3.4 Receptors de Cannabinoides

Els efectes dels endocannabinoides són transduits, majoritàriament, per la seva unió als receptors de cannabinoides CB_1 i CB_2. Tanmateix, altres receptors com GPR55, els PPAR o els canals TRP també poden unir endocannabinoides i produir efectes molt concrets[91,176]. Els receptors de cannabinoides s'expressen, principalment, en les neurones del Sistema Nerviós Central i Perifèric, tot i que també es poden expressar en la melsa, les amígdales i en cèl·lules immunitàries com limfòcits, macròfags i micròglia[176]. L'activació del CB_1R i del CB_2R es troba involucrada en diferents efectes fisiològics cel·lulars, com per exemple la transcripció gènica, la motilitat cel·lular o la funció sinàptica[176]. En aquest sentit, els polimorfismes en els gens dels receptors de cannabinoides estan associats amb malalties com l'esquizofrènia[177]. Ambdós receptors són receptors acoblats a proteïna Gi. La seva activació condueix a la inhibició de l'adenilat ciclasa i conseqüentment a la mobilització del calci intracel·lular. En paral·lel, també activen la via de les MAPK[176].

1.3.4.1 CB_1R

El receptor CB_1 va ser descobert l'any 1990 en mostres de còrtex de rata[178]. En humans, el receptor es troba codificat pel gen CNR1, situat en el cromosoma 6, i en la posició 6q14-q15[179]. El CB_1R està format per 472 aminoàcids i manté una alta homologia entre humans i rosegadors, amb una identitat de seqüència del 97-99% entre espècies[180]. El receptor CB_1 és el GPCR més expressat a nivell de Sistema Nerviós Central, concretament, les àrees on més s'expressa són l'escorça, els ganglis basals, l'hipocamp i el cerebel[181]. La majoria dels receptors CB_1 es troben expressats als axons terminals i als segments preterminals dels axons, però també s'ha detectat en els terminals postsinàptics[91,182].

En escorça i hipocamp el CB_1R s'expressa, principalment, en neurones glutamatèrgiques, mentre que a nivell d'estriat dorsal i ventral, és més abundant en les neurones espinoses mitjanes[183]. El fet que CB_1R s'expressi en els ganglis basals indica que el Sistema Cannabinoide podria tenir un rol important en la regulació de les funcions motores, de fet hi ha estudis que demostren una clara alteració de l'expressió i funcionalitat del CB_1R en malalties neurodegeneratives com l'Alzheimer, el Parkinson o el Huntington[184,185].En el cas del cerebel, el CB_1R s'expressa en fibres paral·leles i trepadores[186].

A part de la seva expressió en el Sistema Nerviós Central, el receptor CB_1 també es localitza en els teixits endocrins, el Sistema Cardiovascular, el Sistema Gastrointestinal, regulant la ingesta i el metabolisme de lípids i sucres[181] i el Sistema Reproductor[181,187]. A nivells inferiors que el CB_2R, el CB_1R també es troba expressats en teixits immunitaris com per exemple la melsa o les amígdales i en cèl·lules com els limfòcits, els macròfags, els monòcits i els neutròfils. Interessantment, el CB_1R també apareix en poblacions de neurones en estat embrionari, suggerint un rol important d'aquesta proteïna en el desenvolupament neuronal[188].

Quan el CB_1R s'uneix al seu lligand endogen, s'acobla a la subunitat $G\alpha_i$. Com a conseqüència, l'activació del receptor provoca la inhibició de l'adenilat ciclasa, produint una disminució dels nivells d'AMPc intracel·lulars[91]. Paral·lelament, també es produeix l'activació de la via de les MAPK i la inhibició de canals de calci regulats per voltatge[188]. Tot i que la subunitat $G\alpha_i$ és la proteïna d'unió per defecte a CB_1R, la interacció entre el receptor de dopamina D_2 i el receptor CB_1 resulta en l'acoblament del receptor cannabinoide a $G\alpha_s$[71]. Aquest canvi d'acoblament a proteïna G ofereix una àmplia diversitat funcional a les cèl·lules controlada per l'oligomerització de GPCR. Un cop produït l'efecte, el receptor pateix un procés de dessensibilització a través de l'acció de les clatrines i la proteïna β-arrestina.

L'alliberació d'endocannabinoides o el tractament amb fitocannabinoides a nivell del SNC inclou efectes terapèutics com l'analgèsia, l'atenuació de les nàusees en malalts de càncer en quimioteràpia o l'alleujament dels espasmes musculars en malalts d'esclerosi múltiple[176]. També hi ha estudis que relacionen la manca d'ingesta i canvis hormonals amb la deleció del CB_1R[189]. En concret, el model de ratolí C57BL/6J KO pel receptor CB_1 presenta un gran nombre de fenotips com per exemple hipoactivitat, hipoalgèsia supraespinal i en general una elevada mortalitat[190]. A dia d'avui, tots els models KO per a CB_1R demostren una pèrdua total dels comportaments induïts per agonistes cannabinoides com la bradicàrdia, la hipotèrmia i la hipolocomoció, confirmant així el rol d'aquest receptor en aquests fenotips. A més, aquests ratolins mostren una resposta reduïda a opiacis, suggerint un paper important del CB_1R en comportaments addictius[191].

1.3.4.2 CB_2R

El receptor de cannabinoides CB_2 va ser descobert al 1993[192]. En humans, aquest receptor està codificat pel gen CNR2, que es troba en el cromosoma 1, en la posició 1p36.11. La seva traducció resulta en la síntesi d'una proteïna de 360 aminoàcids[92]. Ambdós receptors cannabinoides, el CB_1R i el CB_2R, presenten una homologia de seqüència del 44% i un 68% d'identitat en els seus centres d'unió a lligand[92]. Tot i que en un principi es pensava que únicament el CB_1R s'expressava a nivell de Sistema Nerviós Central, estudis recents han demostrat que el CB_2R també ho fa, principalment a nivell de la micròglia, sent una prometedora diana terapèutica per malalties del SNC[193]. No obstant això, la seva expressió és majoritàriament en teixits perifèrics com el Sistema Endocrí, el múscul, el cor, els ronyons, el fetge, el pàncrees i el tracte gastrointestinal[194], però sobretot en el Sistema Immunitari[91]. També s'ha detectat en precursors d'adipòcits i en adipòcits madurs[195], en cèl·lules pulmonars endotelials i en osteoblasts i osteoclasts, modulant la formació d'òs[196]. Altres estudis indiquen l'expressió de CB_2R en queratinòcits, on es produeixen beta-endorfines en resposta al tractament amb agonistes de CB_2R[197]. A més, s'ha vist que el CB_2R podria tenir un rol important en l'espermogènia, ja que els endocannabinoides afavoreix la remodelació de les espermàtides per formar espermatozous. La inactivació del CB_1R produeix una condensació de la cromatina ineficient i dany en cèl·lules germinals[198]. Finalment, també s'ha vist expressió del receptor CB_2 en la medul·la espinal i les neurones perifèriques[194].

En el SNC, el CB_2R s'expressa en l'escorça, l'estriat, l'amígdala, el tàlem, l'hipotàlem, el bulb olfactori, el tronc cerebral i la glàndula pineal[194]; principalment en cèl·lules glials però també en neurones[199]. No obstant això, la major expressió de CB_2R s'ha detectat en les cèl·lules de Purkinje en el cerebel i les cèl·lules piramidals de l'hipocamp[200]. Tot i que tant el CB_1R com el CB_2R s'expressen majoritàriament en les neurones presinàptiques, s'ha descrit que la seva expressió en les neurones postsinàptiques és més gran en el cas del receptor CB_2 que en el del CB_1R[201].

El CB_2R s'expressa en teixits immunitaris com el timus, la melsa o les amígdales en cèl·lules com els limfòcits, els macròfags, els monòcits i els neutròfils[202,194]. Cada vegada hi ha més evidències que el receptor CB_2 participa en processos de neuroinflamació o lesió, on els seus nivells d'expressió es veuen augmentats de manera significativa, sobretot en micròglia, on juga un paper neuroprotector[203]. Quan es dona una lesió i/o un episodi de neuroinflamació a nivell de SNC, s'activaran els receptors de CB_2 expressats en cèl·lules del Sistema Immunitari. Com a conseqüència es produiran una sèrie d'efectes immunosupressors, com la disminució de la producció de citocines proinflamatòries o la migració de cèl·lules activades amb la finalitat de limitar la inflamació i el dany tissular[204].

El receptor CB_2 s'acobla a la subunitat $G\alpha_i$ i com a conseqüència, l'activació del receptor provoca la inhibició de l'adenilat ciclasa, produint una disminució dels nivells d'AMPc intracel·lulars i l'activació de la via de les MAPK[91]. A diferència de CB_1R però, CB_2R sembla tenir una modulació més pobra tant dels canals de potassi com dels canals de calci regulats per voltatge[205]. En paral·lel, els agonistes del CB_2R també activen la ruta del PI3K/Akt i mTOR, associats amb la supervivència i proliferació cel·lular així com en la síntesi de la ceramida, la qual té capacitat proapoptòtica en cèl·lules tumorals[206]. Per aquest motiu, el receptor CB_2 es relaciona amb patologies cancerígenes.

El receptor CB_2 és una atractiva diana terapèutica, ja que la seva activació afecta un gran nombre de respostes tant inflamatòries com relacionades amb la neuroprotecció. Per un costat, en la malaltia d'Alzheimer s'ha detectat un increment de l'expressió del CB_2R en micròglia associada a les plaques amiloides[207]. Tant en esclerosi múltiple com en esclerosi lateral amiotròfica es pot veure com els nivells de CB_2R augmenten en la medul·la espinal[208]. També hi ha estudis que relacionen l'activació del CB_2 amb l'analgèsia i la resposta al dolor, tan agut com neuropàtic en diferents models animals[209].

Estudiant l'efecte immunomodulador dels cannabinoides es va demostrar que el Δ^9-THC era capaç d'inhibir l'activació de les cèl·lules T *helper,* però que en ratolins C57BL/6 sense expressió de CB_2R es perdia aquesta capacitat, indicant que aquest efecte és mediat pel receptor de cannabinoides tipus 2[210].

1.3.4.3 GPR55

Els cannabinoides poden produir els seus efectes a través d'altres receptors a part del CB_1 i el CB_2 (Figura 10). Alguns d'ells són GPCR, com per exemple el GPR55, i d'altres no, com el canal iònic TRPV1 o els receptors nuclears PPAR[211]. Existeix certa controvèrsia i discussió sobre si algun d'aquests receptors podria ser considerat un receptor cannabinoide, però sembla que els compostos cannabinoides no actuen en el seu centre ortostèric i que no uneixen endocannabinoides de manera específica[212]. La controvèrsia s'estén amb alguns autors que afirmen que el Δ^9-THC és capaç d'activar el GPR55, mentre que d'altres afirmen que no produeix cap efecte[211]. També apareixen dubtes a nivell de lligands, ja que certs estudis argumenten que els antagonistes de CB_1R i CB_2R, rimonabant i AM 630, respectivament, també realitzen aquest efecte sobre GPR55, mentre que altres afirmen que es comporten com a agonistes[213]. En conclusió, els lligands que tenen una afinitat acceptable per GPR55 són sintètics. És per aquest motiu que la hipòtesi que el GPR55 sigui un receptor cannabinoide està pràcticament descartada[213].

El GPR55 és un receptor orfe, un receptor pel qual encara no s'ha aconseguit identificar el seu lligand endogen. Estudis recents han suggerit el fosfolípid lisofosfatidilinositol (LPI) com a lligand endogen, però encara romanen dubtes, amb discrepàncies entre diferents estudis[211]. El GPR55 és un GPCR de classe A, que no va ser descobert fins al 1999[178]. El gen que codifica per aquest receptor es troba en el cromosoma 2 d'humans, en la posició 2q37 i expressa una proteïna de 319 aminoàcids[214]. Té una homologia relativament baixa tant amb el CB_1R com amb el CB_2R, 13,5% i 14,4% respectivament[215].

El GPR55 s'expressa tant a Sistema Nerviós Central com a la perifèria. En el cervell, el GPR55 s'expressa principalment en escorça i en estriat, tot i que en el nucli accumbens i l'hipotàlem també s'ha detectat, però en menor grau[216]. A nivell perifèric, el GPR55 es troba expressat principalment en cèl·lules del Sistema Immune, els testicles, el tracte gastrointestinal i el teixit adipós[216]. Però també en el Sistema Respiratori, el teixit endocrí i els ulls[214].

GPR55 pot acoblar-se a diferents proteïnes G, és el que es coneix com un receptor promiscu. Hi ha estudis que indiquen que el GPR55 pot unir-se a $G\alpha_{13}$, a $G\alpha_q$ o a $G\alpha_{12}$[217,215]. Així doncs, l'activació de GPR55 resulta en l'alliberació de Ca^{2+} i el conseqüent increment dels nivells de calci intracel·lular a través de la $G\alpha_q$[218]. A més, l'activació de GPR55 també promou la proliferació a través de l'activació de la via de les MAP cinases i activa factors nuclears com el NFAT (*nuclear factor of activated T cells*) i el NF-κβ (*nuclear factor κβ*)[216].

El GPR55 està involucrat en algunes condicions fisiopatològiques com el càncer, les alteracions vasculars, algunes patologies òssies o la descoordinació motora[219,220,221,222]. A causa d'aquest rol en la funció motora i a la seva elevada expressió en Sistema Nerviós Central, sobretot en estriat s'ha proposat GPR55 com a possible diana terapèutica per malalties com el Parkinson[222,223].

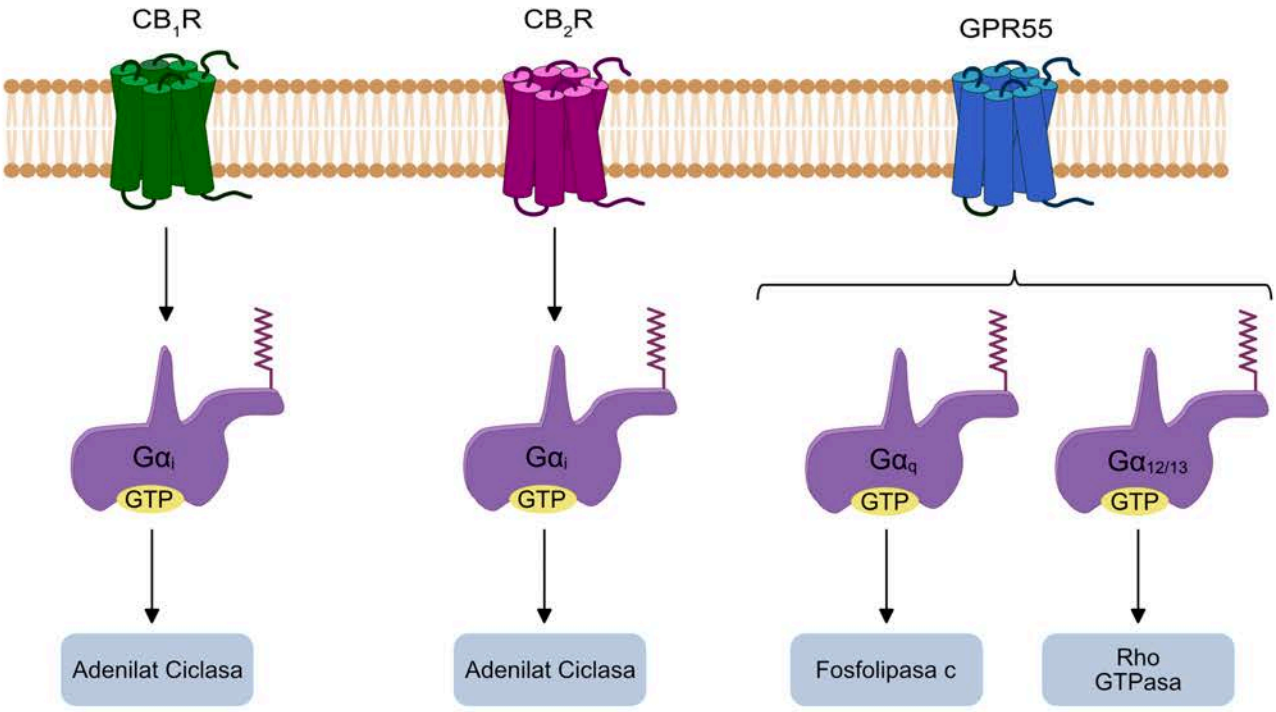

Figura 10: Senyalització dels receptors Cannabinoides i GPR55

1.4 Neuroinflamació i Neuroprotecció

1.4.1 Neuroinflamació

La inflamació és un procés clau del nostre organisme en resposta a infeccions o traumes a nivell cèl·lules i tissular. En un procés d'inflamació, l'objectiu final consisteix a eliminar patògens, afavorir la recuperació cel·lular i potenciar l'angiogènesis[224]. No obstant això, la inflamació a nivell de SNC es pot convertir en un factor de risc que pot conduir a l'aparició de patologies agudes i/o cròniques. És per aquest motiu que les neurones inverteixen molts esforços a combatre aquest risc, eliminant les restes cel·lulars i regulant la secreció de factors neurotròfics i citocines[225].

La neuroinflamació és una resposta en la que intervenen totes les cèl·lules del SNC. Factors ambientals, l'inici d'una malaltia neurodegenerativa o una infecció són condicions que poden activar la micròglia i iniciar un procés de neuroinflamació[225]. Aquests patrons moleculars associats a dany (DAMPs) són detectats per receptors cel·lulars específics com per exemple els *toll-like receptor* (TLR)[226]. L'activació d'aquests receptors activa diverses vies de transducció de senyal com per exemple la fosfoinositol 3-cinasa/proteïna cinasa B (PI3K/AKT), la via de les MAP cinases o la via mTOR. Eventualment, aquestes cascades finalitzaran amb l'activació de NF-κB. NF-κB regula la producció de citocines proinflamatòries com la IL-1β, el TNFα o la IL-6 i quimiocines així com l'activació d'enzims inductibles com l'iNOS, que converteix l'arginina en òxid nítric i COX-

2[227]. Com a resultat, s'inicien episodis de neuroinflamació, producció d'espècies reactives d'oxigen fins a arribar a la mort cel·lular.

La neuroinflamació es troba molt lligada amb la neurodegeneració, la pèrdua progressiva de funcionalitat i mort neuronal en el Sistema Nerviós Central, que causa deteriorament motor i cognitiu[228]. Entre les patologies neurodegeneratives, l'Alzheimer i el Parkinson són les que tenen una major incidència, però també s'inclouen l'ictus, malalties de la medul·la espinal, malalties prièniques, l'esclerosi múltiple o l'esclerosi lateral amiotròfica[229]. La majoria de les malalties neurodegeneratives es caracteritzen per induir l'activació de la resposta immune, activar la micròglia, causant neuroinflamació i posteriorment la mort neuronal.

1.4.2 Neuroprotecció

L'activació de la micròglia és un procés clau per la regulació de la neuroinflamació. Quan la micròglia entra en contacte amb una substància que reconeix com a forana o estranya, s'activa i aquesta activació condueix a la neuroinflamació[126]. Existeix tot un ventall d'estats d'activació de la micròglia. En un extrems trobem el fenotip M1, amb un clar caràcter proinflamatori, i en l'altre un estat alternatiu M2, on la micròglia es comporta com un potent agent antiinflamatori[230] (Figura 11). Aquest fenotip allibera citocines antiinflamatòries com la IL-10 i la IL-12[231]. Un dels marcadors més coneguts de la micròglia M2 és l'enzim Arginasa I, que converteix l'arginina en poliamines i prolines que ajuden a la regeneració cel·lular. Puntualitzar que aquest enzim competeix amb l'iNOS pel mateix substrat, de manera que quan Arginasa I s'expressa, iNOS no pot catalitzar la conversió de l'arginina i com a conseqüència disminueix la producció d'òxid nítric[232].

Des que es va descobrir la planta del cànnabis, s'han atribuït nombrosos efectes físics com psicològics al seu consum. Tant el CBD com el Δ^9-THC, els dos fitocannabinoides més abundants de la planta del cànnabis, han demostrat ser efectius en el tractament de malalties neurodegeneratives com el Parkinson, l'Alzheimer i l'esclerosi múltiple[233]. El fet que diferents compostos cannabinoides tinguin un efecte positiu en patologies que presenten estadis de neuroinflamació, sembla indicar un rol neuroprotector dels receptors de cannabinoides. Per un costat, s'ha descrit que el tractament amb Δ^9-THC fa disminuir la producció de citocines proinflamatòries en micròglia activada per LPS[234]. Per l'altre, el CBD i el CBG també han demostrat tenir efectes antiinflamatoris en models de malalties respiratòries[235], induir la inhibició de l'enzim inductible iNOS i del factor NF-κB i la reducció de les espècies reactives d'oxigen[233].

Les cèl·lules immunitàries com la micròglia expressen ambdós receptors cannabinoides, CB_1R i CB_2R. A més, els nivells d'expressió de CB_2R es veuen fortament incrementats quan aquesta s'activa[236]. Un exemple són els models de dolor crònic produïts per lesió de nervis perifèrics on es veia clarament incrementada l'expressió de CB_2R en la medul·la espinal, coincidint amb l'aparició de micròglia activada[237]. També s'ha descrit una relació directa on l'interferó gamma (IFN-γ), que és una citocina proinflamatòria produïda per les

cèl·lules TH1 T i NK, és capaç d'incrementar l'expressió tant del mRNA com de la proteïna CB_2R[238].

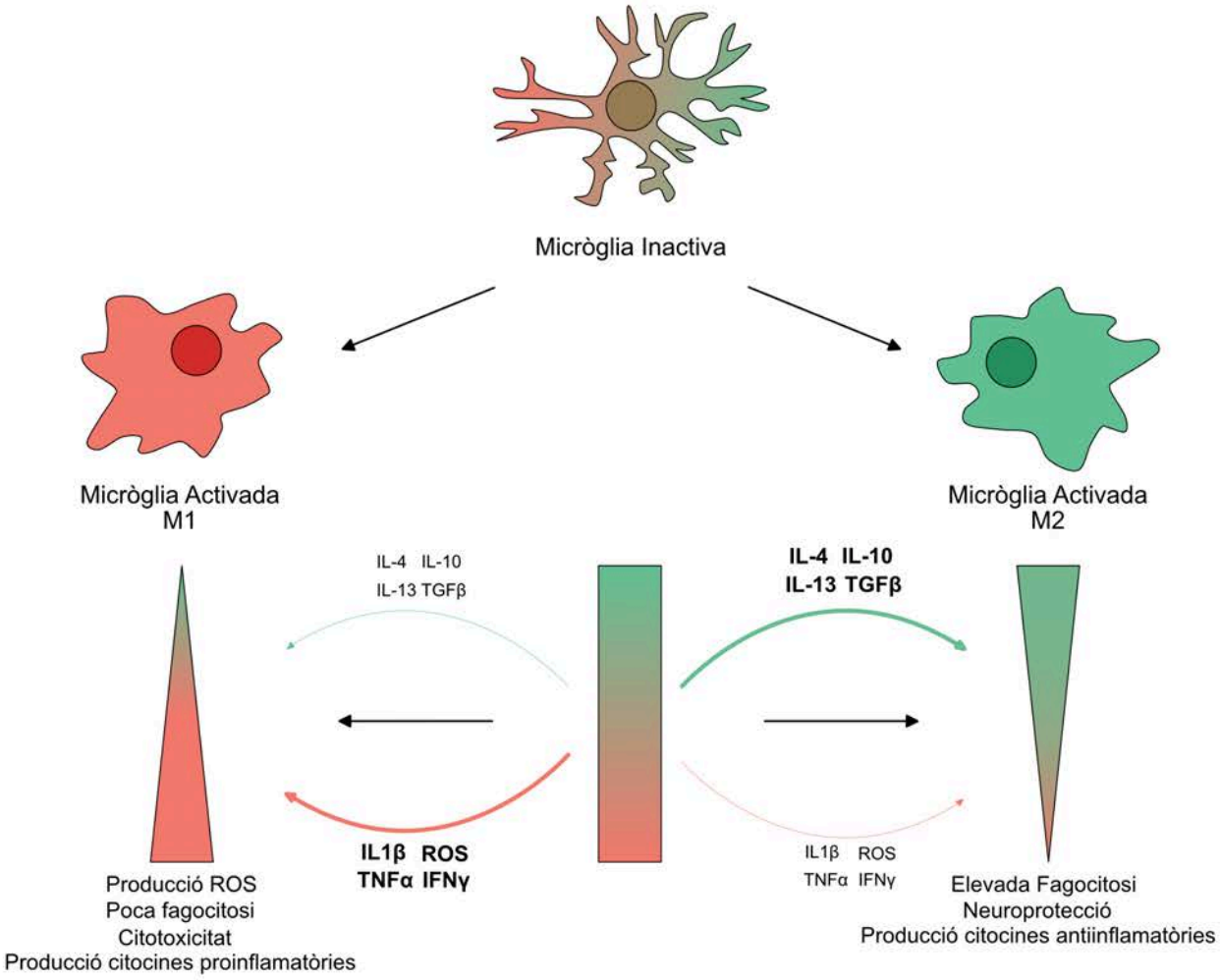

Figura 11: Activació i diferenciació de la micròglia en els fenotips M1 i M2.

En el cas dels astròcits, el grup més abundant de cèl·lules glials del SNC, s'han detectat diferents nivells d'expressió del receptor CB_1 segons el nivell d'activació de les cèl·lules, l'espècie, les zones del SNC i inclús l'edat dels cultius[236]. Pel que fa al CB_2R, els seus nivells d'expressió eren elevats en astròglia model d'esclerosi lateral amiotròfica. Aquest increment d'expressió estava concentrat en els astròcits activats[239]. Ambdós receptors cannabinoides, CB_1R i CB_2R, també s'expressen en oligodendròcits, que curiosament degeneren en la patologia de l'esclerosi múltiple i l'encefalomielitis autoimmune. En aquestes patologies també s'ha detectat una sobreexpressió de CB_2R podent ser utilitzada com a marcador específic per aquestes patologies[240]. Aquest conjunt de resultats sembla indicar que els receptors de cannabinoides, i sobretot el CB_2R, poden ser utilitzats com a una prometedora diana per potenciar els efectes neuroprotectors de les cèl·lules glials.

1.5 Implicació dels cannabinoides en la Patologia de l'Alzheimer

Entre les demències descrites, la malaltia de l'Alzheimer (AD) és la més comú[241]. Es pot definir com una malaltia neurodegenerativa lenta que es caracteritza per l'aparició de plaques amiloides i de cabdells neurofibril·lars en el lòbul temporal medial i estructures adrenocorticals, principalment, a conseqüència de l'acumulació del pèptid β-amiloide (Aβ)[242].

Un dels primers símptomes de la malaltia d'Alzheimer és la pèrdua de memòria a curt termini[243]. A mesura que la malaltia avança, el deteriorament cognitiu es fa evident amb un increment de la dificultat per entendre i desenvolupar el llenguatge tant escrit com oral (afàsia), per efectuar moviments voluntaris (apràxia) i una pèrdua de la percepció (agnòsia) i desorientació[244]. Gradualment, les funcions fisiològiques bàsiques es van perdent, fins a arribar eventualment a la mort. Totes aquestes afectacions cognitives fan que la malaltia d'Alzheimer no només afecti els propis individus, sinó que també ho faci a les seves famílies.

En l'actualitat, hi ha al voltant de 50 milions de persones que pateixen Alzheimer arreu del món, i es preveu que aquest nombre es dobli cada 5 anys, arribant als 152 milions el 2050[245]. La malaltia no només afecta la qualitat de vida de les persones, sinó també a l'economia mundial. El tractament de l'Alzheimer i les seves afectacions derivades, produeixen a la sanitat pública uns costs globals estimats de mil milions de dòlars anuals a nivell mundial[246]. En l'actualitat, no hi ha cura per a aquesta patologia, tan sols tractaments pal·liatius que milloren la simptomatologia.

Hi ha dos tipus de canvis neuropatològics en els malalts d'Alzheimer que es fan evidents a mesura que la malaltia progressa i són claus per al seu diagnòstic. Aquests es classifiquen en lesions positives causades per l'acumulació de plaques amiloides, cabdells neurofibril·lars i altres dipòsits en el cervell dels malalts; i en lesions negatives, causades per la pèrdua de neurones, com són l'atròfia i la mort cel·lular[247]. Tot i això, altres factors com la neuroinflamació, o l'estrès oxidatiu també poden afavorir aquesta neurodegeneració.

1.5.1 Plaques Amiloides

Les plaques amiloides o plaques senils, són dipòsits extracel·lulars de la proteïna β-amiloide (Aβ). La biosíntesi d'aquests dipòsits la realitzen els enzims proteolítics β-secretases, que actuen sobre una proteïna transmembrana anomenada proteïna precursora amiloide (APP)[248]. Aquests enzims tallen l'APP en fragments de diferent longitud: 43, 45, 46, 48, 49, i 51 aminoàcids. Aquests fragments seran processats fins a obtenir la seva forma final: Aβ40 i Aβ42[249].

Hi ha diferents tipus de monòmers Aβ, alguns són insolubles i poden agregar i acumular-se formant plaques amiloides i d'altres són solubles i poden desplaçar-se pel cervell. Els dipòsits d'Aβ tenen un nivell de neurotoxicitat molt important, i la seva acumulació en zones com l'hipocamp, l'amígdala o el còrtex pot induir

l'estimulació d'astròcits i micròglia, causant pèrdua de sinapsis i produint deteriorament cognitiu[250].

1.5.2 Cabdells Neurofibril·lars

Els cabdells neurofibril·lars són filaments anormalment hiperfosforilats de proteïna Tau. Aquesta proteïna es troba associada a l'estabilització de microtúbuls i el manteniment del citoesquelet, sobretot en neurones, i permet el creixement axonal[251]. La proteïna Tau en condicions normals és soluble, però quan es troba hiperfosforilada, pot interaccionar amb altres proteïnes Tau, formant agregats i acumulant-se en axons i dendrites produint la pèrdua de microtúbuls i proteïnes associades a tubulina[252] (Figura 12). La proteïna Tau hiperfosforilada és el principal component dels cabdells neurofibril·lars en el cervell d'un malalt d'Alzheimer, i precisament aquesta proteïna és un marcador dels diferents estadis morfològics d'aquests agregats. La formació dels cabdells s'inicia amb una primera fase on la proteïna Tau hiperfosforilada es va tornant insoluble i s'acumula formant filaments. La segona fase es caracteritza per l'agregació dels filaments de proteïna Tau i finalment, la tercera fase consisteix en l'aparició de cabdells neurofibril·lars, resultant en la mort neuronal i la resistència de la proteïna Tau a la proteòlisi[253].

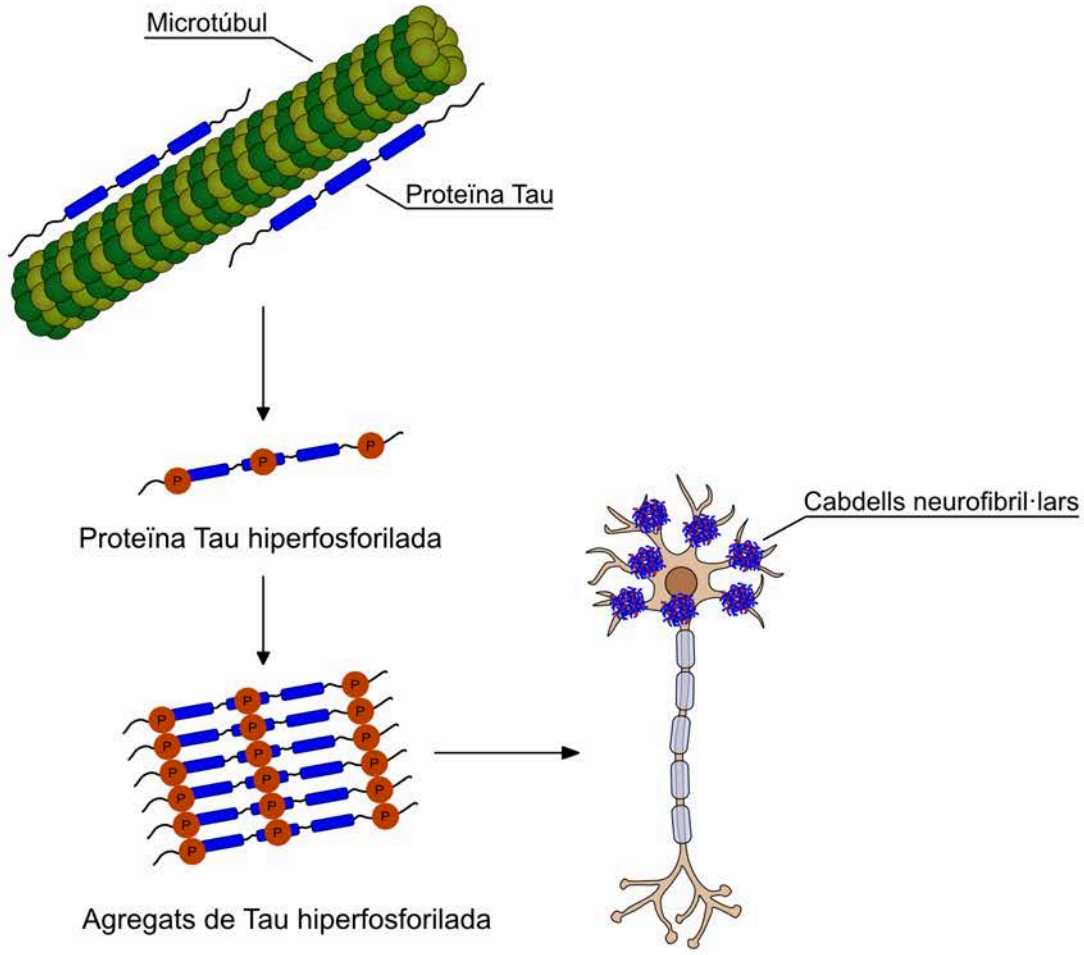

Figura 12: Mecanisme molecular de la formació d'agregats de proteïna Tau.

La malaltia d'Alzheimer (AD) és una patologia que cursa amb neurodegeneració, produint un deteriorament de la independència personal i l'habilitat per dur a terme activitats quotidianes[254]. L'Alzheimer és considerada una malaltia multifactorial, no se'n coneix la causa, però s'han proposat dues hipòtesis, la hipòtesis colinèrgica, i l'amiloide[241].

1.5.3 Hipòtesi Colinèrgica

Al voltant dels anys 70, es va començar a observar que pacients amb Alzheimer presentaven un dèficit colinèrgic presinàptic i neocortical, amb una important degeneració de neurones colinèrgiques, suggerint que la pèrdua de la funció colinèrgica a nivell del Sistema Nerviós Central contribuïa significativament al declivi cognitiu associat a avançats estadis d'Alzheimer amb alteracions cognitives i pèrdua de memòria[255]. El rol essencial de l'acetilcolina en les funcions cognitives va conduir a proposar la hipòtesi colinèrgica. L'acetilcolina és un neurotransmissor implicat en processos com l'aprenentatge, la memòria i la presa de decisions. Es sintetitza al citoplasma de les neurones colinèrgiques per l'enzim colina acetiltransferasa (ChAT) a partir de colina i acetil-coenzim A. Seguidament és transportada fins a les vesícules sinàptiques amb el transportador d'acetilcolina[256].

Estudis publicats demostren que la pèrdua de sinapsis en neurones colinèrgiques està estretament relacionada amb la neurotoxicitat induïda pel pèptid Aβ, degut a la interacció amb neurones colinèrgiques[257]. A més, s'ha vist que el pèptid Aβ provoca una reducció de l'alliberament d'acetilcolina. De fet, es va observar que l'ús d'antagonistes del receptor colinèrgic, com per exemple l'escopolamina, podia induir amnèsia[258]. Aquest efecte es veia contrarestat amb l'ús de compostos que activaven la formació d'acetilcolina.

1.5.4 Hipòtesi Amiloide

Durant dècades es va acceptar que la deposició anormal de pèptid β-amiloide, tenia una forta correlació amb la demència senil. Això va conduir a la creació de la hipòtesi amiloide. És curiós però, que la presència de plaques amiloides també es trobava en cervells sans a edats avançades[259]. Aquest fet va fer plantejar la pregunta de si realment les plaques amiloides eren una causa de la malaltia de l'Alzheimer. En els últims anys s'han proposat hipòtesis alternatives per les formes de l'Alzheimer no hereditari, però actualment, la hipòtesi amiloide segueix essent el mecanisme patològic més acceptat. Aquest model suggereix que la proteòlisi del pèptid amiloide Aβ, produït a partir de la proteïna APP per mitjà de β- i γ-secretases, decreixi amb l'edat o sota condicions patològiques. Aquesta pèrdua de la capacitat de degradar els pèptids Aβ, resulta en l'increment l'acumulació de les formes Aβ40 i Aβ42. Si el ratio Aβ42/Aβ40 augmenta, l'acumulació d'Aβ42 resulta en la inducció de la formació de fibres amiloides[260] (Figura 12). Això produeix neurotoxicitat, inducció de patologies relacionades amb Tau i com a conseqüència, mort cel·lular de neurones i neurodegeneració.

S'ha detectat que les malalties vasculars, certes infeccions, factors mediambientals, lesions cranials i mutacions dels gens com l'APP, el PSEN1 i el PSEN2, són factors de risc de l'Alzheimer, afectant tant al catabolisme com a l'anabolisme del pèptid Aβ i causant una ràpida acumulació de plaques amiloides i accelerant encara més la progressió de la neurodegeneració[261]. Actualment, només hi ha dues classes de fàrmacs aprovats per tractar la malaltia de l'Alzheimer: els inhibidors de l'enzim colinesterasa i els antagonistes del receptor N-metil-D-aspartat (NMDAR)[262]. Aquests compostos només són eficaços en el tractament dels símptomes de la patologia, però no la curen ni la prevenen. Avui en dia, la investigació es centra en la comprensió de la patologia de la malaltia d'Alzheimer, centrant l'atenció en els mecanismes moleculars, com ara el metabolisme anormal de les proteïnes Tau, l'acumulació i agregació del pèptid β-amiloide, la resposta inflamatòria i el dany colinèrgic, amb l'objectiu de desenvolupar tractaments que siguin capaços d'aturar o modificar el curs de la malaltia d'Alzheimer[263].

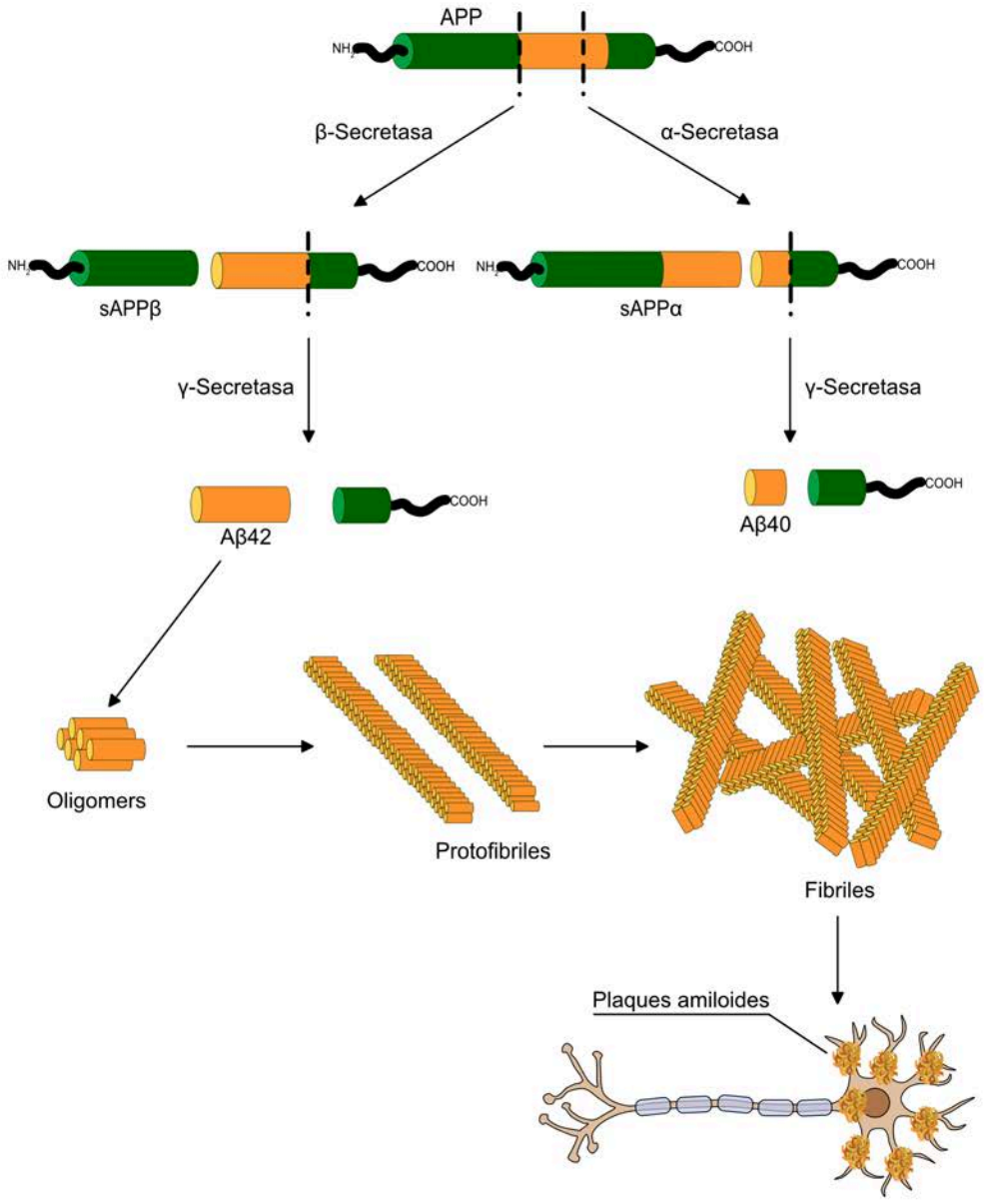

Figura 13: Mecanisme molecular de la formació d'agregats de pèptid β-amiloide a partir de la proteïna APP.

1.5.5 Receptor NMDA

El receptor N-metil-D-aspartat (NMDAR) és un canal iònic que pertany a la família dels receptors ionotròpics[264]. En concret, és un receptor de glutamat que juga un rol excitador en la neurotransmissió sinàptica a nivell de Sistema Nerviós Central. Aquest receptor es troba expressat en els diferents estadis de desenvolupament i està implicat en el desenvolupament cerebral i la plasticitat sinàptica, tenint un rol clau en l'aprenentatge i la memòria[265].

El glutamat és el principal neurotransmissor excitador en el SNC, i pot actuar a través de la unió a receptors inotròpics o metabotròpics[266]. S'han descrit diferents tipus de receptors ionotròpics, els més importants són els receptors AMPA, els receptors Kainat (KA) i els receptors NMDA. Mentre els receptors AMPA i els KA, poden activar-se directament amb glutamat, els receptors NMDA també necessiten de la unió de glicina per poder activar-se[267].

Els receptors NMDA són receptors heterotetramèrics, normalment compostos per dues subunitats capaces d'unir glicina anomenades GluN1 i dues subunitats capaces d'unir glutamat, que poden ser de tipus GluN2A, GluN2B, GluN2C o GluN2D[268]. En humans, l'estructura més comú en sinapsis de neurones d'hipocamp és el complex format per les subunitats GluN1–GluN2A–GluN2B[269]. Les subunitats GluN3A i GluN3B són capaces d'unir glicina i D-serina i també poden formar part del complex tetramèric, expressant-se en diferents zones del SNC, però el seu rol està menys descrit en comparació amb els complexos GluN1– GluN2[270].

Una altra diferència entre els receptors de NMDA i els receptors AMPA/KA consisteix en que el NMDAR en estat basal, es troba constitutivament bloquejat per unió al catió Mg^{2+}, de manera que el Ca^{2+} és incapaç d'entrar a l'interior cel·lular[271]. El receptor es troba bloquejat a nivells fisiològics de Mg^{2+} extracel·lular, però la presència de glutamat al medi, resulta en una ràpida activació dels receptors AMPA i KA que despolaritzen la neurona, reduint el potencial de membrana i eliminant el bloqueig depenent de voltatge produït per l'ió Mg^{2+}, ajudant a despolaritzar encara més la neurona[272].

Quan el receptor NMDA és activat, es produeix l'entrada de ions Ca^{2+} a l'interior cel·lular[264]. El conseqüent increment dels nivells de Ca^{2+} intracel·lular activa un gran ventall de mecanismes de senyalització en la neurona postsinàptica tant en condicions fisiològiques com patològiques[273], afectant directament la transmissió sinàptica, la morfologia neuronal i la plasticitat sinàptica[274] (Figura 14).

Una activació excessiva dels receptors NMDA (NRHyper) té un paper important en la fisiopatologia de malalties del SNC com l'epilèpsia, la isquèmia o diferents traumatismes[274]. En aquestes circumstàncies patològiques, els nivells extracel·lulars de glutamat indueixen una activació descontrolada dels receptors NMDA, donant lloc a un flux constant d'ions Ca^{2+} a l'interior de les neurones i produint en conseqüència la mort neuronal[275]. Aquest detriment de les neurones a causa de l'activació per glutamat es coneix com a excitotoxicitat[276]. Recentment, la hiperestimulació dels receptors AMPA/KA i la seva conseqüent excitotoxicitat, s'ha proposat com a possible explicació en la neurodegeneració

de malalties com l'ELA[277]. La hiperactivació dels receptors de NMDA també es troba associada amb problemes de memòria, dèficit en l'aprenentatge i fins i tot psicosi[274].

Per un altre costat, s'ha descrit la implicació dels receptors de NDMA en la fisiopatologia d'un gran nombre de malalties psiquiàtriques i neurològiques com l'esquizofrènia, l'epilèpsia o la malaltia d'Alzheimer[278]. És per aquest motiu, que durant les últimes dècades, els receptors NMDA han estat estudiats per tal de detallar tant el seu rol fisiològic com patològic en profunditat i poder utilitzar-los com a diana terapèutica en diferents malalties. Com hem descrit amb anterioritat, les set subunitats del receptor de NMDA poden combinar-se de diferents formes i expressar diferents tetràmers depenent de la regió o estadi de desenvolupament de l'individu. La complexitat del receptor es veu augmentada quan tots i cada un d'aquests possibles constructes presenten propietats funcionals i farmacològiques diferents[264].

A mesura que el cervell envelleix, els receptors NMDA perden part de la seva funcionalitat, contribuint a la pèrdua de memòria i dificultant la capacitat d'aprenentatge[279]. Contràriament, aquells individus que patiran Alzheimer (ja sigui tant per factors ambientals com per predisposició genètica) presentaran a més, altres patologies com amiloidogènesi o estrès oxidatiu que afavoriran la hiperactivació dels receptors de NDMA i la seva conseqüent excitotoxicitat[280]. Aquests individus malalts poden entrar en un estat d'hiperactivació persistent, que produirà episodis de mort cel·lular generalitzada, amb la seva conseqüent neurodegeneració, i deteriorament cognitiu[274].

En condicions cròniques d'hiperactivació dels receptors de NMDA, com en el cas de la malaltia d'Alzheimer, l'excitotoxicitat causada per l'alteració dels nivells de glutamat és una causa principal de la neurodegeneració[281]. És per aquest motiu que s'ha proposat que els antagonistes del receptor NMDA podrien ser beneficiosos en condicions d'excitotoxicitat, un clar exemple és el Memantine. Malauradament, els efectes secundaris d'aquests compostos inclouen psicosi, anestesia o inclús mort cel·lular, limitant la utilització d'aquests agents per tractar clínicament les condicions cròniques[282].

El Memantine, un antagonista parcial del receptor NMDA, bloqueja l'activació del receptor normalitzant el Sistema Glutamatèrgic i fent millorar els dèficits tant cognitius com de memòria[243]. Va ser aprovat per tractar l'Alzheimer el 2003 i ha resultat tenir potencial per alleugerir malalties com el Parkinson o la demència vascular. En vista dels bons resultats obtinguts, fent disminuir el deteriorament clínic de l'Alzheimer, s'estan investigant petites modificacions de la seva estructura per produir nous fàrmacs com agents terapèutics per noves malalties neurodegeneratives.

Alguns dels efectes secundaris provocats pels antagonistes d'alta afinitat del receptor NMDA són molt semblants als símptomes que exhibeixen els malalts d'esquizofrènia[283]. De fet, s'ha vist que la hipofuncionalitat del receptor NMDA està estretament relacionada amb l'esquizofrènia. En aquest sentit, la potenciació dels receptors NMDA podria ser beneficiosa per tractar aquestes

malalties cognitives, però l'ús d'agonistes de NMDAR com a agents terapèutics no s'ha estudiat en profunditat, a causa del potencial risc d'excitotoxicitat[264].

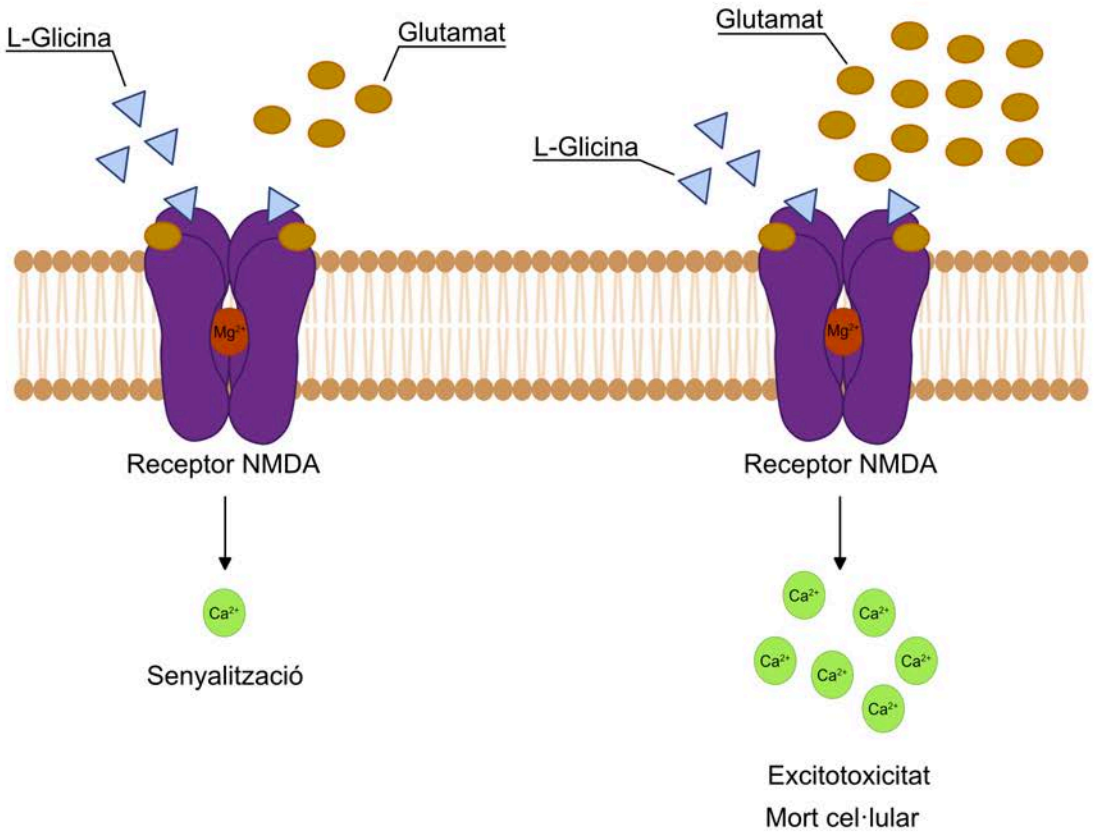

Figura 14: Esquema de l'activació del receptor N-metil-D-aspartat.

1.5.6 Els cannabinoides en la malaltia d'Alzheimer

El Sistema Cannabinoide té un paper molt important en el control de la memòria i les funcions cognitives, estant directament involucrat en la plasticitat neuronal i la resposta sinàptica. En aquest sentit, el consum abusiu de cànnabis, sobretot a edats primerenques, ha estat relacionat amb dificultats en l'aprenentatge i la memòria[284]. Totes aquestes dades han conduït a l'estudi de la regulació del Sistema Cannabinoide pel tractament de malalties neurodegeneratives com l'Alzheimer, esdevenint una estratègia molt prometedora[285].

Estudis recents han demostrat que l'activació tant del receptor CB_1 com del CB_2, ha resultat ser eficaç en la reducció dels dipòsits del pèptid β-amiloide i de proteïna Tau hiperfosforilada, posant de manifest el rol neuroprotector d'aquests receptors[285]. Concretament, s'ha demostrat que baixes dosis de Δ^9-THC produeixen efectes beneficiosos en la demència d'Alzheimer, induint la neurogènesi en l'hipocamp i reduint la toxicitat produïda pel pèptid β-amiloide[286]. Aquests estudis van ser realitzats inicialment en ratolins, però també s'han descrit efectes semblants en estudis tan preclínics com clínics en individus amb demència[285]. Actualment, la recerca de noves estratègies terapèutiques

centrades en els cannabinoides pretenen potenciar els efectes beneficiosos d'aquests compostos evitant l'efecte psicoactiu indesitjat. En aquest sentit, es centren en tres grans camps d'investigació: l'ús de derivats de fitocannabinoides sense efectes psicoactius, com el CBD, l'activació del receptor CB_2 i la modulació dels nivells d'endocannabinoides[287].

1.5.6.1 Tractament amb CBD

El CBD és el segon compost majoritari de la planta del cànnabis, després del Δ^9-THC. És conegut pel seu poder neuroprotector i per no presentar ni efectes psicoactius ni alteracions de l'estat emocional ni cognitiu en ser consumit[288]. S'ha descrit que el CBD pot activar receptors com el TRPV, el receptor de serotonina $5\text{-}HT_{1A}$[289,290].

Encara que l'ús del CBD per tractar l'Alzheimer es troba en estadis molt primerencs d'investigació, amb pocs estudis realitzats, cada vegada hi ha més evidències que suporten el potencial terapèutic del CBD per tractar la simptomatologia associada a l'Alzheimer[291]. En estudis preclínics en cèl·lules PC12 que havien estat tractades amb pèptid β-amiloide, el CBD va demostrar tenir propietats antiinflamatòries, neuroprotectores i antioxidants, reduint dràsticament la toxicitat produïda per Aβ[292]. També s'ha descrit que el CBD pot modular la funció de la micròglia *in vitro,* disminuït l'expressió de citocines proinflamatòries com la IL-6 i el TNF-α en ratolins injectats amb pèptid β-amiloide[293].

El tractament amb CBD també ha demostrat tenir la capacitat de disminuir l'expressió de gens relacionats amb la simptomatologia de l'Alzheimer, com les β- i γ-secretases o gens responsables de la fosforilació de Tau[294]. A més tenint en compte que la hiperfosforilació de la proteïna Tau té un paper protagonista en la malaltia de l'Alzheimer, s'ha demostrat que el CBD inhibeix tant la producció d'òxid nítric com l'agregació de Aβ produït per la fosforilació de Tau[295].

El tractament amb CBD per via oral, pot millorar la memòria i la fisiopatologia de l'Alzheimer en un model de ratolí transgènic per aquesta malaltia[296]. En aquesta línia, en un estudi recent, el consum de cànnabis va demostrar millorar significativament els efectes neuropsiquiàtrics en malalts d'Alzheimer[297].

1.5.6.2 Tractament mitjançant l'activació del CB_2R

Encara que el receptor CB_1 és el receptor de membrana més abundant del SNC, la seva activació està directament relacionada amb l'aparició d'efectes psicoactius indesitjats. Per aquest motiu, el receptor CB_2 és una millor alternativa com a diana terapèutica per a un fàrmac contra l'Alzheimer[285]. El potencial dels agonistes de CB_2R pel tractament de la malaltia d'Alzheimer es veu reflectit en el fet que l'expressió del receptor CB_2 s'incrementa significativament en cervells afectats per la malaltia d'Alzheimer[298]. El poder antiinflamatori dels agonistes de CB_2R ha estat àmpliament d'escrit en diferents models cel·lulars, tant en ratolins transgènics com en models *in vitro* de la patologia d'Alzheimer[299]. Recentment, l'agonista del CB_2R MDA7, va demostrar ser capaç d'afavorir l'eliminació del pèptid β-amiloide, millorant així, la resposta cognitiva[300].

Un altre efecte terapèutic del CB_2R en la patologia d'Alzheimer està relacionat amb la modulació de la hiperfosforilació de la proteïna Tau. De fet, l'administració de JWH-133, agonista sintètic de CB_2R, va resultar ser efectiu en reduir tant la fosforilació excessiva de Tau com l'acumulació d'Aβ en ratolins APP/PS1[301]. Així, en ratolins que sobreexpresaven la proteïna Tau humana es va veure una disminució dels nivells de cabdells neurofibril·lars en presència d'un tractament prolongat amb Sativex®[302].

A causa de la naturalesa multifactorial de la patologia d'Alzheimer, l'ús de fàrmacs multi diana capaços d'actuar sobre més d'una proteïna simultàniament, està resultant ser una molt bona estratègia per tractar aquesta malaltia. Recentment, un fàrmac híbrid que combinava els efectes agonistes de CB_2R amb un inhibidor de l'enzim colinesterasa (ChE), responsable de la degradació de l'acetilcolina, va ser sintetitzat i provat en assajos tant *in vitro* com *in vivo*[303]. Aquest híbrid va demostrar posseir un efecte neuroprotector contra l'estrès oxidatiu induït per glutamat, exhibint millores en la memòria a curt i llarg termini[303]. Un altre híbrid amb una estructura semblant, unint un potent agonista de CB_2R i un inhibidor de l'enzim butirilcolinesterasa va demostrar també, tenir un rol neuroprotector, afavorint l'activació de la micròglia cap a un fenotip neuroprotector M2[304].

1.5.6.3 Tractament mitjançant la modulació dels cannabinoides endògens

La modulació dels nivells dels endocannabinoides (l'anandamida i el 2-AG, principalment) mitjançant el bloqueig dels seus enzims de degradació és una estratègia molt prometedora per tractar l'Alzheimer[305]. La inhibició dels dos enzims principals de degradació de cannabinoides, el FAAH i el MAGL, incrementa els nivells d'endocannabinoides a l'espai sinàptic, induint una sobreestimulació dels receptors CB_1 i CB_2. A més, aquest augment dels nivells d'endocannabinoides no es troba associat amb els efectes secundaris que s'obtenen en presència d'altes dosis d'agonistes de CB_1R[306].

Un dels inhibidors del MAGL, el JZL184, va ser utilitzat en ratolins APdE9, un model de ratolí per la patologia de l'Alzheimer, resultant en una disminució de la resposta proinflamatòria de la micròglia i dels nivells d'Aβ i dels seus precursors[307]. A nivell molecular, la inhibició d'aquests enzims de degradació i el subseqüent increment d'endocannabinoides està correlacionat amb una disminució tant de l'acumulació d'Aβ com de l'expressió de β-secretases[308].

Els nivells d'expressió tant de l'anandamida i del 2-AG i com dels seus enzims de degradació, es veuen alterats amb l'edat, tant en condicions normals com en models de malalties neurodegeratives[309]. En humans, l'anàlisi *post-mortem* de teixit neuronal de pacients d'Alzheimer mostra nivells molt reduïts d'anandamida comparats amb els grups control[310]. Contràriament, cultius de la línia cel·lular de micròglia BV-2 tractats amb β-amiloide, presentaven alts nivells d'expressió de l'enzim FAAH, posant de manifest el rol del Sistema Cannabinoide en la neuroinflamació de la patologia d'Alzheimer[311].

1.6 Implicació dels cannabinoides en la patologia de l'ictus

L'ictus és un accident cerebrovascular causat per la falta de reg sanguini en un punt concret del SNC[312]. Aquest bloqueig en la irrigació cerebral evita que certes àrees del SNC rebin l'oxigen i els nutrients necessaris de la sang. Sense oxigen ni glucosa, les cèl·lules neuronals comencen a morir en pocs minuts.

Els símptomes de l'ictus poden variar des d'una lleugera debilitat fins a la paràlisi d'una part de la cara o del cos. Altres senyals que caracteritzen l'ictus són la pèrdua de visió, el mal de cap sobtat, els problemes en la parla com en la comprensió i la incapacitat motora d'algunes parts del cos[313]. L'ictus pot causar dany permanent o irreversible en el SNC, produint discapacitat o inclús la mort.

La patologia de l'ictus és la segona causa de mort i la tercera causa d'incapacitat arreu del món[314]. Des del 1990 fins al 2019, els casos d'ictus s'han vist incrementats en un 70%, mentre que els casos de mort per ictus han augmentat un 43%. Els casos de discapacitat després de patir un ictus també han incrementat en un 143% en aquest mateix període[314]. En els països en vies de desenvolupament aquest increment ha estat encara més marcat, amb un augment del 86% en els casos de mort per ictus.

S'han descrit dos tipus diferents d'ictus, el més comú és l'isquèmic, causat per la interrupció del reg sanguini a una determinada regió cerebral. Aquest tipus d'ictus és el majoritari i engloba el 85% del total de casos[315]. El 15% restant són els ictus de tipus hemorràgic, que són provocats pel trencament d'un vas sanguini en el SNC, conduint a la disminució del reg sanguini (Figura 15). Quan els símptomes tenen una durada inferior a una o dues hores, es considera que l'ictus és de tipus transitori (TIA)[316].

1.6.1 Ictus isquèmic

L'ictus isquèmic és el tipus d'ictus més comú, i es dona quan una artèria que irriga el SNC és bloquejada, produint la mort del teixit neuronal i desencadenant en deficiències neurològiques importants[317]. Hi ha quatre tipus d'ictus isquèmic: l'arterioesclerosi de vasos grans, l'arterioesclerosi de vasos petits, l'ictus cardioembòlic, i l'ictus criptogènic[315]. Tant l'arterioesclerosi de vasos grans com la de vasos petits, són provocades per una acumulació excessiva de greix, colesterol i altres components del reg sanguini, fent que els vasos s'endureixin i perdin capacitat de transport del flux sanguini, arribant a obstruir-se completament[318]. En el cas de l'ictus cardioembòlic, l'obstrucció de l'artèria cerebral és deguda a un coàgul provinent del cor, que es desenganxa i viatja pel torrent sanguini fins a quedar-se encallat en algun estretament dels vasos sanguinis[319]. En darrer lloc, l'ictus criptogènic és un ictus d'origen desconegut, un calaix de sastre on se situen tots aquells casos on després d'investigar les causes no es troba cap explicació possible i es considera impossible determinar-ne l'origen[320].

Els costos derivats de l'ictus isquèmic, igual que en el de tipus hemorràgic també són molt elevats. Es calcula que el cost per pacient durant els primers dos anys posteriors a l'accident són d'entre 37.000 i 39.000 euros[321]. El cost emocional tant per les famílies com pels pacients és devastador. Certament, la majoria de pacients que han patit un ictus, tenen més por de la discapacitat que produeix l'ictus que de la pròpia mort[317].

Sigui quin sigui el tipus d'ictus, el temps és sempre un factor crític. Es considera que per cada minut que passa després d'un ictus isquèmic de tipus arterioescleròtic sense tractar, moren aproximadament dos milions de neurones[315]. És per aquest motiu que juntament amb la ràpida detecció de l'ictus, la recuperació cognitiva i neurològica en malalts d'ictus és un tema prioritari en la societat que atreu l'atenció d'investigadors, del govern i de la indústria.

1.6.2 Ictus hemorràgic

L'ictus hemorràgic és una de les principals causes de mort a escala mundial. És el tipus d'ictus més agressiu i amb seqüeles més greus i persistents[322]. Hi ha dos tipus d'ictus hemorràgic: l'intracerebral (ICH), on el sagnat es dona en el parènquima cerebral, i el subaracnoide (SAH), on hi ha sagnat en l'espai comprés entre la capa interna i la capa mitja del teixit que recobreix l'encèfal[323].

El ICH és l'ictus hemorràgic més comú i engloba el 10% de tots els casos d'ictus[324]. La taxa de mortalitat és del 40% en el primer mes, del 54% en el primer any i del 70% en els 5 anys següents des que es pateix l'ictus. Malauradament, únicament entre el 12 i el 39% dels que sobreviuen aconsegueixen una independència funcional a llarg termini[325].

El tractament de la patologia de l'ictus i el cost de la incapacitació dels pacients que queden greument afectats generen una gran pèrdua a nivell econòmic, produint un gran impacte en l'economia mundial. El cost derivat de l'ictus a nivell global és de més de 721 bilions de dòlars (el 0,66% del PIB mundial)[326]. Un estudi que avaluava el cost econòmic durant els dos primers anys després d'un ictus tipus ICH va calcular un cost d'entre 40.000 i 50.000 euros per pacient, posant de manifest que la incidència de l'ICH es troba estretament relacionada amb alts costs individuals[321].

El tipus SAH correspon al 5% del total dels casos d'ictus i té una taxa de mortalitat d'entre el 25 i el 50% i una taxa de discapacitat del 50%, a causa de les conseqüències del sagnat o del trencament d'un aneurisma[327].

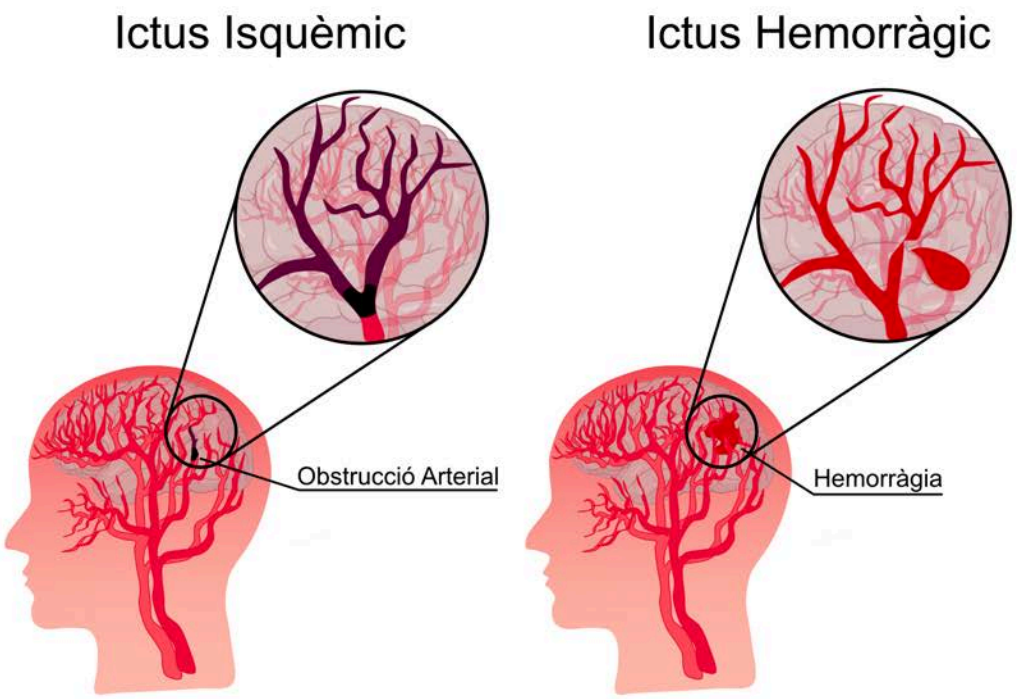

Figura 15: Esquema dels tipus d'ictus més comuns.

1.6.3 Els receptors de serotonina

Els receptors de serotonina (5-HTR) formen part de la família dels GPCR, exceptuant el receptor 3 que és un canal iònic. S'expressen principalment en el Sistema Nerviós Central, però també en el Perifèric[328]. El lligand endogen dels receptors 5-HT és la serotonina.

La serotonina és un neurotransmissor molt relacionat amb el control de les emocions, com per exemple la satisfacció, la felicitat i l'optimisme. Els nivells de serotonina es veuen disminuïts en la patologia de depressió, i la gran majoria de fàrmacs antidepressius són inhibidors selectius de la recaptació de serotonina (SSRI), actuant sobre els transportadors de serotonina (SERT), inhibint-los fent augmentar els nivells de serotonina a l'espai sinàptic.

En un inici, es va determinar l'existència d'únicament dos tipus de receptors de serotonina, que van ser anomenats com a D i M[329]. Des de llavors, la combinació d'aproximacions moleculars i farmacològiques han anat aportant noves dades fins a completar l'espectre actual dels receptors 5-HT. El consens actual indica l'existència de 7 tipus diferents de receptors de serotonina (5-HT_{1-7}) donant lloc a un total de 14 subtipus de receptors[330].

Tots els subtipus dels receptors de serotonina s'expressen al Sistema Nerviós Central i en teixits perifèrics, amb l'excepció dels receptors 5-HT_{1E}, 5-HT_{2C} i 5-HT6, els quals únicament es troben expressats fora del Sistema Nerviós Central. Alguns receptors de serotonina, entre ells el 5-HT_{2C}, el 5-HT_3, el 5-HT_4 i el 5-HT_7 tenen diferents isoformes, diferents proteïnes que comparteixen una mateixa

funció[330]. Aquesta gran diversitat de receptors només és comparable a la d'altres grans famílies de neurotransmisors com els receptors de glutamat[330].

En el genoma humà, els receptors de serotonina estan codificats per 17 gens, sintetitzant 12 receptors GPCR i 5 canals iònics[331]. La desregulació dels nivells d'expressió dels receptors de serotonina està associada a marcades alteracions del comportament[332]. A més, hi ha estudis que demostren la relació directa entre els polimorfismes dels gens dels receptors de serotonina i certes malalties psiquiàtriques[333]. Aquesta àrea d'investigació dels receptors 5-HT es troba en expansió, incorporant estudis epigenètics dels gens 5-HTR per intentar entendre millor com l'alteració natural d'aquests gens es troba relacionada amb la vulnerabilitat de l'individu a patir malalties del SNC[334].

La família de receptors 5-HT_1 inclou els receptors 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, i 5-HT_{1F}. Tots ells són GPCR que s'acoblen a proteïna Gi, inhibint l'adenilat ciclasa i com a conseqüència fent disminuir els nivells d'AMPc intracel·lulars[330]. La família de receptors 5-HT_5, inclou els receptors 5-HT_{5A} i 5-HT_{5b}, que també s'acoblen a proteïna Gi[335]. D'altra banda, la família de receptors 5-HT_2, que inclou els receptors 5-HT_{2A}, 5-HT_{2B} i 5-$HT_{2C,}$ s'acobla a la proteïna $G_{q/11}$, activant la fosfolipasa C, incrementant la formació d'inositol trifosfat i diacilglicerol i mobilitzant els nivells de calci intracel·lulars. La resta de receptors metabotròpics (5-HT_4, 5-HT_6 i 5-HT_7) són proteïnes excitadores, amb efectes mediats per la proteïna Gs, que activa l'adenilat ciclasa, incrementant els nivells d'AMPc intracel·lulars.

Les funcions del Sistema Serotoninèrgic són molt àmplies, amb una forta implicació en l'agregació de les plaquetes i la regulació de la motilitat gastrointestinal[336]. També modulen la pressió sanguínia i tenen un rol important en la patologia d'hipertensió pulmonar[337]. A nivell del SNC es troben implicats en un gran nombre de funcions comportamentals, modulant l'estat d'ànim, les emocions i la son, i en funcions fisiològiques com la temperatura corporal i l'emesis[338].

1.6.4 El receptor 5-HT_{1A}

El receptor 5-HT_{1A} és el subtipus de receptor de serotonina més estudiat i més ben caracteritzat i una molt bona diana terapèutica a causa de la seva implicació en patologies relacionades amb disfuncions de la neurotransmissió serotoninèrgica, com l'ansietat, la depressió o l'esquizofrènia[339]. A més, el receptor 5-HT_{1A} controla un gran nombre de cascades de senyalització com són l'activació i la inhibició de diferents enzims com la NO sintasa o la NADPH oxidasa, els canals de K^+ i Ca^{2+} o la via de les MAPK[340].

El receptor 5-HT_{1A} està àmpliament distribuït pel SNC amb un augment dels nivells d'expressió en les neurones de l'hipocamp, el còrtex prefrontal i certes zones de l'encèfal com el mesencèfal, el bulb raquidi i el pont[341] tant a nivell presinàptic com postsinàptic. La seva localització posa de manifest la seva rellevància en la neurotransmissió serotoninèrgica, on juga un paper clau. El receptor 5-HT_{1A} també s'expressa en astròcits i microglia, on està implicat en la

neuroprotecció contra l'estrès oxidatiu i juga un paper important en malalties neurodegeneratives com el Parkinson[342].

Els receptors serotoninèrgics presinàptics es classifiquen com a autoreceptors somatodendrítics i tenen la funció de mediar una inhibició per feedback negatiu del Sistema Serotoninèrgic, regulant l'alliberació de serotonina i modulant la síntesi de neurotransmissors i la seva alliberació al terminal sinàptic[343]. La sobreexpressió dels autoreceptors 5-HT_{1A} està implicada en la reducció de la neurotransmissió serotoninèrgica, que es troba associada amb la depressió i les tendències suïcides[344].

Els nivells de neurotransmissors com la serotonina o la dopamina es veuen alterats en un episodi d'isquèmia i poden contribuir a la fisiopatologia de l'ictus[345]. El paper que juga la serotonina en la patologia de l'ictus està encara en vies d'estudi, però hi ha evidencies que condueixen a pensar que la potenciació del seu efecte en l'hipocamp, resulta en la neuroprotecció contra el dany neuronal després d'un episodi d'isquèmia[345]. A més, diversos estudis han demostrat que els inhibidors selectius de la reabsorció de serotonina i els agonistes del propi receptor 5-HT_{1A} redueixen el dany cerebral i ajuden a la recuperació funcional després d'un ictus[346].

L'agonista selectiu del 5-HT_{1A}R, el Repinotan, va mostrar un gran poder neuroprotector en models animals que havien patit isquèmia. El Repinotan afavoreix la reducció de les zones infartades del cervell en episodis d'ictus, tant a causa de l'oclusió d'una artèria com davant d'un origen traumàtic[347]. D'altra banda, l'antagonista del 5-HT_{1A}R, WAY 100635 bloquejava aquests efectes, indicant que aquests efectes neuroprotectors eren induïts pel receptor 5-HT_{1A}. El mecanisme de neuroprotecció proposat es basava en la hiperpolarització neuronal induïda pel Repinotan mitjançant l'activació del GPCR[348]. Aquesta hiperpolarització provoca que algunes neurones glutamatèrgiques no s'activin, evitant així, la excitotoxicitat.

El receptor 5-HT_{1A} té un gran nombre de lligands coneguts, entre els quals es troba el 8-OH-DPAT, un dels agonistes totals més ben descrits i més utilitzats en la seva caracterització farmacològica[349]. Al principi, el 8-OH-DPAT es va considerar un agonista selectiu del receptor 5-HT_{1A}, però més endavant es va veure que també tenia afinitat pel 5-HT_7R[350]. Entre els lligands de 5-HT_{1A} trobem també el cannabidiol o CBD. Diversos estudis clínics indiquen que el principal component no psicoactiu del cànnabis, interacciona també amb el 5-HT_{1A}R, produint efectes analgèsics i ansiolítics[351]. Primerament, es va demostrar que el CBD és capaç de desplaçar l'agonista [3H]8-OH-DPAT del receptor 5-HT_{1A} humà de forma dependent en cultius de cèl·lules d'ovari de hàmster Xinès (CHO)[352]. Seguidament, en termes de transducció de senyal, es va demostrar que: El CBD fa incrementar la unió de [35S]-GTPγS al receptor acoblat a proteïna G de la mateixa manera que ho fan els agonistes de 5-HT_{1A} i que el CBD provoca la disminució dels nivells d'AMPc de manera molt similar a aquests agonistes[122].

1.6.5 Efecte dels cannabinoides en l'ictus

Actualment, les teràpies convencionals per tractar l'ictus es basen en tractaments de teràpia ocupacional i sobretot en fisioteràpia, per tal de millorar les seqüeles i la qualitat de vida[353]. Tot i això, aquests tractaments únicament produeixen canvis molt limitats en quant a la reorganització i la plasticitat neuronal. És per aquest motiu, que s'han dut a terme una gran quantitat d'estudis per desenvolupar nous mètodes que promoguin la neuroplasticitat i incrementin l'eficàcia en la rehabilitació de la patologia de l'ictus[354].

Amb aquesta finalitat, l'ús d'agents farmacològics com els SSRIs, entre ells la Fluoxatina, que milloren l'excitabilitat neuronal i promouen canvis en la plasticitat, està augmentant cada vegada més, demostrant que aquest tipus de fàrmacs millorant la rehabilitació motora en els malalts d'ictus[355]. La majoria de les estratègies terapèutiques relacionades amb els cannabinoides estan centrades en l'ús de derivats de fitocannabinoides sense efectes psicoactius com el CBD i l'activació tant del receptor CB_1 com del receptor CB_2.

1.6.5.1 Efecte del CBD en l'ictus

Cada vegada hi ha més estudis que demostren que el poder neuroprotector del CBD també juga un efecte important en la patologia de l'ictus. Un anàlisi de més de 34 estudis preclínics que examinaven l'efecte del CBD després d'un episodi d'ictus va concloure que el CBD reduïa significativament la mida de l'infart i millorava la recuperació funcional, produint els seus efectes tant a través de CB_1R, CB_2R com de $5\text{-}HT_{1A}R$[356,357].

El grup del Dr. Reza Bigdeli va demostrar que la infusió de CBD, durant 5 dies, al ventricle lateral mitjançant una cànula implantada quirúrgicament era capaç de reduir el dany neuronal, l'edema i la permeabilitat de la barrera hematoencefàlica en un model animal d'ictus isquèmic[358]. En la mateixa línia, en un model animal d'isquèmia cerebral el CBD va resultar ser efectiu en la reducció de la neurodegeneració en l'hipocamp, així com en la disminució de factors neurotròpics i la resposta glial proinflamatòria produïda per la inducció de l'episodi d'ictus[359].

Per un altre costat, s'ha descrit, recentment, que els efectes neuroprotectors del CBD post ictus podrien ser dirigits per la seva activitat antagonista sobre el receptor sigma-1[360]. Concretament, els antagonistes de sigma-1 inhibeixen l'activitat del NMDAR, indicant que el CBD podria exercir els seus efectes neuroprotectors contrarestant els efectes de l'excitotoxicitat per NMDA en el SNC.

Hi ha estudis que indiquen que la teràpia conjunta amb CBD i hipotèrmia en porcs nounats que han patit un episodi d'hipòxia, és capaç de reduir la inflamació, l'estrès oxidatiu i el dany cel·lular. L'efecte observat amb la teràpia combinatòria és significativament més potent que l'efecte d'ambdós tractaments de manera aïllada[361]. Resultats similars es van obtenir amb ratolins nounats, allargant l'interval d'actuació del tractament amb CBD, passant de tan sols 30 min, fins 18 hores després de l'episodi d'ictus[362].

El rol neuroprotector del CBD es troba cada vegada més acceptat en la comunitat científica, però calen més estudis per acabar de determinar el seu paper en la hipòxia isquèmica, tant en un context d'ictus en edat adulta com en ictus neonatal.

1.6.5.2 Efecte neuroprotector del CB_1R

Un estudi desenvolupat pel grup de la Dra. Sophie Parmentier-Batteur va observar que en models de ratolí KO pel receptor CB_1, que havien estat sotmesos a una isquèmia permanent o transitòria, s'evidenciaven infarts més grans, dèficits neurològics més greus i nivells d'excitotoxicitat per NMDAR més elevats respecte el model WT, demostrant que el Sistema Cannabinoide té un gran potencial en la teràpia contra l'ictus[363]. En la mateixa línia, en autòpsies de cervells humans que havien patit un ictus isquèmic es va detectar un increment en l'expressió de CB_1R en la zona de l'infart[364].

L'administració d'antagonistes del receptor CB_1 30 min abans d'induir un episodi d'isquèmia, va demostrar ser eficaç en reduir el dany cerebral en models de rata, indicant que el bloqueig del receptor CB_1 reverteix els efectes causats per l'ictus[365]. Aquests efectes dels antagonistes de CB_1R no només s'observen quan s'administren en forma de pretractament, sinó que l'administració de SR141716A després de la inducció d'un episodi d'ictus isquèmic en un model de ratolí va obtenir els mateixos resultats[366]. En la mateixa direcció, hi ha estudis que suggereixen que els agonistes de CB_1R també poden jugar un paper important en la neuroprotecció contra l'ictus, reduint el deteriorament causat per la isquèmia tant en neurones com astròcits, així com millorant la recuperació motora[367].

1.6.5.3 Efecte neuroprotector de CB_2R

De la mateixa manera que el CB_1R, el receptor CB_2 també ha demostrat ser una nova diana terapèutica a tenir en compte en el tractament de l'ictus. En un model animal d'ictus de rata, quan els agonistes del CB_2R s'utilitzaven com a pretractament, aquests eren capaços de suprimir la neurodegeneració associada a la isquèmia. No obstant això, aquest efecte es veia disminuït si s'aplicava entre 2-5 dies després de l'episodi d'ictus[368].

A més de les publicacions que indiquen que els agonistes del receptor CB_2 indueixen la neuroprotecció, hi ha d'altres estudis que proposen que els antagonistes de CB_2R poden fer l'efecte contrari. Per exemple, es va detectar que l'administració d'antagonistes de CB_2R en un model crònic d'ictus produïen una disminució de la migració de neuroblasts en zones amb dany neuronal amb un empitjorament tant de la capacitat sensorial com motora 28 dies després de l'infart en comparació amb un model control[369].

Un dels temes més discutits sobre el potencial terapèutic dels agonistes de CB_2R en la patologia de l'ictus és la durada dels seus efectes beneficiosos. S'ha vist que l'agonista del receptor CB_2, GW405833, deixava de mostrar efectes positius tant a nivell histològic com de comportament 15 dies després de l'episodi en un

model animal d'ictus isquèmic[370]. Això suggereix que la majoria dels efectes neuroprotectors associats als agonistes de CB_2R tenen un efecte primerenc i temporal, requerint d'una investigació més sòlida per assentar les propietats i la durada d'aquests compostos en la patologia de l'ictus.

El mecanisme exacte pel qual els agonistes de CB_2R indueixen un efecte neuroprotector està encara sent estudiat. Una possible explicació recau en la modulació de la resposta immune activada per l'ictus[371]. S'ha descrit un increment de l'expressió del receptor CB_2 tant les àrees amb lesions isquèmiques del còrtex cerebral com en la micròglia reclutada al voltant de les zones infartades[372]. Aquests resultats suggereixen que el CB_2R regula tant la migració com l'activació de la micròglia, així com la resposta inflamatòria post ictus.

A més, hi ha estudis que demostren que la manca d'activitat del CB_2R també podria promoure la inflamació. Estudis en ratolins KO pel receptor CB_2 tenien nivells més elevats de factors proinflamatoris com el factor induïble per hipòxia (HIF-1A) o TIM-3 en micròglia infiltrada[373]. Actualment s'ha avançat molt en la recerca al voltant del receptor CB2 en l'ictus, però encara queden moltes incògnites per descriure i compostos per analitzar per arribar a detallar el mecanisme d'acció i possibles aplicacions terapèutiques.

1.7 Implicació dels cannabinoides en la Patologia de la Obesitat

L'obesitat es defineix com l'acumulació anormal o excessiva de greix o teixit adipós que impedeix una bona qualitat de vida[374]. L'obesitat està associada amb el desenvolupament de malalties cardiovasculars, diabetis mellitus i hipertensió així com amb alguns tipus de càncers, malalties musculoesquelètiques i una salut mental pobre, tenint efectes negatius en la qualitat de vida i en la productivitat laboral, a més d'elevats costos mèdics[375]. L'obesitat és una malaltia multifactorial molt complexa. És la segona causa de mortalitat evitable més comú per darrere del consum de tabac[374]. La prevalença de l'obesitat s'ha duplicat arreu del món des de l'any 1980, arribant a l'extrem on es calcula que aproximadament un terç de la població mundial es considera obesa[376].

L'obesitat és el resultat d'un balanç positiu crònic d'energia, és a dir, quan l'energia que s'obté és major a la que es consumeix. L'excés d'energia es converteix en triglicèrids, que s'acumulen en el teixit adipós, expandint-se, incrementant els nivells greix i produint un augment del pes corporal[377]. Tot i això, l'obesitat no és provocada, únicament, per una mala alimentació, també pot derivar de problemes hormonals o inclús de factors genètics[378].

Estudis sobre la tendència de l'obesitat indiquen que la taxa d'obesitat ha incrementat tant en adults com en nens de totes les edats i en ambdós sexes, independentment de l'ètnia, la localització geogràfica o l'estatus socioeconòmic[376]. En països en vies de desenvolupament, l'obesitat és més prevalent en adults de mitjana edat, sobretot dones, mentre que en països desenvolupats afecta ambdós sexes de totes les edats, indistintament[379].

L'índex de massa corporal (IMC) s'utilitza normalment per definir el sobrepès i l'obesitat en estudis epidemiològics. L'índex de massa corporal es calcula dividint el pes corporal en Kg entre el quadrat de l'alçada en metres. L'OMS defineix un rang normal d'IMC d'entre 18,5 i 24,9 kg/m^2. Un IMC igual o més gran a 25 kg/m^2 es considera sobrepès, un IMC més gran de 30 kg/m^2 indica obesitat, mentre que un IMC superior a 40 kg/m^2 es defineix com a obesitat severa[376].

L'obesitat no únicament comporta costs en la salut de les persones que la pateixen, també comporta uns costs importants al sistema sanitari. A Espanya els costs mèdics associats amb l'obesitat l'any 2016 van ser de 1900 milions, i es calcula que al 2030 seran de 3000 milions[380]. A Europa, els costs tant directes com indirectes atribuïbles al tractament de l'obesitat van ser el 4% del total dels costos sanitaris a l'any 2020[381].

1.7.1 L'Obesitat Infantil

L'obesitat infantil és un problema en quant a la salut pública arreu del món. Es calcula que aproximadament, un de cada 3 nens pateix obesitat o sobrepès[382]. L'increment de la prevalença de l'obesitat infantil està associada amb l'aparició de malalties abans considerades "d'adults", com per exemple diabetis tipus 2, hipertensió o apnea del son i que actualment també se'n detecten casos en infants.

La globalització dels sistemes *fast-food*, que produeixen menjar més econòmic però també més processat i promouen un hiperconsum d'aliments rics en energia però pobres en nutrients, han estat identificats com un dels principals responsables d'aquesta epidèmia d'obesitat, tant en infants com en adults[379]. La reducció de l'activitat física, ja sigui per un increment en el sedentarisme o per una modernització del nostre estil de vida, també hi juga un paper molt important. De fet, la majoria d'infants obesos no tenen una patologia endocrina o una predisposició genètica[377].

La prevalença de l'obesitat ha incrementat dràsticament en infants i adolescents. Un estudi de 2019 indica que a Europa, en infants d'entre 6 i 11 anys, la incidència de l'obesitat s'ha duplicat des de 1950[383]. Hi ha estudis que indiquen una relació directa entre malalties mentals en edat adulta i obesitat infantil[384]. Malalties com la depressió, l'ansietat o la hiperactivitat, poden tenir una forta predisposició en adults que de petits van patir obesitat.

Un alt percentatge dels infants que pateixen obesitat o sobrepès, ho segueixen patint en edat adulta. Com més gran és l'infant amb obesitat, més persistent serà l'obesitat en l'edat adulta[385]. D'aquesta manera, la majoria d'adolescents amb obesitat ho seguiran sent en edat adulta. En un estudi realitzat amb 1333 individus dels Estats Units, es va observar que el 71% dels adolescents amb obesitat severa van seguir patint-la en edat adulta, mentre que en casos de sobrepès o obesitat no severa, únicament el 8%[386].

El problema de l'obesitat infantil pot ser tractat amb una dieta adequada i amb un increment significatiu de l'activitat física, però no és l'única solució. El que els infants aprenen a casa sobre menjar sa i fer exercici, així com prendre unes bones decisions nutricionals, és el que farà que a poc a poc vagi decreixent el problema de l'obesitat infantil i, eventualment, es redueixin els efectes consecutius en l'obesitat adulta. L'obesitat parental també juga un paper important: els infants amb pares que pateixen obesitat tenen més del doble de possibilitats de patir obesitat[387]. És per aquest motiu que una bona educació alimentària a casa és clau per mantenir un estil de vida saludable i evitar la majoria dels problemes relacionats amb l'obesitat[388].

1.7.2 La Grelina

La grelina és una hormona peptídica de 28 aminoàcids, coneguda també com el lligand endogen del receptor secretagog de l'hormona de creixement (GHS-R1a)[389]. La grelina es troba codificada pel gen "Preproghrelin", que a la vegada codifica per un altre pèptid de 23 aminoàcids[390]. Perquè la grelina es trobi en estat actiu necessita de l'acilació d'una cadena alifàtica, normalment de 8 carbonis, tot i que també ho pot ser de 10, a la serina en posició 3[391]. Aquesta reacció la du a terme l'enzim grelina O-acil-transferasa (GOAT)[392].

Els lípids que la GOAT utilitza per a l'activació de la grelina són majoritàriament obtinguts de la ingesta diària de greixos, aprofitant que la majoria de cèl·lules productores de grelina es troben a l'estómac, tenint contacte directe amb els lípids ingerits[393]. Concretament, el substrat preferit de la GOAT són els àcids grassos provinents de les cadenes alifàtiques dels triglicèrids de la dieta, que poden ser absorbits i entrar a la circulació directament, sense ser digerits per les lipases o els àcids biliars. Tot i això, també hi ha una proporció de lípids que són sintetitzats *de novo* per a que l'enzim GOAT pugui usar-los per acilar la grelina[394]. Estudis de mutacions sobre la regió de reconeixement de la GOAT van demostrar que la glicina 1, la serina 3 i la fenilalanina 4 són aminoàcids crítics per una bona acilació de la grelina[395].

En ser descoberta, es va observar que la grelina, a part de tenir la capacitat d'estimular la secreció de l'hormona del creixement des de la glàndula pituïtària, també tenia una funció inductora de la sensació de gana, regulant, així, la ingesta d'aliments i l'obtenció d'energia[389]. Consistentment amb aquest rol, la grelina és produïda a les glàndules gàstriques en condicions de dejú i s'allibera al torrent sanguini, mentre que després d'un àpat o en condicions d'excedent d'energia els nivells de grelina decreixen, regulant la sensació de gana i l'homeòstasi energètica i modulant el metabolisme mitjançant l'activació de circuits orexigènics[396,397].

Cada vegada hi ha més evidències que la grelina té un ventall de participació més ampli en les funcions biològiques, fent que la visió tradicional de la grelina com a "hormona de la gana" estigui quedant obsoleta. Tot i ser produïda majoritàriament a l'estómac, les seves accions no es limiten, únicament, a l'aparell gastrointestinal. Entre les seves implicacions, tant a nivell del SNC com a la perifèria, s'ha descrit la regulació de la motilitat gastrointestinal, de la

secreció d'àcids gàstrics, de la son, del sistema de recompensa, de la vasodilatació, de la contracció cardíaca i de la supressió de la termogènesi d'adipòcits marrons[398,399] (Figura 16).

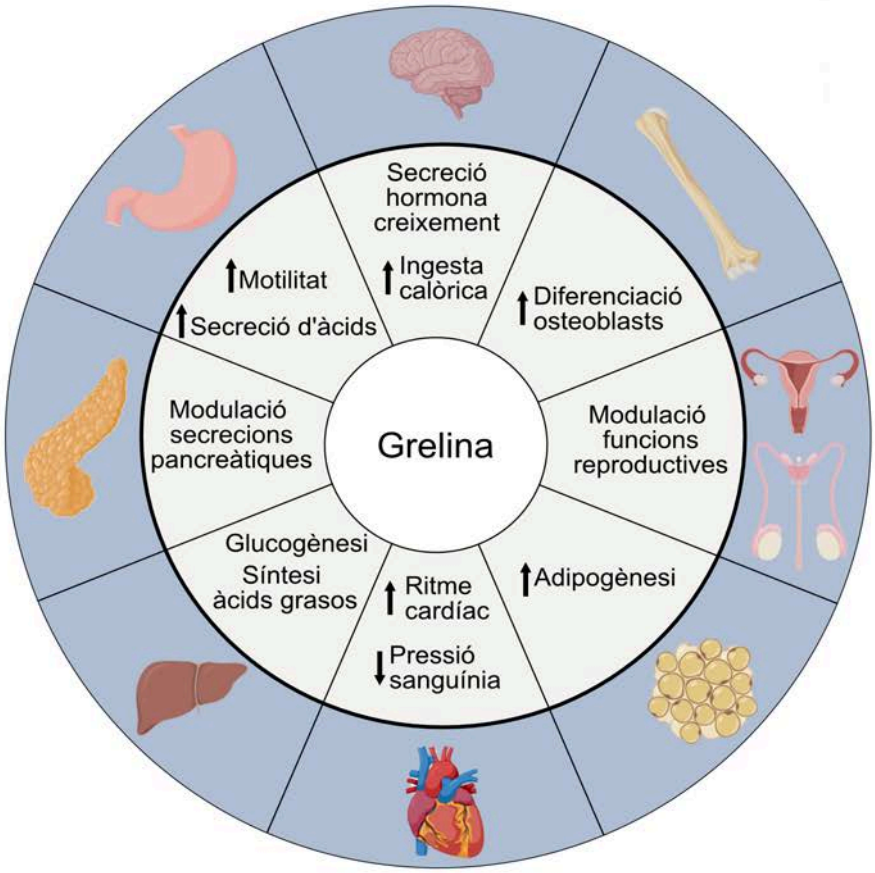

Figura 16: Esquema de les diferents accions que du a terme la grelina en diferents teixits del nostre organisme.

1.7.3 El receptor GHS-R1a

El receptor de grelina és un receptor acoblat a proteïna G, àmpliament expressat en el Sistema Nerviós Central, sobretot en l'hipotàlem, el tàlem, el còrtex i l'hipocamp[400]. El receptor GHS-R1a també s'expressa en teixit perifèric, com per exemple, l'aparell gastrointestinal, on s'ha vist expressió del receptor a l'estómac i també a l'intestí prim[401]. Mitjançant assaigs d'immunohistoquímica, es va detectar expressió del receptor en els ovaris i els testicles, així com en els òrgans endocrins com per exemple les glàndules adrenals, el pàncrees, les tiroides i sobretot, la glàndula pituïtària[402,403].

El gen del receptor de grelina codifica per dos mRNA diferents, donant lloc a la variant 1a i la variant 1b, produïdes per *splicing* alternatiu[404]. La variant 1a està codificada pels exons 1 i 2, produint una proteïna funcional amb 7 dominis transmembrana i alta afinitat per la unió de la grelina, mentre que la variant 1b únicament està codificada per l'exó 1, donant lloc a una proteïna truncada de 5 dominis transmembrana que és incapaç d'unir grelina[405]. Aquesta variant

s'anomena GHS-R1b i va considerar-se inactiva durant molt de temps, però recentment s'ha vist que té un rol modulador molt important sobre el receptor GHS-R1a. Tant és així, que a nivells d'expressió baixos afavoreix el reclutament del GHS-R1a a nivell de membrana plasmàtica, mentre que a nivells d'expressió elevats redueix l'activitat del GHS-R1a de manera molt significativa. Ambdós efectes són provocats per la interacció directa entre els dos receptors de grelina[406].

La via principal de senyalització del receptor GHS-R1a és a través de l'acoblament a la proteïna Gq, encara que també pot acoblar-se a les proteïnes Gi/o i G13[407]. Durant molts anys el receptor de grelina ha estat investigat com a diana terapèutica per tractar l'obesitat i altres malalties metabòliques. Concretament, s'ha detectat que ratolins KO pel receptor de grelina tenen una menor eficiència alimentària (calculada com a pes guanyat per quantitat de menjar ingerit) comparat amb un ratolí WT[408]. Tot això indica que un ratolí KO acumula menys energia comparat amb un ratolí control quan es troben consumint la mateixa quantitat de calories. En conseqüència, aquests ratolins tenen un fenotip més prim que els WT[409].

Paradoxalment a aquestes observacions, quan els ratolins KO pel receptor de grelina són alimentats amb una dieta alta en greixos, pateixen un canvi de preferència en quant a la font d'energia, utilitzant els greixos com a substrats metabòlics i reduint dràsticament la seva activitat locomotora, sobretot durant la nit[410]. També s'ha observat que la deleció del receptor de grelina produeix nivells de glucosa en sang més baixos, tant en ratolins alimentats amb una dieta alta en greixos com amb una dieta estàndard[411]. No obstant això, els nivells d'insulina en ratolins KO pel receptor GHS-R1a no es veuen incrementats, indicant que la deleció del receptor de grelina produeix una major sensibilitat a la insulina comparat amb un ratolí WT[412]. A més dels efectes sobre el pes corporal, s'ha vist que els ratolins KO pel receptor de grelina tenen una disminució del ritme respiratori i un increment de la taxa metabòlica basal[413]. Aquest recull de dades indica que el receptor de grelina té un rol en el metabolisme energètic més complex que el d'únicament regular la sensació de la gana.

Tant la grelina com el receptor GHS-R1a han estat estudiats pel tractament de malalties metabòliques com l'obesitat o altres alteracions del pes corporal a causa del seu rol en la regulació energètica, la ingesta calòrica i l'adipogènesi. Com que l'activació dels receptors GHS-R1a comporta l'activació dels circuits orexigènics, s'han investigat diverses estratègies per intentar bloquejar la senyalització del receptor de grelina i dissenyar un tractament per malalties com l'obesitat. En aquesta línia s'han utilitzat antagonistes i agonistes inversos del receptor GHS-R1a per bloquejar l'alta activitat constitutiva del receptor[414]. Aquests compostos han estat provats tant *in vitro* com *in vivo*, però malauradament, cap d'ells es troba actualment disponible al mercat, principalment degut als seus efectes secundaris a llarg plaç[415].

Com que els principals efectes de la grelina en quant a la regulació energètica i la ingesta calòrica són a través del receptor GHS-R1a expressat a l'hipotàlem, la majoria de fàrmacs contra l'obesitat han estat centrats en el seu efecte en el SNC. Una cirurgia de *bypass* gàstric és la teràpia més eficient per a un malalt

d'obesitat mòrbida, ja que no només limita la ingesta d'aliments sinó que també produeix una disminució dràstica de l'alliberació de grelina al torrent sanguini[416]. L'elevada dificultat per trobar fàrmacs eficients a nivell de SNC per tractar l'obesitat, ha conduït a centrar l'atenció en l'estómac, intentant reduir la producció de grelina com a nova estratègia per combatre aquesta patologia.

En aquesta mateixa línia, una alternativa als fàrmacs contra l'obesitat consisteix en l'acilació de la grelina. Més del 80% de la grelina que circula pel torrent sanguini ho fa en la seva forma inactiva, sense l'acilació de la serina en posició 3[417]. La grelina només indueix la ingesta d'aliments i la sensació de gana en presència d'aquesta modificació posttraduccional. La disrupció de l'enzim GOAT, dona lloc a l'absència total de grelina acilada en la circulació[418]. La presència de GO-CoA-Tat, un inhibidor de GOAT, va resultar efectiu en reduir l'increment de pes en ratolins així com en millorar la tolerància a la glucosa[419]. Actualment, la millor estratègia d'actuació sobre la grelina encara no està clara, degut a la seva implicació en funcions vitals com són la motilitat gastrointestinal o la regulació cardíaca.

1.7.4 Cannabinoides i obesitat

Tot i el rebuig que hi ha en l'opinió pública sobre el consum de cànnabis, cada vegada hi ha més estudis que demostren el potencial terapèutic dels cannabinoides, així com la implicació del Sistema Cannabinoide en processos com la formació i remodelació del teixit adipós o la regulació del consum calòric. És una realitat que l'estimulació de la sensació de gana, especialment per aliments dolços i gustosos, es veu induïda pel consum de cànnabis[420]. És per aquesta propietat, precisament, que s'ha proposat un rol important del Sistema Cannabinoide en la patologia de l'obesitat.

1.7.4.1 Tractament amb CBD

El teixit adipós es diferencia en dos tipus segons les seves característiques. El teixit adipós marró (TAM) es caracteritza per estar format per petites vesícules lipídiques i una alta densitat de mitocòndries, que li ofereixen una coloració marronosa. En contraposició, el teixit adipós blanc (TAB) es caracteritza per contenir una única vesícula lipídica de gran mida que ocupa la major part de la cèl·lula[421].

El teixit adipós marró es troba involucrat en la regulació de l'homeòstasi, la termogènesi i el consum calòric, tan en repòs com en activitat. Aquesta funció està regulada per la proteïna desacobladora de mitocòndries 1 (UCP1), que atura la fosforilació oxidativa fent que es produeixi calor en comptes d'ATP, fenomen que es coneix com a termogènesi[422]. D'altra banda, el teixit adipós blanc es troba involucrats en l'emmagatzematge de greixos i la secreció d'hormones.

Recentment s'ha descobert que el teixit adipós blanc pot adquirir propietats semblants a les del teixit adipós marró. Un clar exemple és l'expressió de la proteïna UCP1 en el teixit adipós blanc, fenomen conegut com a *browning*,

produint en conseqüència, un increment del consum energètic i del consum metabòlic dels greixos per mitjà de la termogènesi[423]. Aquesta conversió del teixit adipós blanc al teixit adipós marró, i la seva posterior activació està demostrant ser una potencial diana per tractar l'obesitat.

Hi ha un gran nombre de compostos que poden afavorir aquest fenomen de *browning* dels adipòcits blancs, el mentol, la curcumina o inclús el te verd han resultat ser capaços d'induir l'expressió de la proteïna UPC1, amb els seus subseqüents efectes beneficiosos per la salut[424].

Un dels compostos que ha resultat ser capaç d'induir la conversió del TAB en TAM és el CBD. S'ha descrit que el CBD pot induir l'expressió de gens marcadors específics de teixit adipós marró com l'Ucp1, el Cited1, el Tmem26 o el Prdm16, a la vegada que incrementa l'expressió de proteïnes com UCP1 en adipòcits 3T3-L1[425].

El CBD és capaç d'activar el receptor PPARγ, que té un rol molt important en la regulació de l'homeòstasi de la glucosa i el metabolisme dels lípids[426]. Estudis recents han demostrat que l'activació de PPARγ, es troba estretament relacionada amb la regulació de la diferenciació dels adipòcits i l'emmagatzematge de lípids, produint un increment en la lipòlisi i la termogènesi i una disminució de la lipogènesi. En conseqüència, el CBD podria ser un agent terapèutic molt prometedor per la prevenció i el tractament de l'obesitat[423].

L'activació dels receptors PPARγ produeix una potent resposta antiinflamatòria capaç d'activar la micròglia, induint la diferenciació cap a un fenotip antiinflamatori M2. Conseqüentment, es promou la síntesi de citocines antiinflamatòries i se suprimeix l'expressió de factors proinflamatoris (Figura 17).

L'obesitat està estretament relacionada amb la inflamació. L'excés de macronutrients en el teixit adipós estimula l'alliberació de mediadors inflamatoris com per exemple la IL-6 i redueix la producció d'adiponectina, predisposant un estat proinflamatori i un elevat estrès oxidatiu[427].

Un gran nombre d'estudis també relacionen l'obesitat amb un increment de la proteïna C reactiva. Amb cada grau d'obesitat, la proteïna C reactiva incrementava de forma directament proporcional i independentment de l'ètnia i el sexe[427]. La relació entre l'obesitat i la producció de mediadors proinflamatoris, així com l'increment de la proteïna C reactiva s'ha descrit tant en adults com en infants.

Així doncs, un tractament amb CBD també seria efectiu en intentar reduir la inflamació i l'estrès oxidatiu derivat tant de l'obesitat com de qualsevol altra situació inflamatòria com per exemple una infecció.

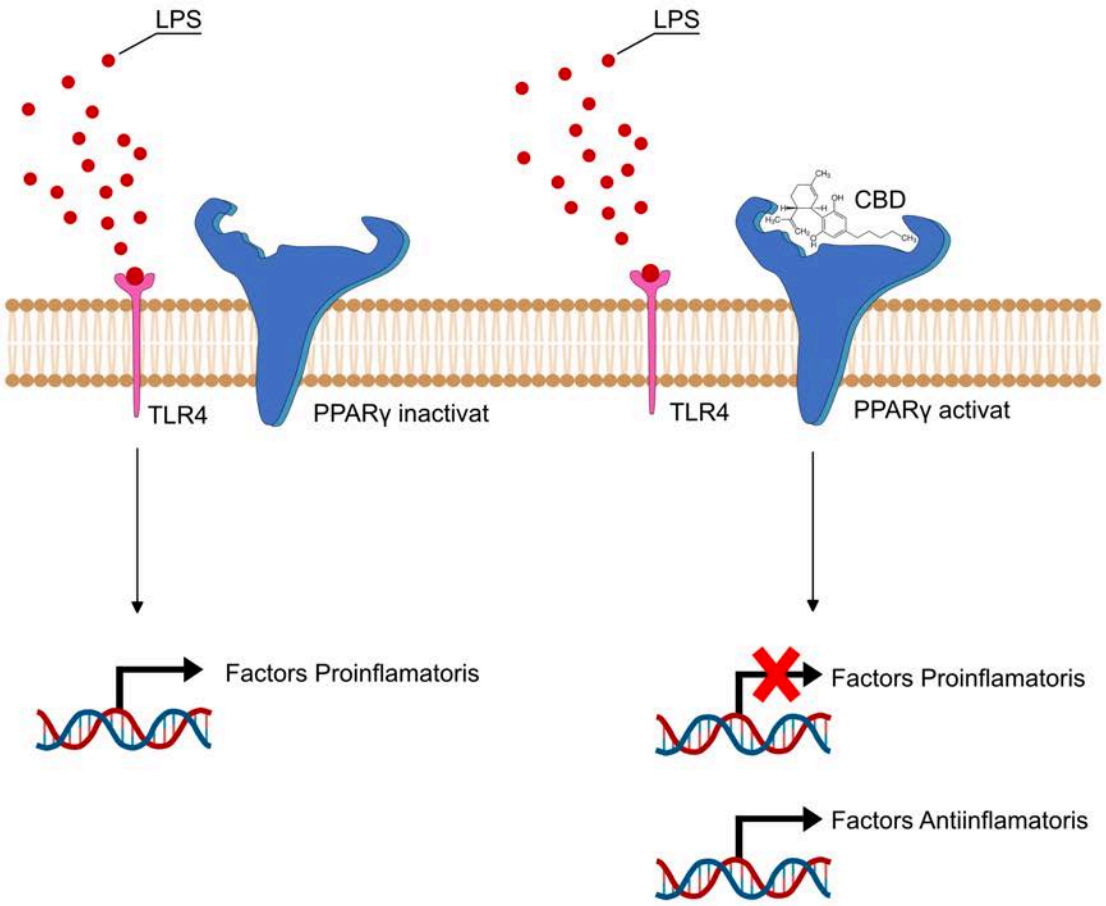

Figura 17: Esquema de l'acció antiinflamatòria que produeix el CBD a través del receptor PPARγ.

1.7.4.2 El rol del CB_2R en l'obesitat

Cada vegada hi ha més evidències que el receptor CB_2 pot tenir un rol important en l'homeòstasi energètica. El fet que el CB_2R s'expressi en l'hipotàlem, on també es troba el receptor GHS-R1a, responsable de la sensació de gana i en major mesura, en teixits metabòlicament actius com el fetge, el pàncrees i el teixit adipós, posa de manifest el seu paper en quant a la regulació metabòlica i energètica[428].

La inhibició del CB_2R via una injecció intracerebroventricular d'AM 630, el seu antagonista específic, va produir un increment significatiu en la ingesta calòrica de ratolins no obesos[429]. De manera semblant, la deleció del receptor CB_2 va induir un fenotip d'obesitat en ratolins d'edat avançada amb un increment en la ingesta calòrica i hipertròfia del teixit adipós[430]. D'altra banda, l'administració de l'agonista específic de CB_2R, JWH-133, provocava la millora de la tolerància a la glucosa en rates no obeses, indicant que el receptor CB_2 podria tenir un rol important en la millora de l'obesitat associada a diabetis, a causa del seu efecte en l'homeòstasi de la glucosa[431].

Altres agonistes del receptor CB_2, com per exemple el Δ^9-THC, han demostrat un millora de la sensibilitat a la insulina en adipòcits resistents[426]. A més, estudis recents han demostrat que el CBG és capaç d'estimular la sensació de gana en rates, sense presentar efectes secundaris durant l'administració[432].

El fet que el receptor CB_2 no s'expressi en regions del cervell que afecten l'estat d'ànim, obre la possibilitat d'utilitzar-lo com a diana per tractar l'obesitat, atenuant la ingesta calòrica i limitant l'acumulació de greix, produint el mínim d'efectes psicològics[433].

1.7.4.3 El Sistema Cannabinoide com a diana pel tractament de l'obesitat

En els últims anys, el Sistema Cannabinoide s'ha reivindicat com un agent clau en la regulació energètica, trobant-se implicat en el control del metabolisme i la ingesta calòrica, entre d'altres. Tant és així, que en pacients amb càncer que estaven rebent quimioteràpia, se'ls receptaven cannabinoides per estimular la gana i pal·liar així els efectes secundaris del tractament[434].

S'ha detectat que la sobreactivació del receptor CB_1 es troba estretament relacionada amb el desenvolupament de l'obesitat i la resistència a la insulina, sent una diana molt prometedora per diferents desordres metabòlics[435]. En aquesta línia, es va recórrer a la utilització d'antagonistes del CB_1R com el rimonabant per tractar la patologia de l'obesitat. L'administració crònica de rimonabant tant en ratolins com en humans obesos reduïa el pes i el greix corporal, millorant la homeòstasi de la glucosa i reduint la sensibilitat a la insulina[436]. Malauradament, els efectes neuropsiquiàtrics secundaris derivats de la seva administració van portar a la retirada del mercat del rimonabant i a la resta d'antagonistes del receptor CB_1[435]. Aquest fet va conduir a focalitzar l'atenció en altres elements del Sistema Cannabinoide, on recentment s'han identificat diferents mecanismes d'acció pels quals els endocannabinoides regulen tant el metabolisme com el balanç energètic, demostrant ser una eina potencial per tractar l'obesitat i d'altres desordres metabòlics. S'ha observat que l'anandamida estimulava la ingesta en ratolins SABRA-M a una dosi considerablement baixa d'1 μg/kg[437]. També s'ha vist que el 2-AG incrementa de manera dosi-dependent, la ingesta calòrica. Aquest efecte es veia atenuat per l'antagonista SR-141716A[438].

La injecció de 2-AG a l'hipotàlem, així com la inhibició de l'enzim FAAH estimulen la ingesta d'aliments en rates. Aquest efecte desapareix en presència d'antagonistes del CB_1R[439]. En models animals obesos, tant els nivells d'anandamida com els de 2-AG en hipotàlem eren elevats, contribuint a la hiperfàgia[440]. A més, en ratolins alimentats amb una dieta alta en greixos, es van veure nivells elevats de 2-AG i d'anandamida així com un increment dels enzims de síntesi d'aquests, indicant que l'obesitat indueix canvis en el Sistema Cannabinoide més enllà de les àrees involucrades directament en la regulació metabòlica[441]. És cert que el tractament amb leptina, feia reduir significativament els nivells hipotalàmics d'aquests endocannabinoides, indicant que també podrien estar relacionats amb la inducció a la gana regulada per aquesta hormona[442].

Els nivells en sang de 2-AG són més elevats en humans que pateixen obesitat en comparació amb persones no obeses i es troben correlacionats directament amb els nivells de greix visceral[443]. En canvi, els nivells d'anandamida en sang són significativament més elevats en malalts amb desordres alimentaris com l'anorèxia comparat amb individus sans[444].

En persones obeses, la reducció del pes corporal després de canvis en l'estil de vida com la dieta o l'esport, resulta en una disminució dels nivells d'anandamida i de 2-AG en sang. A més, la disminució dels nivells d'endocannabinoides es correlaciona amb la pèrdua de greix visceral[445].

Els nivells elevats de 2-AG i anandamida no només s'observen al torrent sanguini. Analitzant el teixit adipós de pacients obesos també es van detectar nivells més elevats d'aquests endocannabinoides. Totes aquestes observacions suggereixen que un Sistema Cannabinoides sobreactivat afavoreix el desenvolupament de l'obesitat[446].

Tot i que encara queda molt camí per investigar i entendre el rol del Sistema Cannabinoide en el context de la regulació del metabolisme a través dels endocannabinoides, les evidències són molt clares i les possibilitats d'aquests compostos semblen múltiples[447].


OBJECTIUS

2. Objectius

Els receptors de cannabinoides CB_1 i CB_2 són els dos receptors acoblats a proteïna G (GPCR) que componen el Sistema Cannabinoide. Des dels anys noranta, es coneix que els GPCR poden interaccionar formant estructures de major ordre com homòmers o heteròmers. La interacció entre dos GPCR indueix a la formació de complexos amb propietats diferents de les que expressaven els receptors individualment. A dia d'avui, un gran nombre de receptors, tant ionotròpics com metabotròpics, han estat identificats com a proteïnes capaces de formar complexos heteromèrics amb els receptors de cannabinoides. Entre aquests receptors es troba el receptor inotròpic NMDA. El receptor NMDA té un paper clau en la transmissió de senyal induïda pel glutamat en el Sistema Nerviós Central, tant a nivell de neurotransmissió com en desenvolupament i neurogènesi. Paral·lelament, el receptor NMDA també es troba relacionat amb l'excitotoxicitat induïda per nivells elevats de glutamat extracel·lular, que acabarà produint una acumulació tòxica d'ions al citoplasma de les neurones. En aquesta línia, es va hipotetitzar que el NMDAR podria ser una possible diana per tractar certes malalties neurodegeneratives com la malaltia de l'Alzheimer, ja que la reducció de la sobreactivació del NMDAR, suprimiria l'excitotoxicitat. Amb aquests antecedents, el primer i segon objectiu d'aquesta Tesi Doctoral van ser els següents:

Objectiu 1. Demostrar la formació de l'heteròmer NMDAR-CB_2R en cultius primaris de neurones i micròglia i caracteritzar l'efecte dels agonistes cannabinoides sobre la senyalització del NMDAR en el model animal $APP_{Sw/Ind}$ de la malaltia d'Alzheimer.

Objectiu 2. Demostrar la implicació dels sensors de calci calmodulina, calneuron-1, NCS1 i caldendrina en la senyalització del NMDAR en cèl·lules HEK-293T transfectades, cultius primaris de neurones i micròglia. I estudiar el rol dels sensors de calci sobre l'efecte induït per les proteïnes α-sinucleïna, Tau, i pTau en ratolins control i model d'Alzheimer.

La formació de complexes entre els receptors del Sistema Cannabinoide i altres receptors implicats en diverses patologies ha conduït a què en els darrers anys, estigui havent-hi un interès creixent en l'ús dels cannabinoides pel tractament de diferents malalties. Un exemple d'aquests compostos és el Cannabidiol (CBD), el segon fitocannabinoide més abundant de la planta del cànnabis. El CBD ha estat considerat des de fa temps com una molècula amb caràcter neuroprotector i degut a l'absència d'efectes psicoactius associats al seu consum, ha esdevingut un principi actiu molt atractiu per diverses malalties. Una d'aquestes malalties on el CBD ha resultat ser útil és l'ictus isquèmic. El receptor $5HT_{1A}$ és un GPCR molt implicat en la patologia de l'ictus. Els agonistes de $5HT_{1A}$R van mostrar un gran poder neuroprotector en models animals que havien patit isquèmia amb hipòxia. Per tal d'investigar l'efecte del CBD en la malaltia de l'ictus i la malaltia d'Alzheimer ens vam plantejar els següents objectius d'aquesta Tesi:

Objectiu 3. Determinar els nivells d'expressió de l'heteròmer CB_2R-$5HT_{1A}R$ en un model animal d'ictus isquèmic. Demostrar l'efecte dels cannabinoides CBD i CBG en un model de deprivació d'oxigen i glucosa i en el model animal d'ictus isquèmic així com determinar el seu efecte en termes de polarització de la micròglia cap a un fenotip proinflamatori M1 o antiinflamatori M2, així com la seva capacitat d'activar astròcits i oligodendròcits en el model animal d'ictus isquèmic.

Objectiu 4. Determinar l'efecte del CBD en termes de transport axonal de les proteïnes Aβ, α-sinucleïna, Tau, i pTau, de polarització de la micròglia, de regulació de l'expressió dels receptors cannabinoides així com la seva capacitat de produir una millora cognitiva, quant a la memòria de reconeixement en el model animal 5xFAD de la patologia d'Alzheimer.

Un dels efectes més coneguts del consum de cànnabis és el de la inducció de la gana. La regulació de la gana és un procés molt complex en el qual intervenen molts factors: Entre ells, la grelina és una hormona que té un paper protagonista. Per tal d'investigar la implicació del Sistema Cannabinoide en la patologia de l'obesitat ens vam plantejar el cinquè objectiu d'aquesta Tesi Doctoral.

Objectiu 5. Caracteritzar les interaccions moleculars i funcionals entre els receptors CB_1 i GHS-R1a i els receptors CB_2 i GHS-R1a i determinar la implicació funcional dels heteròmers en cultius primaris de la progènie d'una ratolina alimentada amb dieta alta en greixos.

Els receptors cannabinoides es troben implicats en un gran nombre de processos fisiològics, i com hem vist, les seves interaccions proteïna-proteïna amb altres GPCR estan estretament relacionades amb patologies amb un component neuroinflamatori. Recentment, s'ha proposat el receptor GPR55 com a possible tercer receptor cannabinoide, que pretén explicar aquells efectes que no s'expliquen a través de CB_1R i CB_2R. Per tal d'estudiar la interacció entre els receptors del Sistema Cannabinoide amb el receptor GPR55 ens vam plantejar l'últim objectiu d'aquesta Tesi Doctoral:

Objectiu 6. Determinar els nivells d'expressió dels heteròmers CB_1R-GPR55 i CB_2R-GPR55 en neurones d'estriat en seccions de cervells de *Macaca fascicularis* control i Parkinsonians amb discinèsia induïda pel tractament amb Levodopa.

RESULTATS

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La Tesi Doctoral de Jaume Lillo Jové titulada "Noves evidències de l'efecte neuroprotector del Sistema Cannabinoide a nivell del Sistema Nerviós Central" es presenta com un compendi de publicacions.

L'article **"N-Methyl-D-aspartate (NMDA) and cannabinoid CB_2 receptors form functional complexes in cells of the central nervous system: insights into the therapeutic potential of neuronal and microglial NMDA receptors"** ha estat publicat a la revista *Alzheimer's research & therapy* que té un factor d'impacte l'any de la seva publicació (2021) de 8.831. L'article **"*N*-Methyl-D-Aspartate Receptor Link to the MAP Kinase Pathway in Cortical and Hippocampal Neurons and Microglia Is Dependent on Calcium Sensors and Is Blocked by α-Synuclein, Tau, and Phospho-Tau in Non-transgenic and Transgenic $APP_{Sw,Ind}$ Mice"** ha estat publicat a la revista *Frontiers in molecular neuroscience* que té un factor d'impacte l'any de la seva publicació (2018) de 3.720. L'article **"Regulation of Expression of Cannabinoid CB_2 and Serotonin $5HT_{1A}$ Receptor Complexes by Cannabinoids in Animal Models of Hypoxia and in Oxygen/Glucose-Deprived Neurons"** ha estat publicat a la revista *International journal of molecular sciences* que té un factor d'impacte a l'any 20221 de 6.208. L'article **"Cannabidiol skews microglia towards a neuroprotective phenotype in a model of neonatal hypoxia-ischemia"** ha estat enviat i pendent de revisió a la revista *Frontiers In Molecular Neuroscience* que té un factor d'impacte a l'any 2021 de 6.261. L'article **"Cannabidiol decreases pTau and Aβ axonal transport and improves spatial memory in 5xFAD mice model of Alzheimer's disease"** esta en vies de redacció i revisió i vol ser enviat a la revista *Nature Communications* que té un factor d'impacte a l'any 2021 de 17.694. L'article **"Ghrelin and Cannabinoid Functional Interactions Mediated by Ghrelin/CB_1 Receptor Heteromers That Are Upregulated in the Striatum From Offspring of Mice Under a High-Fat Diet"** ha estat publicat a la revista *International journal of molecular sciences* que té un factor d'impacte l'any de la seva publicació (2021) de 6.208. L'article **"Identification of the Ghrelin and Cannabinoid CB_2 Receptor Heteromer Functionality and Marked Upregulation in Striatal Neurons from Offspring of Mice under a High-Fat Diet"** ha estat publicat a la revista *Frontiers in cellular neuroscience* que té un factor d'impacte l'any de la seva publicació (2021) de 6.147. L'article **"Expression of GPR55 and either cannabinoid CB_1 or CB_2 heteroreceptor complexes in the caudate, putamen, and accumbens nuclei of control, parkinsonian, and dyskinetic non-human primates"** ha estat publicat a la revista *Brain structure and function* que té un factor d'impacte l'any de la seva publicació (2020) de 3.270.

En l'estudi "**N-Methyl-D-aspartate (NMDA) and cannabinoid CB_2 receptors form functional complexes in cells of the central nervous system: insights into the therapeutic potential of neuronal and microglial NMDA receptors"** el doctorand Jaume Lillo Jové va realitzar la preparació dels cultius primaris de micròglia i neurones d'hipocamp d'animals control i animals model de la malaltia d'Alzheimer i va participar en la realització dels assaigs de senyalització que es van desenvolupar en aquests cultius. En l'estudi **"*N*-Methyl-D-Aspartate Receptor Link to the MAP Kinase Pathway in Cortical and Hippocampal Neurons and Microglia Is Dependent on Calcium Sensors and Is Blocked by α-Synuclein, Tau, and Phospho-Tau in Non-transgenic and Transgenic $APP_{Sw,Ind}$ Mice"** el doctorand Jaume Lillo Jové va participar en la preparació dels cultius primaris de neurones i micròglia, en animals control i en un model d'Alzheimer. També es va encarregar de realitzar la funcionalitat de NMDAR en presencia de α-sinucleïna, Tau i pTau tant en neurones com micròglia en animals control i en el model d'Alzheimer d'estudi. En l'estudi **"Regulation of Expression of Cannabinoid CB_2 and Serotonin $5HT_{1A}$ Receptor Complexes by Cannabinoids in Animal Models of Hypoxia and in Oxygen/Glucose-Deprived Neurons"** el doctorand Jaume Lillo Jové va realitzar l'assaig d'immunocitoquímica i l'assaig de BRET per determinar la interacció entre CB_2R i $5HT_{1A}R$. També es va encarregar dels tractaments amb els fitocannabinoides d'estudi i dels assaigs de funcionalitat de la via de l'AMPc i β-arrestina 2. També va desenvolupar la preparació del model de deprivació d'oxigen i glucosa (GOD) i l'assaig de PLA en aquest model GOD i en un model animal d'ictus isquèmic. En l'estudi "**Cannabidiol skews microglia towards a neuroprotective phenotype in a model of neonatal hypoxia-ischemia"** el doctorand Jaume Lillo Jové va realitzar l'obtenció de seccions de cervell d'animals model d'ictus isquèmic mitjançant el criòstat, els assaigs d'immunohistoquímica amb els diferents marcadors i la seva quantificació. En l'estudi "**Cannabidiol decreases pTau and Aβ axonal transport and improves spatial memory in 5xFAD mice model of Alzheimer's disease"** el doctorand Jaume Lillo Jové va realitzar l'obtenció de seccions de cervell d'animals model 5xFAD mitjançant el criòstat, i l'anàlisi d'aquestes mitjançant assaigs d'immunohistoquímica amb diferents marcadors. També va participar en l'estudi de transport de proteïnes Aβ, α-sinucleïna, Tau i pTau en presència i absència de CBD, els assaigs de viabilitat cel·lular, la quantificació dels nivells d'expressió del mRNA de CB_1R i CB_2R en escorça i hipocamp i la detecció de la formació de neurites en diferents condicions d'assaig. En l'estudi "**Ghrelin and Cannabinoid Functional Interactions Mediated by Ghrelin/CB_1 Receptor Heteromers That Are Upregulated in the Striatum From Offspring of Mice Under a High-Fat Diet"** el doctorand Jaume Lillo Jové va realitzar, la preparació dels cultius primaris de neurones tant en animals control com en un model alimentat amb una dieta alta en greixos (HFD) i l'assaig de funcionalitat i el de PLA en animals control i en animals HFD. En l'estudi "**Identification of the Ghrelin and Cannabinoid CB_2 Receptor Heteromer Functionality and Marked Upregulation in Striatal Neurons from Offspring of Mice under a High-Fat Diet"** el doctorand Jaume Lillo Jové va realitzar l'assaig d'immunocitoquímica i l'assaig de BRET per

determinar la interacció entre CB_2R i GHS-R1a, el clonatge de les hemiproteïnes GHS-R1a-nRLuc, GHS-R1acRLuc, CB_2R-nYFP i CB_2R-cYFP, l'assaig de complementació molecular (BiFC) i l'assaig de funcionalitat en la via de l'AMPc i del calci intracel·lular. En l'estudi "**Expression of GPR55 and either cannabinoid CB_1 or CB_2 heteroreceptor complexes in the caudate, putamen, and accumbens nuclei of control, parkinsonian, and dyskinetic non-human primates"** el doctorand Jaume Lillo Jové va realitzar l'assaig de PLA en seccions de cervell de macacos Parkinsonians, discinètics i control, que van ser llegides en un microscopi confocal, i seguidament es va quantificar l'expressió dels heteròmers CB_1R-GPR55 i CB_2R-GPR55 mitjançant l'extensió de *software* "*Andy's Algorithms*" de FIJI.

Barcelona, a 20 de Gener de 2023

Dr. Rafael Franco Fernández
Director i Tutor

Dra.Gemma Navarro Brugal
Directora

Els resultats d'aquesta Tesi Doctoral estan reflectits en els següents articles:

3.1 Rafael Rivas-Santisteban, Alejandro Lillo, **Jaume Lillo**, Joan-Biel Rebassa, Joan S Contestí, Carlos A Saura, Rafael Franco, Gemma Navarro. **N-Methyl-D-aspartate (NMDA) and cannabinoid CB_2 receptors form functional complexes in cells of the central nervous system: insights into the therapeutic potential of neuronal and microglial NMDA receptors.**

Article publicat a la revista *Alzheimer's research & therapy*. Novembre 2021.

3.2 Rafael Franco, David Aguinaga, Irene Reyes, Enric I Canela, **Jaume Lillo**, Airi Tarutani, Masato Hasegawa, Anna Del Ser-Badia, José A Del Rio, Michael R Kreutz, Carlos A Saura, Gemma Navarro. ***N*-Methyl-D-Aspartate Receptor Link to the MAP Kinase Pathway in Cortical and Hippocampal Neurons and Microglia Is Dependent on Calcium Sensors and Is Blocked by α-Synuclein, Tau, and Phospho-Tau in Non-transgenic and Transgenic $APP_{Sw,Ind}$ Mice.**

Article publicat a la revista *Frontiers in molecular neuroscience*. Agost 2018.

3.3 **Jaume Lillo**, Iu Raïch, Laura Silva, David A Zafra, Alejandro Lillo, Carlos Ferreiro-Vera, Verónica Sánchez de Medina, José Martínez-Orgado, Rafael Franco, Gemma Navarro. **Regulation of Expression of Cannabinoid CB_2 and Serotonin $5HT_{1A}$ Receptor Complexes by Cannabinoids in Animal Models of Hypoxia and in Oxygen/Glucose-Deprived Neurons.**

Article publicat a la revista *International journal of molecular sciences*. Agost 2022.

3.4 **Jaume Lillo**, Iu Raïch, Laura Silva, David A. Zafra, Alejandro Lillo, Carlos Ferreiro-Vera, Verónica Sánchez de Medina, José Martínez-Orgado, Gemma Navarro, Rafael Franco. **Cannabidiol skews microglia towards a neuroprotective phenotype in a model of neonatal hypoxia-ischemia**.

Article pendent de revisió a la revista *Frontiers In Molecular Neuroscience*.

3.5 **Jaume Lillo**†, Iu Raïch†, Joan Biel Rebassa, Christian Griñán-Ferré, Mercé Pallàs, Rafael Franco, Gemma Navarro. **Cannabidiol decreases pTau and AB axonal transport and improves spatial memory in 5xFAD mice model of Alzheimer's disease.**

Article en vies de redacció i revisió per ser enviat a la revista *Nature Communications*

3.6 Alejandro Lillo†, **Jaume Lillo**†, Iu Raïch, Cristina Miralpeix, Francesc Dosrius, Rafael Franco, Gemma Navarro. **Ghrelin and Cannabinoid Functional Interactions Mediated by Ghrelin/CB_1 Receptor Heteromers That Are Upregulated in the Striatum From Offspring of Mice Under a High-Fat Diet**.

Article publicat a la revista *International journal of molecular sciences.* Desembre 2021.

3.7 **Jaume Lillo**†, Alejandro Lillo†, David A. Zafra, Cristina Miralpeix, Rafael Rivas-Santisteban, Núria Casals, Gemma Navarro, Rafael Franco. **Identification of the Ghrelin and Cannabinoid CB_2 Receptor Heteromer Functionality and Marked Upregulation in Striatal Neurons from Offspring of Mice under a High-Fat Diet.**

Article publicat a la revista *Frontiers in cellular neuroscience.* Agost 2021.

3.8 Eva Martínez-Pinilla, Alberto J Rico, Rafael Rivas-Santisteban, **Jaume Lillo**, Elvira Roda, Gemma Navarro, José Luis Lanciego, Rafael Franco **Expression of GPR55 and either cannabinoid CB_1 or CB_2 heteroreceptor complexes in the caudate, putamen, and accumbens nuclei of control, parkinsonian, and dyskinetic non-human primates.**

Article publicat a la revista *Brain structure and function.* Setembre 2020.

3.1 N-Methyl-D-aspartate (NMDA) and cannabinoid CB_2 receptors form functional complexes in cells of the central nervous system: insights into the therapeutic potential of neuronal and microglial NMDA receptors.

Rafael Rivas-Santisteban, Alejandro Lillo, **Jaume Lillo**, Joan-Biel Rebassa, Joan S Contestí, Carlos A Saura, Rafael Franco, Gemma Navarro.

Article publicat a la revista *Alzheimer's research & therapy*. Novembre 2021; 13(1):184.

El receptor cannabinoide de tipus 2 (CB_2R) és un component clau en la senyalització del Sistema Cannabinoide. Està àmpliament descrit que la seva activació comporta propietats antiinflamatòries i neuroprotectores. D'altra banda el receptor N-metil-D-aspartat (NMDAR) és una diana de gran interès per millorar la fisiopatologia de la malaltia d'Alzheimer (AD). L'objectiu del nostre estudi va ser detectar la formació de complexos heteromèrics entre els receptors NMDA i CB_2 i observar la funcionalitat d'aquest heteròmer. L'assaig per transferència d'energia per ressonància bioluminescent (BRET) en un model heteròleg d'expressió va confirmar la formació de complexos CB_2R-NMDAR. Mitjançant la tècnica de *Proximity Ligation Assay* (PLA) vam detectar un increment significatiu de l'expressió de l'heteròmer CB_2R-NMDAR en cultius primaris de neurones d'hipocamp i micròglia de ratolí en un model d'AD ($APP_{Sw/Ind}$) en comparació amb ratolins control. Es va caracteritzar la funcionalitat de l'heteròmer CB_2R-NMDAR mitjançant l'assaig de determinació dels nivells d'AMPc intracel·lular, calci intracel·lular i de redistribució dinàmica de massa (DMR). De manera interessant, es va observar un *crosstalk* negatiu al produir-se la coactivació d'ambdós receptors amb els seus agonistes específics. En conclusió, l'activació específica del CB_2R, a la vegada que disminueix la senyalització induïda per NMDAR, pot suposar un tractament amb gran potencial en malalts d'Alzheimer, millorant la seva qualitat de vida.

Alzheimer's Research & Therapy

RESEARCH

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N-Methyl-D-aspartate (NMDA) and cannabinoid CB_2 receptors form functional complexes in cells of the central nervous system: insights into the therapeutic potential of neuronal and microglial NMDA receptors

Rafael Rivas-Santisteban[1,2], Alejandro Lillo[3,4], Jaume Lillo[1,2], Joan-Biel Rebassa[4], Joan S. Contestí[4], Carlos A. Saura[3], Rafael Franco[1,2,5*†] and Gemma Navarro[1,3,4*†]

Abstract

Background: The cannabinoid CB_2 receptor (CB_2R), which is a target to afford neuroprotection, and N-methyl-D-aspartate (NMDA) ionotropic glutamate receptors, which are key in mediating excitatory neurotransmission, are expressed in both neurons and glia. As NMDA receptors are the target of current medication in Alzheimer's disease patients and with the aim of finding neuromodulators of their actions that could provide benefits in dementia, we hypothesized that cannabinoids could modulate NMDA function.

Methods: Immunocytochemistry was used to analyze the colocalization between CB_2 and NMDA receptors; bioluminescence resonance energy transfer was used to detect CB_2-NMDA receptor complexes. Calcium and cAMP determination, mitogen-activated protein kinase (MAPK) pathway activation, and label-free assays were performed to characterize signaling in homologous and heterologous systems. Proximity ligation assays were used to quantify CB_2-NMDA heteromer expression in mouse primary cultures and in the brain of $APP_{Sw/Ind}$ transgenic mice, an Alzheimer's disease model expressing the Indiana and Swedish mutated version of the human amyloid precursor protein (APP).

Results: In a heterologous system, we identified CB_2-NMDA complexes with a particular heteromer print consisting of impairment by cannabinoids of NMDA receptor function. The print was detected in activated primary microglia treated with lipopolysaccharide and interferon-γ. CB_2R activation blunted NMDA receptor-mediated signaling in primary hippocampal neurons from $APP_{Sw/Ind}$ mice. Furthermore, imaging studies showed that in brain slices and in primary cells (microglia or neurons) from $APP_{Sw/Ind}$ mice, there was a marked overexpression of macromolecular CB_2-NMDA receptor complexes thus becoming a tool to modulate excessive glutamate input by cannabinoids.

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Conclusions: The results indicate a negative cross-talk in CB_2-NMDA complexes signaling. The expression of the CB_2-NMDA receptor heteromers increases in both microglia and neurons from the $APP_{Sw/Ind}$ transgenic mice, compared with levels in samples from age-matched control mice.

Keywords: Alzheimer's disease, Neuroprotection, G-protein-coupled receptors, Excitotoxicity

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder affecting more than 46 million people worldwide. The most affected neurons are located in the ascending cholinergic system whose somas are situated in Meinert's basal nucleus, thereafter, neurodegeneration in hippocampal, amygdala, and neocortex areas leads to the pathological AD features [1–3]. The main excitatory neurotransmitter, glutamate, is crucial for the physiological state of the brain. Excitatory glutamatergic neurotransmission is required for neuronal survival and synaptic plasticity; however, aberrant activity promotes excitotoxicity and cell death [4, 5]. Ionotropic ligand-gated glutamate receptors are the main mediators of glutamate action in the central nervous system (CNS). In addition, glutamate can activate the so-called metabotropic receptors that are not channels but G protein-coupled receptors (GPCRs). Three ionotropic glutamate receptors have been discovered, namely kainate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and N-methyl-D-aspartate (NMDA) receptors. The NMDA receptor (NMDAR) plays an important role in neuronal plasticity and learning mechanisms. Memantine, a drug approved for AD therapy [6, 7], targets NMDARs, which are multimers composed of different subunits whose consensual nomenclature is GluN1, GluN2A, GluN2B, GluN2C, GluN2D, GluN3A, and GluN3B [8]. A combination of these subunits leads to different tetrameric functional NMDARs. Moreover, the combination of different subunits leads to NMDARs with different functional and pharmacological properties. Activation of synaptic NMDARs has been reported to control synaptic plasticity and stimulate cell survival, while activation of extrasynaptic NMDARs promotes cell death and thus contributes to the etiology of AD. The limited effect of memantine in AD is likely due to an allosteric effect on extrasynaptic NMDARs [4, 5, 9].

Cannabinoid receptors are widely expressed in the CNS, not only in neurons but also in astrocytes, microglia, and oligodendrocytes. There are two cannabinoid receptors, CB_1 and CB_2, that belong to the superfamily of GPCRs. CB_1 is considered the most abundant GPCR in the CNS and is expressed in many different neuronal types. The expression of the CB_2 receptor (CB_2R) is restricted to some neuronal populations, e.g., in the globus pallidus [10] or the cerebellum [11], but is expressed in other neural cell types [12–17]. GPCRs may interact to form homo and heterodimers which for many of the receptors in the superfamily constitute the real functional units [18, 19]. Heteromers have different functionality than homomers, and different heterodimers have different signaling properties thus adding diversity to the action of neurotransmitters/neuromodulators on GPCRs. CB_1 and CB_2 receptors may form functional heteromers that are heavily expressed in the neurons of the globus pallidus [10, 20, 21] and that have a relevant function in activated microglia [20, 22]. CB_2R and CB_1-CB_2 receptor heteromers are considered to exert neuroprotective actions [23–27]; they have been proposed as targets to delay progression of Parkinson's disease [22].

The NMDAR plays a central role in the CNS excitatory neurotransmission, being also a therapeutic target to combat AD. Receptor function deregulation is, at least in part, responsible for the progression of the disease. Based on our previous experience, NMDAR function may be regulated by GPCRs that may, eventually, establish direct interaction with the ionotropic receptor [28–30]. As interest in the CB_2R is increasing due to its potential to combat neurodegenerative diseases, the aim of this study was to discover CB_2R-mediated mechanisms of regulation of NMDAR function. We found that NMDAR function can be modulated by interaction with the CB_2R and that the resulting complexes interact in neurons and microglia in control animals and in AD models.

Results

The NMDAR interacts with the CB_2R in a heterologous expression system

The NMDA receptor plays a central role in excitatory neurotransmission, being also a therapeutic target to combat AD. Receptor function deregulation is, at least in part, responsible for the progression of the disease. In this regard, it would be interesting to discover membrane proteins capable of interacting with NMDA receptors and being able to modulate their functionality. We considered that CB_2Rs could be candidates for receptor-receptor interactions. Accordingly, the human embryonic kidney HEK-293T cell model was transfected with the cDNAs for CB_2R fused to YFP, for the GluN1 NMDAR subunit fused to Renilla luciferase

(RLuc) and for the GluN2B subunit. Expression of GluN1 and GluN2B protomers leads to the assembly of a tetrameric structure, which is required for NMDA receptor functionality. Immunocytochemical assays showed that the fusion protein containing the cannabinoid receptor and the yellow fluorescent protein CB_2R-YFP, detected by YFP's own fluorescence, was expressed at the plasma membrane and also intracellularly (Fig. 1A). Qualitatively, similar expression was detected for the NMDA-RLuc receptor by using a specific anti-RLuc antibody (Fig. 1B). Moreover, a high level of colocalization between the two receptors was observed in the plasma membrane and in intracellular organelles (yellow in Fig. 1C). The results are suggestive of possible direct interactions. To demonstrate the hypothesis of a physical interaction between CB_2 and NMDA receptors, HEK-293T cells were transfected with a constant amount of cDNA for GluN1-RLuc and GluN2B and increasing amounts of cDNA for CB_2R-YFP. The saturable curve obtained in Bioluminescence Energy Transfer (BRET) experiments was consistent with a physical interacting between CB_2R and GluN1 and the formation of CB_2-NMDA receptor complexes ($BRET_{max}$=460 ± 10 mBU $BRET_{50}$ = 10 ± 2; Fig. 1D, E). When HEK-293T cells were transfected with a constant amount of GluN1-RLuc cDNA and increasing amounts of cDNA for the ghrelin GHS1a receptor 1a fused to YFP (GHS-R1a-YFP) or with a constant amount of angiotensin AT_1 receptor-RLuc cDNA (AT1R-RLuc) and increasing amounts of CB2R-YFP cDNA (CB_2R-YFP), the linear relationship between the BRET donor/acceptor ratio indicated a lack of interaction of those pair of proteins (negative controls; Fig. 1E right).

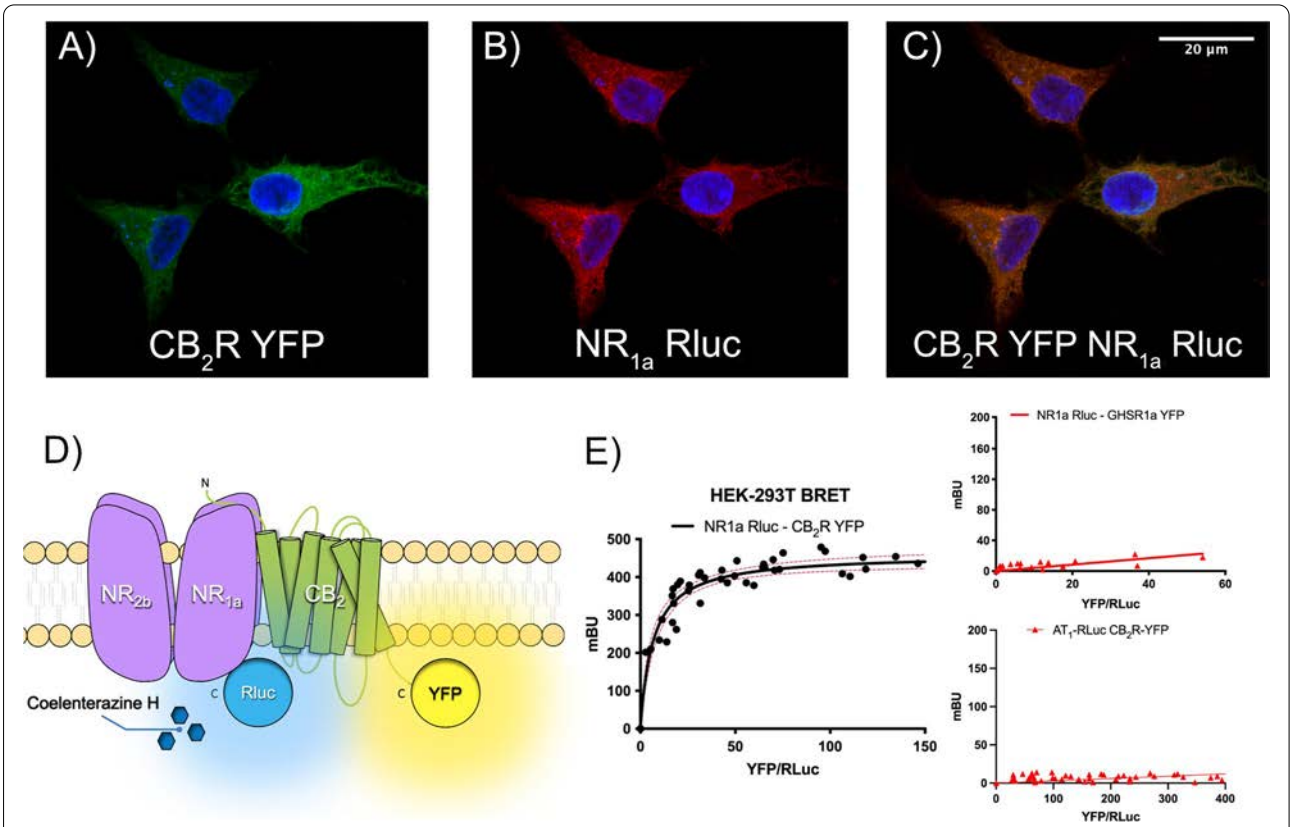

Fig. 1 The NMDA receptor interacts with the cannabinoid CB_2 receptor in a heterologous expression system. **A**–**C** Immunocytochemistry performed in HEK-293T cells expressing CB_2-YFP (**A**) (1 µg cDNA), that was detected by its own fluorescence (green), and GluN1-Rluc receptor (1 µg cDNA) (**B**), that was detected by a mouse monoclonal anti-Rluc antibody and a secondary Cy3-conjugated anti-mouse IgG antibody (red). Colocalization is shown in yellow (**C**). Cell nuclei were stained with Hoechst (blue). Images are taken near the bottom of the cell, i.e. it mainly includes the membrane in contact with the glass of the plate. Scale bar: 20 µm. **D** Schematic representation of BRET assay: the occurrence of energy transfer depends on the distance between the BRET donor (Rluc) and the BRET acceptor (YFP). **E** BRET assays were performed in HEK-293T cells transfected with a constant amount of cDNAs for GluN1-Rluc (0.25 µg), GluN2B (0.15 µg), and increasing amounts of cDNA for CB_2R-YFP (0.25 to 1.25 µg) (left) or, as negative controls, using $GHSR_{1a}$R-YFP (0.25 to 1.25 µg) as acceptor (right top)), or using AT_1R-Rluc (0.5 µg) as donor and CB_2R-YFP (0.25 to 1.25 µg) as acceptor (right bottom). Values are the mean ± S.E.M. of 6 independent experiments performed in duplicates

CB_2R activation impairs signaling via the NMDA receptor expressed in HEK-293T cells

In HEK-293T cells only expressing GluN1 and GluN2B subunits, forskolin-stimulated intracellular cyclic-adenylic acid (cAMP) levels were not modified upon treatment with either NMDA or a NMDAR selective antagonist, MK-801 (Fig. 2A). The concentration of NMDA and MK-801was selected according to our previous experience [28–30] and on optimization of dosages based on early reports on NMDAR agonists and antagonists [31, 32]. As G_i is the cognate protein coupled to the CB_2R, activation of the receptor leads to adenylate cyclase activity inhibition and a decrease of intracellular cAMP levels. Such canonical functionality was confirmed in HEK-293T cells expressing CB_2R treated with forskolin and, afterwards, with a selective CB_2R agonist, JWH-133. The decrease in forskolin-induced cAMP levels was mediated by the cannabinoid receptor as it was completely counteracted by the pretreatment with a selective antagonist, SR 144528 (Fig. 2B). In HEK-293T cells expressing GluN1, GluN2B and CB_2R, agonist activation of the cannabinoid receptor produced a significant decrease in forskolin-induced cAMP levels, that was counteracted by the activation of the NMDAR (Fig. 2C). This phenomenon is usually described as negative cross-talk and can serve as a print/pattern to identify CB_2-NMDA receptor complexes in natural sources. The small decrease upon NMDAR activation was not significant. The CB_2R antagonist, SR-144528, blocked CB_2R activation while the NMDAR antagonist produced no effect (Fig. 2C). As a control of specificity, we performed similar assays in cells coexpressing the NMDAR and the ghrelin GHS1a receptor without observing any cross-regulation (Supplementary Figure S1).

Next, we determined mitogen-activated protein kinase (MAPK) pathway activation by means of assays addressing the phosphorylation degree of extracellular signal-regulated kinases, ERK1/2. It should be noted that both NMDAR and CB_2R activation leads to engagement of the MAPK pathway. Then, in HEK-293T cells expressing GluN1 and GluN2B, NMDA activation induced an effect that was counteracted by MK-801 pretreatment (Fig. 2D); analogously, in HEK-293T expressing the CB_2R, JWH-133 produced a significant effect that disappeared by

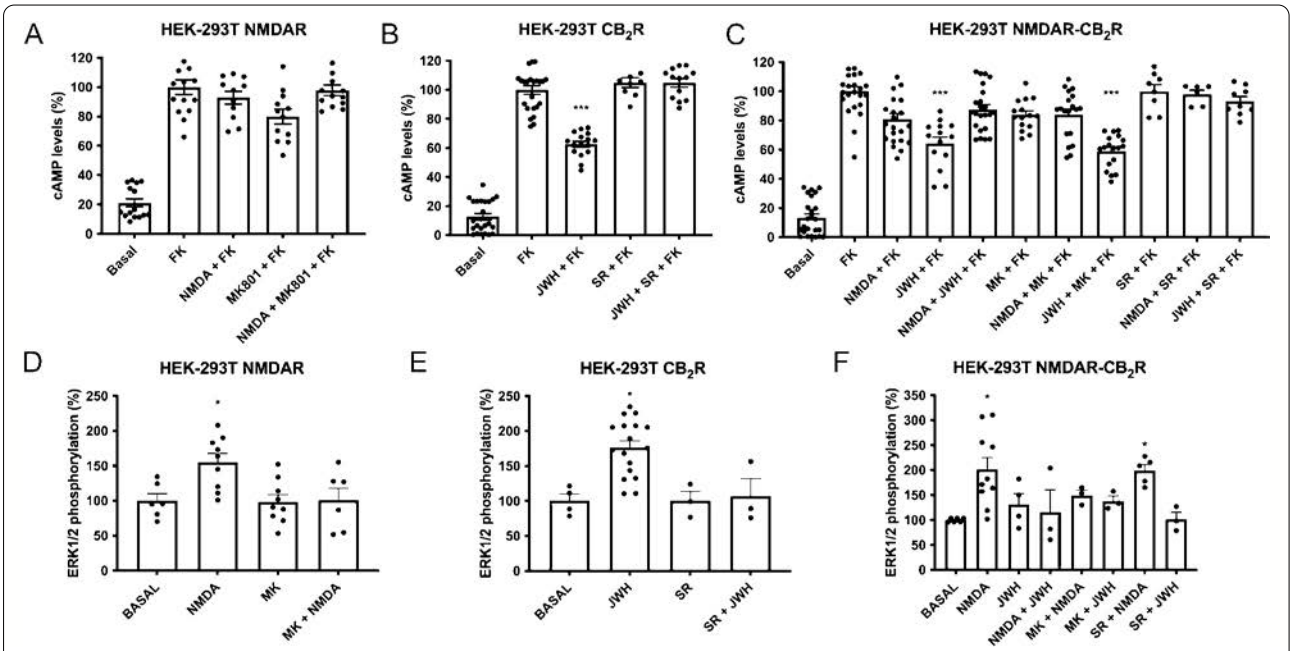

Fig. 2 Signaling in HEK-293T cells expressing NMDA-CB_2R heteromers. HEK-293T cells transfected with the cDNAs for two protomers of the NMDA receptor: GluN1 (1 μg) and GluN2B (0.75 μg) and/or with the cDNA for the CB_2R (1 μg), and were treated with selective agonists (15 μM NMDA for NMDAR and/or 100 nM JWH-133 for CB_2R). When indicated cells were pretreated with selective receptor antagonists (1 μM MK-801 for NMDA or 1 μM SR-144528 for CB_2R). **A**–**C** Intracellular cAMP levels were determined by TR-FRET as described in Methods. As G_i coupling was assessed, decreases in cAMP levels were determined in cells previously treated with 0.5 μM forskolin (15 min). Values are the mean ± S.E.M. of 6 independent experiments performed in triplicates. In cAMP one-way ANOVA followed by Bonferroni's multiple comparison post hoc test were used for statistical analysis (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus forskolin treatment). ANOVA summary: **A** F: 67.6, p<0.001; **B** F: 238.0, p<0.001; **C** F: 62.5 p<0.001. **D**–**F** ERK1/2 phosphorylation was analyzed using an AlphaScreen®SureFire® kit (Perkin Elmer). Values are the mean ± S.E.M. of 5 independent experiments performed in triplicates. One-way ANOVA followed by Bonferroni's multiple comparison post hoc test were used for statistical analysis (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus vehicle treatment). ANOVA summary: **D** F: 5.4, p<0.005; **E** F: 8.3, p<0.001; **F** F: 3.8, p<0.005

the pretreatment with SR-144528 (Fig. 2E). In cells coexpressing the two receptors, NMDA induced a circa 2-fold increase in ERK1/2 phosphorylation; in contrast, CB_2R activation led to a non-significant response. Furthermore, coactivation with both agonists impeded the link of the CB_2-NMDA receptor complex to the MAPK pathway (Fig. 2F). Finally, antagonist pre-treatment did not lead to cross-antagonism, i.e., the antagonist of one receptor did not block the effect of the agonist of the partner receptor in the heteromeric complex.

Activation of NMDA by glutamate results in the opening of the ligand-gated ion channel to allow calcium influx. In HEK-293T cells expressing NMDAR the increase in cytoplasmic calcium caused by NMDA was inhibited in cells pretreated with the antagonist MK-801 (Fig. 3A). As expected, JWH-133 did not produce any calcium signal in HEK-293T cells expressing the CB_2R (Fig. 3B). However, this CB_2R agonist blocked the NMDA-induced effect in HEK-293T cells coexpressing NMDAR and CB_2R, thus showing a negative cross-talk. In addition, the CB_2R antagonist, SR-144528, potentiated the NMDA-induced effect. These results indicate that, when forming complexes with CB_2Rs, NMDA receptor activation is restrained by CB_2R activation and is restored when the CB_2R is blocked by its antagonist (Fig. 3C).

Finally, the label-free technique dynamic mass redistribution (DMR) that underscores ligand-induced changes due to multiple pathways and cellular events was applied [33]. First, in cells expressing CB_2R or NMDAR redistribution of mass occurred upon agonist treatment (Fig. 3D, E), the effects were partially inhibited by pretreatment with selective antagonists. In cotransfected cells, it was demonstrated that coactivation induced no additive effect, while the CB_2R antagonist, SR-144528, potentiated NMDA activation (Fig. 3F).

To sum up, heteromers constituted by the two receptors (CB_2-NMDA-Hets) show a negative cross-talk that may disappear when the partner receptor is blocked by an antagonist. It should be noted that the blockade of CB_2R markedly potentiates the ligand-gated ionotropic action subsequent to NMDAR activation.

CB_2R activation blocks NMDA signaling in activated microglia

The cannabinoid receptor CB_2R is upregulated in activated microglia, thus being a promising target for neuroprotection. Then, to evaluate the possible role of the CB_2R in regulating NMDAR function, mouse primary microglia were activated (48 h) with 1 μM LPS and 200 U/ml IFN-γ and treated with receptor ligands. Treatment of non-activated cells with the CB_2R agonist,

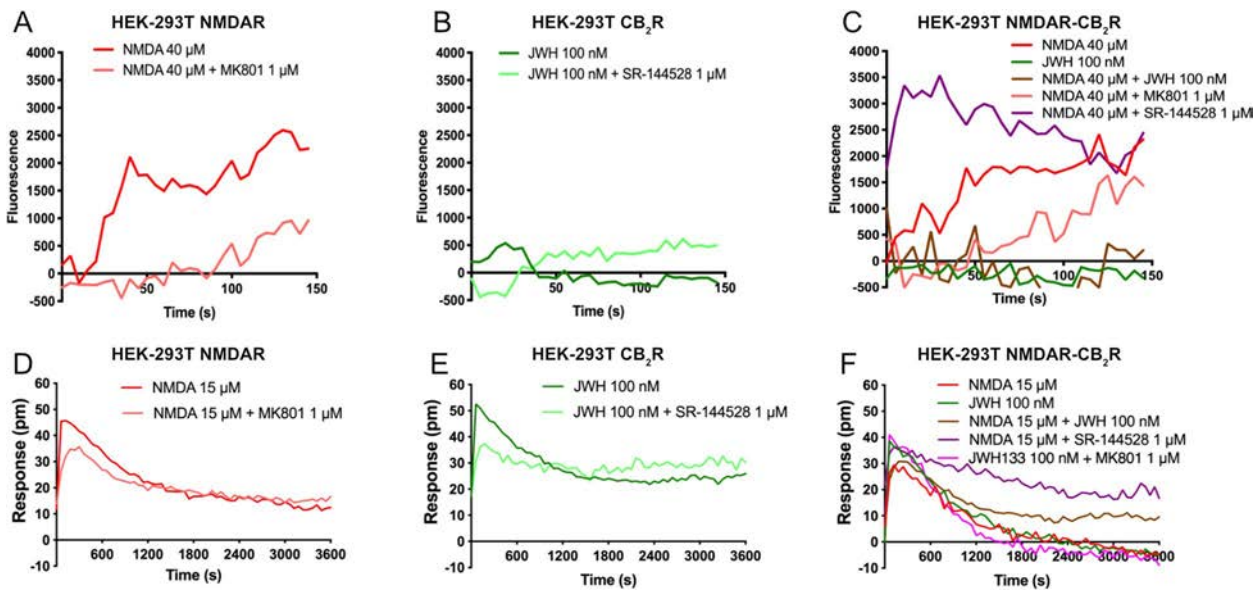

Fig. 3 DMR and intracellular calcium mobilization in HEK-293T cells expressing NMDA-CB_2R heteromers. HEK-293T cells were transfected with the cDNAs for two protomers of the NMDA receptor: GluN1 (1 μg) and GluN2B (0.75 μg) and/or with the cDNA for the CB_2R (1 μg); for calcium assays, cells were also transfected with the cDNA for the engineered calcium sensor, 6GCaMP (1 μg) (**D**–**F**). Receptors were activated using selective agonists (40 μM NMDA (for calcium detection assays) or 15 μM NDMA (for DMR assays) for NMDAR and/or 100 nM JWH-133 for CB_2R). When indicated cells were pretreated with selective receptor antagonists (1 μM MK-801 for NMDA or 1 μM SR-144528 for CB_2R). Real-time calcium-induced fluorescence (**A-C)** or DMR readings (**D-F**) were collected. Values in each figure are from a representative experiment out of 5 independently performed

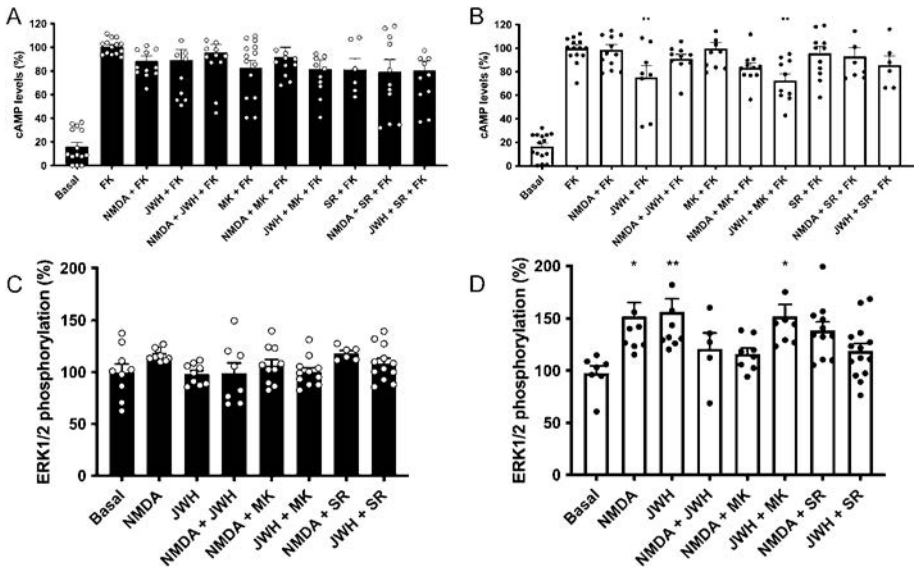

Fig. 4 Signaling in primary microglia activated with LPS and IFN-γ. Primary microglial cells were incubated for 48 h in the absence (black bars) or in the presence (white bars) of 1 μM LPS and 200 U/mL IFN-γ. Cells were treated with selective agonists (15 μM NMDA for NMDA channel, and/or 100 nM JWH-133 for CB_2R) and cAMP levels and MAPK pathway activation were determined. As G_i coupling was assessed, decreases in cAMP levels were determined in cells previously treated with 0.5 μM forskolin (15 min). When indicated cells were pretreated with selective receptor antagonists (1 μM MK-801 for NMDA or 1 μM SR-144528 for CB_2R). Values are the mean ± S.E.M. of 5 independent experiments performed in triplicates. One-way ANOVA followed by Bonferroni's multiple comparison post hoc test were used for statistical analysis (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus forskolin treatment in cAMP determinations or versus vehicle treatment (basal) in ERK phosphorylation assays). ANOVA summary: **A** F: 12.0, $p<0.001$; **B** F: 30.0, $p<0.001$, **C** F: 1.8, $p<0.093$, **D** F: 4.1, $p<0.001$

JWH-133, did not produce any significant decrease of forskolin-induced cAMP levels (Fig. 4A). This is likely due to the low CB_2R expression levels in resting microglia. In contrast, in activated microglial cell cultures, in which the CB_2R is upregulated, JWH-133 treatment induced a significant decrease of forskolin-induced cAMP levels which was completely blocked by the CB_2R antagonist, SR144528, but not by NMDAR antagonist, MK-801. However, no effect was found when the two agonists were added together, i.e., a negative cross-talk was detected (Fig. 4B). These results were similar to those observed in transfected HEK-293T cells.

In resting cells the effect on MAPK pathway activation of either JWH-133 or NMDA was not significant (Fig. 4C). In activated microglia, the significant effect of both CB_2R and NMDAR agonists on increasing ERK1/2 phosphorylation was, however, significantly decreased when the two agonists were added together (Fig. 4D). Pretreatment with the NMDAR antagonist, MK-801, did not block the effect of JWH-133, whereas the CB_2R antagonist, SR-144528, slightly decreased the NMDAR-mediated effect. These results are consistent with the occurrence of CB_2-NMDA-Hets in activated microglia in which CB_2R activation exerts a negative regulation over the NMDAR link to the MAPK pathway.

Differential levels of CB_2-NMDA-Hets in neurons and microglia from $APP_{Sw/Ind}$ mice

Finally, we investigated the levels and cross-talk of CB_2R/NMDAR complexes in primary hippocampal neurons of control and $APP_{Sw/Ind}$ mice, a transgenic line that expresses the human amyloid precursor protein (APP) harboring the Indiana and Swedish mutations. First, the expression of CB_2-NMDAR-Hets was determined by in situ PLA (see the "Materials and Methods" section). Compared with control mice, CB_2-NMDA-Hets expression was circa two-fold higher in primary neurons and circa 2.6-fold higher in microglia from $APP_{Sw/Ind}$ mice (Fig. 5A–F). These results demonstrate that CB_2R-NMDA receptor complexes are aberrantly increased in primary neurons and microglia of $APP_{Sw/Ind}$ mice.

CB_2R activation blocks NMDAR-mediated signaling in primary hippocampal neurons from $APP_{Sw/Ind}$ mice

Intracellular cAMP levels were determined in primary neurons treated with forskolin and with selective receptor agonists. Whereas CB_2R activation produced a significant 30% decrease of forskolin-induced cAMP levels,

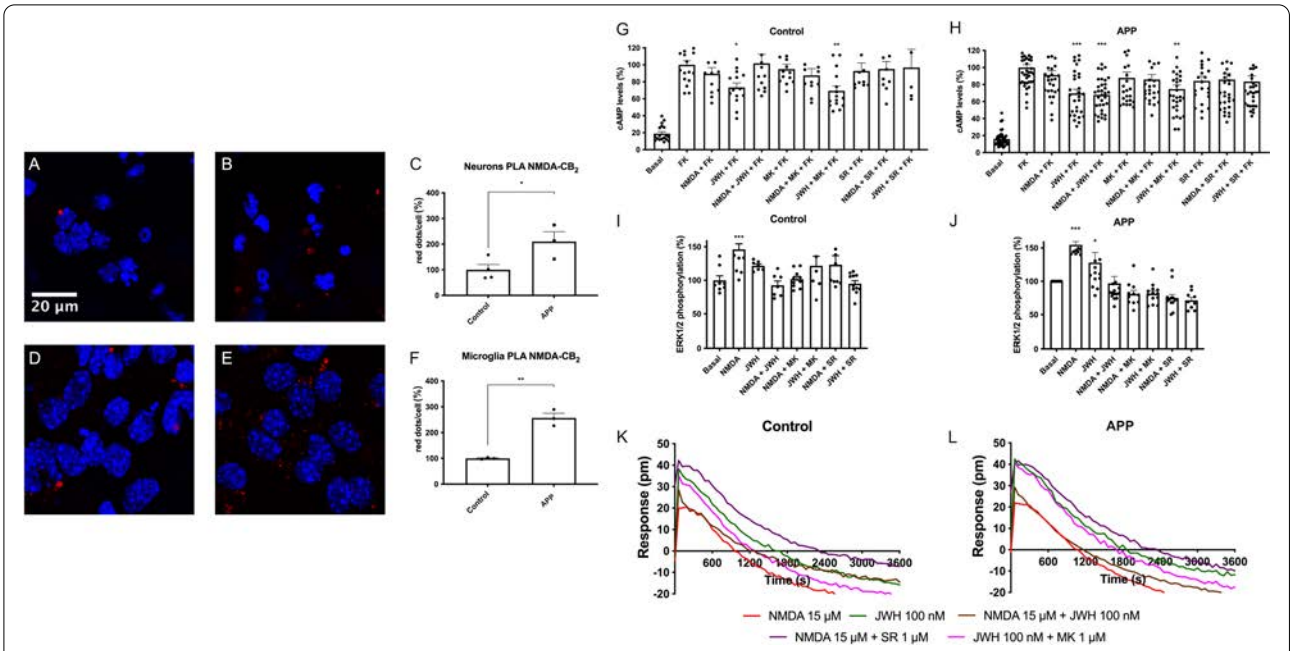

Fig. 5 NMDA-CB_2R heteromer levels and functionality in $APP_{Sw/Ind}$ neurons. **A**–**F** Expression of NMDA-CB_2R heteromers in mouse primary neurons (**A**, **B**) and microglia (**D**, **E**) of wild type (**A**, **D**) and $APP_{Sw/Ind}$ transgenic mice (B, E) as determined by PLA (see Materials and Methods) using specific primary antibodies against the GluN1 subunit and against the CB_2R receptor. Confocal microscopy images (stacks of 3 consecutive panels) show heteroreceptor complexes as red clusters over Hoechst-stained nuclei (blue). Three independent experiments were performed using, for each condition, 5 preparations per session. Bar graphs show the amount of red dots/cell in $APP_{Sw/Ind}$ mice and control animals (*$p < 0.05$, **$p < 0.01$; Student's *t* test versus the control condition). **G**–**L** Primary neurons from control and $APP_{Sw/Ind}$ mice were treated with selective agonists (15 µM NMDA for NMDA channel, and/or 100 nM JWH-133 for CB_2R) and cAMP levels (**G**, **H**), ERK1/2 phosphorylation (**I**, **J**), and DMR (**K**, **L**) assays were determined. When indicated cells were pretreated with selective receptor antagonists (1 µM MK-801 for NMDA or 1 µM SR-144528 for CB_2R). Values are the mean ± S.E.M. of 5 independent experiments performed in triplicates. One-way ANOVA followed by Bonferroni's multiple comparison post hoc test were used for statistical analysis (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus forskolin treatment in cAMP determinations or versus vehicle treatment (basal) in ERK phosphorylation assays). ANOVA summary: **G** *F*: 14.5, $p<0.001$; **H** *F*: 25.3, $p<0.001$, **I** *F*: 5.6, $p<0.001$, **J** 13.2, $p<0.001$

NMDA did not generate any significant effect but counteracted the activation of the cannabinoid receptor (Fig. 5G). Thus, the negative cross-talk observed in transfected HEK-293T cells was also noticeable in primary neurons, thus suggesting the occurrence of functional CB_2-NMDA-Hets in hippocampal neurons. A similar phenomenon was also observed upon analysis of MAPK pathway activation: JWH-133 and NMDA induced ERK1/2 phosphorylation, which was undetectable in cells simultaneously treated with the two agonists (Fig. 5I). In addition, as observed in HEK-293T cells, pretreatment with the CB_2R selective antagonist blocked the JWH-133-induced signal while exerting no significant effect on NMDAR activation. Reciprocally, the NMDAR antagonist reverted the effect of NMDA but not that due to JWH-133. Finally, similar results were obtained in DMR label-free assays (Fig. 5K), i.e., NMDA receptor activation blocked CB_2R function and vice versa. Moreover, as observed in transfected HEK-293T cells, SR144528 pretreatment potentiated the NMDAR action.

Finally, we investigated the expression and cross-talk of cannabinoid CB_2 and NMDA receptors in primary neurons from control and $APP_{Sw/Ind}$ mice. Interestingly, it was observed that CB_2R activity was potentiated in $APP_{Sw/Ind}$ neurons, and contrary to control mice, it was not blocked by cotreatment with NMDA (see Fig. 5H). Upon analysis of MAPK pathway activation, JWH-133 and NMDA induced ERK1/2 phosphorylation, which was undetectable in cells simultaneously treated with the two agonists (Fig. 5J). Remarkably, the pretreatment with the CB_2R selective antagonist blocked the NMDAR-mediated effect in samples from control animals, and with more potency than in $APP_{Sw/Ind}$ neurons. This cross-antagonism was found in the opposite direction, i.e. the antagonist of the NMDAR completely blocked the JWH-133 effect. Finally, in dynamic mass redistribution assays, a negative cross-talk was identifiable when the two agonists were added together. This phenomenon, which was observed in neurons from both control and $APP_{Sw/}$

$_{Ind}$ mice (Fig. 5L), indicates that NMDAR activation blocks CB_2R function and vice versa.

CB_2-NMDA-Hets expression increase in cortical slices from $APP_{Sw/Ind}$ compared to those from age-matched mice

As neuronal cultures may change the phenotype upon a time in culture, we wanted to assess in cortical slices of adult mice the expression and functionality of the CB_2-NMDA-Hets, with special focus in the $APP_{Sw/Ind}$ AD model. The PLA results (Fig. 6A–F) demonstrated a higher expression of the CB_2-NMDA-Het in slices from adult $APP_{Sw/Ind}$ mice (compared with those from age-matched control mice). On the other hand, treatment with the selective CB_2R agonist, JWH-133, lead to a marked decrease in CB_2-NMDA-Het expression, not only in $APP_{Sw/Ind}$ slices but also in those from control mice. Functionality was assessed by means of assays of ERK1/2 phosphorylation in adult mice to find, in slices from control animals, that both JWH-133 and NMDA led to MAPK pathway activation and that there was neither an additive effect nor cross-antagonism (Fig. 6G). In contrast, the MAPK pathway was slightly activated when slices from $APP_{Sw/Ind}$ mice brain were incubated with JWH-133. The slices were refractory to the action of exogenous NMDA, irrespective of the presence or absence of JWH-133 (Fig. 6H). These results indicate that the link of the NMDAR to the MAPK pathway is blocked in brain cortical slices of adult $APP_{Sw/Ind}$ mice.

Discussion

Ionotropic glutamate receptors are essential for the proper functioning of the mammalian CNS. The NMDA receptor is key to many aspects of glutamate-mediated actions in the nervous system, for excitatory neurotransmission, but also for development and neurogenesis. However, a dark side is related to excitotoxicity due to excessive levels of extracellular glutamate and the subsequent accumulation of toxic levels of ions in the cytoplasm of neurons. In this sense, it

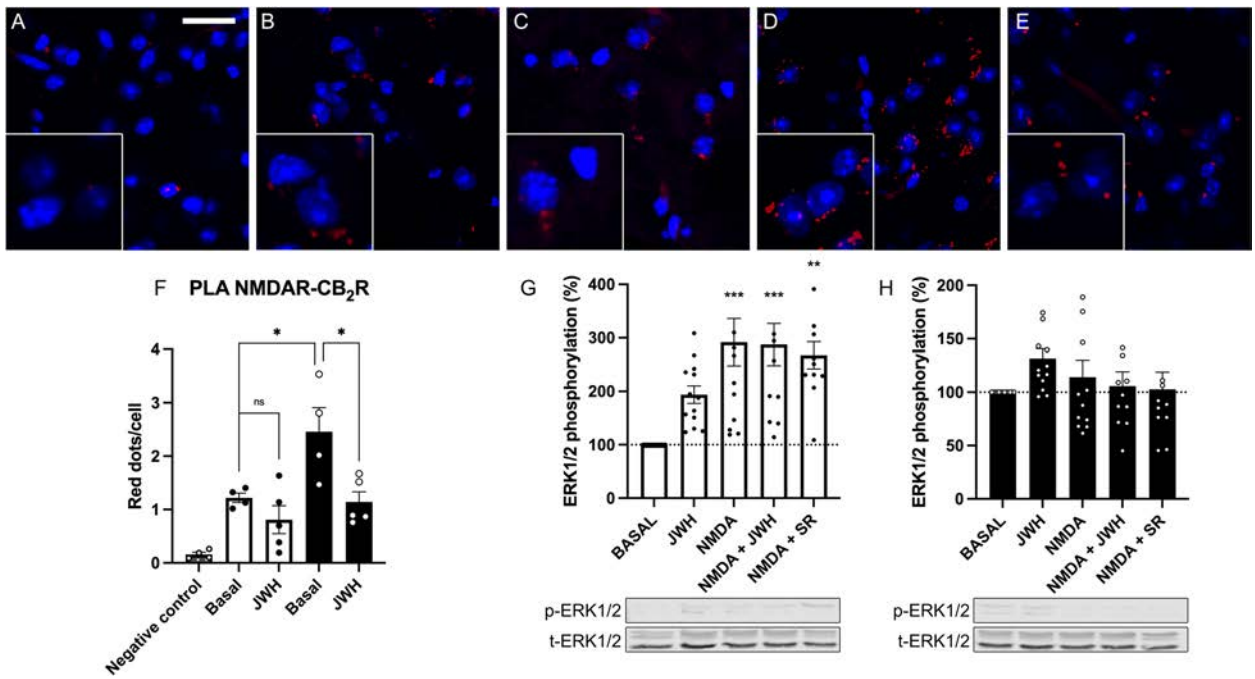

Fig. 6 NMDA-CB_2R heteromer levels and functionality in adult $APP_{Sw/Ind}$ mice brain slices. **A**–**F** Expression of NMDA-CB_2R heteromers in adult brain (brain cortex) slices from control (**B**, **C**) and $APP_{Sw/Ind}$ transgenic mice (**D**, **E**) as determined by PLA (see the "Materials and Methods" section) using specific primary antibodies against the GluN1 subunit and against the CB_2R receptor. For negative control, only the anti-GluN1 antibody was used (**A**). Confocal microscopy images (stacks of 3 consecutive panels) show heteroreceptor complexes as red clusters; nuclei were stained with Hoechst (blue). Three independent experiments were performed using, for each condition, 5 preparations per session. Scale bar: 40 µm. Bar graphs (**F**) show the amount of red dots/cell in $APP_{Sw/Ind}$ mice and control animals (*$p < 0.05$; One-way ANOVA followed by Bonferroni's multiple comparison *post* hoc test were used for statistical analysis). MAPK phosphorylation assays were performed in control (**G**) and $APP_{Sw/Ind}$ (**H**) transgenic mice brain slices. Slices were treated with selective agonists (15 µM NMDA, and/or 100 nM JWH-133). When indicated, slices were pretreated with the CB_2R selective receptor antagonist (1 µM SR-144528). Results are expressed as a percentage over basal and are the extracellular signal regulated (ERK) 1/2 phosphorylation mean ± SEM signals of three independent experiments performed in triplicates. ANOVA summary: **F** *F*: 10.4, $p<0.001$, **G** *F*: 7.7, $p<0.001$, **H** *F*: 1.2 , n.s

was hypothesized that NMDA would be a target of neurodegenerative diseases since, in fact, among the few existing drugs to combat AD, a negative allosteric NMDAR modulator, Memantine, was approved several years ago. Although the main interest has logically focused on neurons, NMDARs are expressed in glial cells, where they play a critical role in maintaining brain homeostasis. This work was based on the hypothesis that the action of NMDAR could be regulated by cannabinoid receptors and we focused on neurons and microglia, whose activation phenotype affects the progression of AD.

Although the different GPCRs tend to form dimers and oligomers, it was assumed that the multimeric structure of ionotropic receptors prevented the addition of more proteins to form macromolecular complexes with particular physiological properties. In fact, few examples of direct interactions between ionotropic receptors and GPCRs have been reported. We had previously demonstrated the interaction of a GPCR, which is a target for neuroprotection, the adenosine A_{2A} receptor, and NMDAR. This complex appears to have a relevant role in activated microglia where these complexes, which are expressed in the microglia of WT animals, are markedly upregulated in cells of AD transgenic mice [29].

In this report, we first addressed the possibility of a direct interaction between CB_2R and NMDAR, and the results were positive, that is, these two receptors can form complexes that alter the effect exerted by NMDA or CB_2R agonists. This finding is remarkable and confirms that GPCRs that are relevant to maintaining a correct neuroprotective balance (the adenosine A_{2A} receptor is a significant example ([29]) can interact with ionotropic receptors. Although CB_2R is less abundant in neurons than the cannabinoid CB_1 receptor, it can be found in neurons in different brain regions, and here we were able to find CB_2-NMDA-Hets in primary hippocampal neurons (Fig. 5). In microglia, heteromers were present but at a lower level at resting than in activated cells; this is likely due to the increase in CB_2R, whose expression in resting conditions was quite low.

In the heterologous expression system, the most noticeable property of the CB_2-NMDA-Hets was the negative cross-talk, namely simultaneous treatment with the two agonists led to the absence of response in either the Gi/adenylate cyclase/cAMP pathway or the MAPK pathway. Because CB_2R agonists could not, in the heteromeric context, significantly phosphorylate ERK1/2, the blocking effect appears to be direct, that is, due to intra-CB_2-NMDA-Het allosteric interactions and conformational changes upon binding of cannabinoids to the CB_2R. Perhaps the most relevant effect was the reduction by CB_2R agonists of the ionotropic function of the NMDAR. The cross-antagonism found in complexes formed by two GPCRs was not found in HEK-293T cells expressing CB_2 and NMDA receptors. This contrast with the $A_{2A}R$-NMDAR properties whose structure allows detecting cross-antagonism when GPCR-GPCR heteromer formation is suspected [19, 34, 35].

Importantly, CB_2-NMDA-Hets were also detected in hippocampal neurons, although the glutamate/cannabinoid relationships are more complex. In WT animals, the findings related to Gi-coupled actions of the CB_2R were similar to those found in the heterologous cells; basically, there was a negative cross-talk. However, this cross-talk was not found in cells from the $APP_{Sw/Ind}$ transgenic mice. On the one hand, these findings show that hippocampal neurons from WT and transgenic mice are different, already, in the early steps of CNS development; the AD-like phenotype takes months to be detectable. On the other hand, the results may indicate a lack of complexes in neurons from the AD mouse model. The cross-antagonism detected in samples from those mice, i.e., the antagonist of one receptor blunted the link to the MAPK pathway and vice versa, shows that CB_2-NMDA-Hets are present. Thus the most reasonable hypothesis is that different populations of receptors coexist and that the CB_2-NMDA-Het is one of them. This hypothesis could explain why NMDA does not affect the JWH-133 effect on cAMP levels; perhaps Gi-coupled to CB_2R are not interacting with NMDARs in neurons from transgenic animals or the CB_2-NMDA-Het is not well coupled to the Gi. Remarkably, the negative cross-talk in the link to the MAPK pathway occurs in both neurons from WT and from $APP_{Sw/Ind}$ animals. The presence of complexes was confirmed by PLA, which furthermore showed that the expression of the CB_2-NMDA-Het increases in both microglia and neurons from the transgenic mice (compared with levels in WT mice).

NMDAR is a target to combat AD. However, drugs that directly affect its function are not effective in the medium/long term [7]. Finding GPCRs that can interact and modulate NMDAR-mediated function holds promise for innovative treatments targeting neurons, microglia, or both. In the case of $A_{2A}R$, impairment of NMDAR function by $A_{2A}R$ antagonists is an attractive possibility. In the present study, cannabinoids could provide equivalent benefits by significantly reducing the effect of agonists that activate NMDAR. With the exception of Δ^9-tetrahydrocannabinol (THC), which produces CB_1R-mediated psychotropic effects, most of the natural cannabinoids studied so far are generally safe. Additionally, there is an increased interest in cannabinoids as potential drugs to combat a variety of diseases [36–38].

A deleterious factor in neurodegenerative diseases, including AD, is excitotoxicity, that is, the aberrant

increase in cytoplasmic Ca^{2+} levels after excessive stimulation of NMDAR by extracellular glutamate [39]. Since allosteric modulators that act directly on NMDAR do not provide much help to AD patients, an interacting GPCR-mediated allosteric modulation is an attractive possibility to explore further. In the case of CB_2R, its complex pharmacology can be an added value to find the best way to regulate the NMDAR function. In fact, cannabinoids show multiple modes of binding and biased signaling due to the wormhole-like structure of their orthosteric site and due to the existence of various non-orthosteric binding sites. Multiple modes of cannabinoid binding to CB_2R lead to specific receptor conformations underlying functional selectivity (biased agonism) [40] and, ultimately, differentially regulating NMDAR function. As an example, we have designed bitopic ligands that bind to an exosite located at the entrance of the structure that connects the orthosteric site with the lipid bilayer [41]. These findings constitute a selective advantage since the expression of CB_2-NMDA-Het increases in neurons and microglia of $APP_{Sw/Ind}$ transgenic mice.

Study limitations

The main and most common limitation is related to the AD transgenic model. There is not any model that could appropriately cover the non-familial cases of the disease. Another limitation is that modulation cannot be tested in human neurons and that the occurrence of the dimer has to be confirmed using human brain samples. However, the $APP_{Sw/Ind}$ transgenic mice is one of the most used animal models of AD. The results of the study strongly indicate that cannabinoids negatively modulate the NMDAR function like memantine does. Then it would be possible to design a clinical trial looking for extra efficacy of one of the known safe cannabinoids acting on CB_2 receptors when combined with memantine.

Conclusions

In conclusion, the present study demonstrates that CB_2 and NMDA receptors form CB_2R-NMDAR complexes that are expressed in cells of the CNS. CB_2R-NMDAR complexes are novel functional units with singular signaling properties. Particularly, activation of the cannabinoid receptor reduces the signaling output of NMDA. In addition, the level of expression of the CB_2-NMDA complexes increases in both microglia and neurons from the $APP_{Sw/Ind}$ transgenic mice AD model, compared with levels in WT mice.

Materials and methods

Reagents

Lipopolysaccharide (LPS), interferon-γ (IFN-γ), JWH-133 (JWH), and SR-144528 (SR) were purchased from Sigma-Aldrich (St Louis, MO, USA). N-Methyl-D-aspartate (NMDA), MK-801 (MK), and forskolin (FK) were purchased from Tocris (Bristol, UK). Tau and p-Tau proteins were kindly provided by Prof. J. Avila (CBM, UAM-CSIC, Madrid, Spain). Detailed descriptions of the elaboration and processing of proteins can be found elsewhere [42, 43]

HEK-293T cells and primary cultures

Human embryonic kidney HEK-293T (lot 612968) cells were acquired from the American Type Culture Collection (ATCC). They were amplified and frozen in liquid nitrogen in several aliquots. Cells from each aliquot were used until passage 12.

HEK-293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 2 mM L-glutamine, 100 µg/ml sodium pyruvate, 100 U/ml penicillin/streptomycin, MEM non-essential amino acids solution (1/100), and 5% (v/v) heat-inactivated fetal bovine serum (FBS) (all supplements were from Invitrogen, Paisley, Scotland, UK). Cells were maintained at 37 °C in a humid atmosphere of 5% CO_2.

To prepare mice hippocampal primary microglial cells, the brain was removed from C57BL/6 mice of 2–4 days of age. Microglial cells were isolated as described in [44]. Briefly, the brain was dissected, carefully stripped of its meninges and the hippocampus was digested with 0.25% trypsin for 20 min at 37 °C. Trypsinization was stopped by washing the tissue. Cells were brought to a cell suspension by passage through 0.9 mm and 0.5 mm nails followed by passage through a 100 µm pore mesh. Glial cells were resuspended in medium and seeded at a density of 1×10^6 cells/ml in 6-well plates for cyclic adenylic acid (cAMP) assays, in 12-well plates with coverslips for in situ proximity ligation assays (PLA) and in 96-well plates for mitogen-activated protein kinase (MAPK) activation experiments. Cultures were grown in DMEM medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, MEM non-wssential amino acids preparation (1/100) and 5% (v/v) heat-inactivated fetal bovine serum (FBS) (Invitrogen, Paisley, Scotland, UK) and maintained at 37°C in humidified 5% CO_2 atmosphere and, unless otherwise stated, medium was replaced once a week.

For culturing primary neurons, the hippocampus from mouse embryos (E19) was removed and the neurons were isolated as described by [45] [45] and plated at a density of circa 120,000 cells/cm^2. Cells were grown in a neurobasal medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, and 2% (v/v) B27 supplement (Gibco) in a 6-, 12- or 96-well plate for 12 days. Cultures were maintained at 37ºC in a humidified 5% CO_2 atmosphere and the medium was replaced every 4–5 days.

Immunodetection of specific markers (Neu N for neurons and CD-11b for microglia) showed that neuronal preparations contained >98% neurons and microglia preparations contained, at least, 98% microglial cells [28].

Preparation of brain cortex slices

Mouse brains were extracted in a cold chamber at a temperature of 4°C. Brain slices (BS) with a thickness of 500 μm were made with the aid of a mouse coronal matrix (Agnthos, ref. 69-2165) and subsequently, the cortical region was isolated. Brain slices were maintained in Krebs's buffer (124 mM NaCl, 4 mM KCl, 1,25 mM KH_2PO_4, 1,5 mM $MgSO_4$, 10 mM glucose, 26 mM $NaHCO_3$, 1,5 mM $CaCl_2$ and carbogen). Brain slices were incubated for 2 h at 32 °C in a humidified 5% CO_2 atmosphere, Krebs's buffer was replaced once in the first 30 min. After that, BSC were treated or not for 15 min with the selective antagonist for CB_2R (SR-144528 (1 μM)) followed by 15 min treatment with the selective agonists (NMDA (15 μM) and/or JWH-133 (100 nM)). After treatment, the slices were immediately frozen in dry ice to stop the metabolic activity.

On the one hand, ERK1/2 phosphorylation was determined by Western blot. Samples were sonicated on ice with 10-s pulse, 20-s rest, and 10-s pulse, using lysis buffer for tissue disaggregation and cell lysis. Final lysates total protein was adjusted to 2 μg/μL with SDS and lysis buffer using BCA quantification. Equivalent amounts of protein (40 μg) were subjected to electrophoresis (10% SDS-polyacrylamide gel) and transferred onto PVDF membranes (Immobilon-FL PVDF membrane, MERK, St. Louis, MO, USA) for 30 min using Trans-Blot Turbo system (Bio-Rad). Then, the membranes were blocked for 2 h at room temperature (constant shaking) with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE, USA) and labeled with a mixture of primary mouse anti-phospho-ERK 1/2 antibody (1:2500, MERK, Ref. M8159), primary rabbit anti-ERK 1/2 antibody (1:40,000, MERK, Ref. M5670), which recognizes both phosphorylated and non-phosphorylated ERK1/2 overnight at 4 °C with shaking. Then, the membranes were washed three times with PBS containing 0.05% tween for 10 min and subsequently were incubated by the addition of a mixture of IRDye 800 anti-mouse antibody (1:10,000, MERK, Ref. 92632210) and IRDye 680 anti-rabbit antibody (1:10,000, MERK, Ref. 926-68071) for 2 h at room temperature, light-protected. Membranes were washed 3 times with PBS-tween 0.05% for 10 minutes and once with PBS and left to dry. Bands were analyzed using Odyssey infrared scanner (LI-COR Biosciences). Band densities were quantified using Fiji software, and the level of phosphorylated ERK1/2 was normalized using the total ERK 1/2 protein band intensities. Results obtained are represented as the percent over basal (non-stimulated cells).

$APP_{Sw/Ind}$ transgenic mice

$APP_{Sw/Ind}$ transgenic mice (line J9; C57BL/6 background) expressing the human APP695 harboring the FAD-linked Swedish (K670N/M671L) and Indiana (V717F) mutations under the platelet-derived growth factor subunit B (PDGFβ) promoter were obtained by crossing heterozygous $APP_{Sw/Ind}$ to non-transgenic (control) mice [46]. Control and $APP_{Sw;Ind}$ embryos (E16.5) and adult mice (6 months) were genotyped individually, and hippocampus/cortex dissected and prepared for microglia and neuron primary cultures as described elsewhere [22, 28]. All experimental procedures were conducted according to the approved protocols from the Animal and Human Ethical Committee of the Universitat Autònoma de Barcelona (CEEAH 2895) and Generalitat de Catalunya (10571) following the experimental European Union guidelines and regulations (2010/63/EU)

Fusion proteins

Human cDNAs for the GluN1 NMDA receptor subunit, for the CB_2 receptor, and for the ghrelin GHS1a receptor, all cloned into pcDNA3.1 were amplified without their stop codons using sense and antisense primers harboring either BamHI and HindIII restriction sites to amplify GluN1, BamHI, and KpnI restriction sites to amplify CB_2 receptor or EcoRI and KpnI restriction sites to amplify GHS1a receptor. Amplified fragments were then subcloned to be in frame with an enhanced yellow fluorescent protein (pEYFP-N1; Clontech, Heidelberg, Germany) or a RLuc (pRLuc-N1; PerkinElmer, Wellesley, MA) on the C-terminal end of the receptor to produce GluN1-RLuc, CB_2R-YFP, and GHSR1a-YFP fusion proteins.

Cell transfection

HEK-293T cells were transiently transfected with the corresponding cDNA by the PEI (PolyEthylenImine, Sigma-Aldrich) method. Briefly, the corresponding cDNA diluted in 150 mM NaCl was mixed with PEI (5.5 mM in nitrogen residues) also prepared in 150 mM NaCl for 10 min. The cDNA-PEI complexes were transferred to HEK-293T cells and were incubated for 4 h in a serum-starved medium. Then, the medium was replaced by a fresh supplemented culture medium, and cells were maintained at 37 °C in a humid atmosphere of 5% CO_2. 48 h after transfection, cells were washed, detached, and resuspended in the assay buffer.

Immunocytochemistry

HEK-293T cells were seeded on glass coverslips in 12-well plates. Twenty-four hours after, cells were transfected with CB_2-YFP cDNA (1 μg), GluN1-RLuc cDNA (1 μg), and GluN2B cDNA (0.75 μg). Forty-eight hours after, cells were fixed in 4% paraformaldehyde for 15 min and washed twice with PBS containing 20 mM glycine before permeabilization with PBS-glycine containing 0.2% Triton X-100 (5 min incubation). Cells were blocked during 1 h with PBS containing 1% bovine serum albumin. HEK-293T cells were labeled with a mouse anti-RLuc antibody (1/100; Millipore, Darmstadt, Germany) and subsequently treated with Cy3-conjugated anti-mouse (1/200; Jackson ImmunoResearch (red)) antibody (1 h each). The CB_2R-YFP expression was detected by the YFP's own fluorescence. Nuclei were stained with Hoechst (1/100 from stock 1 mg/mL; Sigma-Aldrich). Samples were washed several times and mounted with 30% Mowiol (Calbiochem).

Images were obtained in a Zeiss LSM 880 confocal microscope (ZEISS, Germany) with the 40X and 63X oil objectives.

Bioluminescence resonance energy transfer (BRET) assay

For BRET assay, HEK-293T cells were transiently cotransfected with a constant amount of cDNA encoding for GluN1-RLuc (0.25 μg) and GluN2B (0.15 μg) and with increasing amounts of cDNA corresponding to CB_2R-YFP (0.25 to 1.25 μg). As a negative control, HEK-293T cells were transiently cotransfected with a constant amount of cDNA encoding for GluN1-RLuc (0.25 μg) and GluN2B (0.15 μg) and with increasing amounts of cDNA corresponding to GHSR1a-YFP (0.25 to 1.5 μg). To control the cell number, sample protein concentration was determined using a Bradford assay kit (Bio-Rad, Munich, Germany) using bovine serum albumin (BSA) dilutions as standards. To quantify fluorescent proteins, cells (20 μg of total protein) were distributed in 96-well microplates (black plates with a transparent bottom) and fluorescence was read in a Fluostar Optima Fluorimeter (BMG Labtech, Offenburg, Germany) equipped with a high-energy xenon flash lamp, using a 10-nm bandwidth excitation filter at 485 nm. For BRET measurements, the equivalent of 20 μg of total protein cell suspension was distributed in 96-well white microplates with a white bottom (Corning 3600, Corning, NY). BRET was determined one minute after adding coelenterazine H (Molecular Probes, Eugene, OR), using a Mithras LB 940 plate reader (Berthold Technologies, DLReady, Germany), which allows the integration of the signals detected in the short-wavelength filter at 485 nm and the long-wavelength filter at 530 nm. To quantify GluN1-RLuc expression, luminescence readings were obtained 10 min after the addition of 5 μM coelenterazine H. MilliBRET units (mBU) are defined as:

$$\mathrm{mBU} = \left[\frac{\lambda_{530}(\text{long} - \text{wavelength emission})}{\lambda_{485}(\text{short} - \text{wavelength emission})} - C_f \right] \times 1000$$

where C_f corresponds to [(long-wavelength emission)/(short-wavelength emission)] for the RLuc construct expressed alone in the same experiment.

cAMP level determination

The analysis of cAMP levels was performed in HEK-293T cells cotransfected with the cDNA for two subunits of the NMDA receptor, GluN1 (1 μg) and GluN2B (0.75 μg) or/and/or the cDNA for the CB_2R (1 μg). Similar assays were also performed in primary microglia and primary neurons prepared from wild-type mice or the transgenic $APP_{Sw/Ind}$ AD mice model. In the case of microglia cells were first activated using 1 μM LPS and 200 U/mL IFN-γ (48 h). Two hours before the experiment, the medium was substituted by serum-starved DMEM medium. Cells growing in a medium containing 50 μM zardaverine were distributed in 384-well microplates (2000 HEK-293T cells or 4000 hippocampal neurons or microglial cells per well) followed by the stimulation with the NMDA and/or CB_2R agonists (NMDA (15 μM) and/or JWH-133 (100 nM)) for 15 min before adding 0.5 μM forskolin or vehicle for an additional 15 min period. When indicated cells were pre-treated (15 min) with the NMDA or CB_2R antagonists, respectively, MK-801 (1 μM) or SR-144528 (1 μM). Homogeneous time-resolved fluorescence energy transfer (HTRF) measures were performed using the Lance Ultra cAMP kit (PerkinElmer). Fluorescence at 665 nm was analyzed on a PHERAstar Flagship microplate reader equipped with an HTRF optical module (BMG Labtech). A standard curve for cAMP was obtained in each experiment.

MAP kinase pathway activation is measured by ERK1/2 phosphorylation

Hippocampal neurons, microglial cells, or HEK-293T cells cotransfected with the cDNA for the protomers of the NMDA receptor, GluN1 (1 μg) and GluN2B (0.75 μg), and/or with the cDNA for CB_2R (1 μg) were plated in transparent Deltalab 96-well microplates. Primary microglial cells were activated by incubating cells with 1 μM LPS and 200 U/mL IFN-γ during 48 h. Two hours

before the experiment, the medium was substituted by serum-starved DMEM medium. Cells were treated or not for 10 min with the selective antagonists (MK-801 (1 μM) or SR-144528 (1 μM)) followed by 7 min treatment with the selective agonists (NMDA (15 μM) and/or JWH-133 (100 nM)). Cells were then washed twice with cold PBS before the addition of lysis buffer (15 min treatment). Ten microliters of each supernatant was placed in white ProxiPlate 384-well microplates and ERK1/2 phosphorylation was determined using an AlphaScreen®SureFire® kit (Perkin Elmer) following the instructions of the supplier and using an EnSpire® Multimode Plate Reader (PerkinElmer).

Detection of cytoplasmic calcium levels

HEK-293T cells were cotransfected with the cDNA for the protomers of the NMDA receptor channel GluN1 (1 μg) and GluN2B (0.75 μg), with thee cDNA for CB_2R (1 μg) and with the cDNA for the GCaMP6 calcium sensor (1 μg) [47] by the use of PEI method (Section "Cell Transfection"). 48 hours after transfection, HEK-293T cells plated in 6-well black, clear bottom plates, were incubated with Mg^{2+}-free Locke's buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5.6 mM glucose, 5 mM HEPES, 10 μM glycine, pH 7.4). Online recordings were performed right after the addition of agonists. When indicated cells were pre-treated with receptor antagonists for 10 min. Fluorescence emission intensity due to complexes GCaMP6 was recorded at 515 nm upon excitation at 488 nm on the EnSpire® Multimode Plate Reader for 150 s every 5 s at 100 flashes per well.

Dynamic mass redistribution (DMR) label-free assays

Cell signaling was explored using an EnSpire® Multimode Plate Reader (PerkinElmer) by a label-free technology. Cellular cytoskeleton redistribution induced upon receptor activation was detected by illuminating the underside of the plate with polychromatic light and measured as changes in wavelength of the reflected monochromatic light. The magnitude of this wavelength shift (in picometers) is directly proportional to the amount of DMR. To determine the label-free DMR signal, 10,000 HEK-293T cells cotransfected with cDNAs for the protomers of the NMDA receptor channel, GluN1 (1 μg) and GluN2B (0.75 μg) and/or with the cDNA for the CB_2R (1 μg). Similar assays were performed using 10,000 primary neurons from wild type or transgenic $APP_{Sw/Ind}$ mice. Transparent 384-well fibronectin-coated microplates were used until obtaining 70-80% confluent monolayers (kept in the incubator for 24 h). Previous to the assay, cells were washed twice with assay buffer (HBSS with 20 mM HEPES, pH 7.15, 0.1% DMSO) and incubated in the reader with assay buffer for 2 h at 24 °C. Hereafter, the sensor plate was scanned and a baseline optical signature was recorded for 10 min before adding 10 μL of selective agonists (NMDA (15 μM) and/or JWH-133 (100 nM)) also dissolved in assay buffer. When indicated cells were pre-treated with antagonists (MK-801 (1 μM) or SR-144528 (1 μM); 10 μL in volume). Real-time DMR responses were monitored for a minimum of 3600 s.

Proximity Ligation Assay (PLA)

Detection in natural sources of clusters formed by the NMDA and CB_2 receptors was addressed in slices of in primary hippocampal microglia and hippocampal neurons of wild type mice or the transgenic $APP_{Sw/Ind}$ mice model. When assays were performed in slices they were embedded in O.C.T. compound (OCT; Tissue Tek Products, Ames Division, Miles Laboratories, Inc., Elkhart, IN, USA) to allow cryostat sectioning (Leica CM3050S; 40 μm-thick sections). When using cells, they were grown on glass coverslips, were fixed in 4% paraformaldehyde for 15 min, washed twice with PBS containing 20 mM glycine to quench the aldehyde groups, permeabilized with the same buffer containing 0.05% Triton X-100 between 5 and 15 min and washed with PBS. After 1 h incubation at 37 °C with the blocking solution in a pre-heated humidity chamber, samples were incubated overnight at 4 °C with a mixture of a rabbit monoclonal anti-GluN1 antibody (1/100, ab52177, Abcam, Cambridge, UK) and a mouse monoclonal anti-CB_2R antibody (1/100, sc-293188, Santa Cruz Biotechnology, TX, USA). Nuclei were stained with Hoechst (1/100 from 1 mg/mL stock; Sigma-Aldrich). The antibodies were validated following the method in the technical brochure of the vendor with fairly similar results. Cells were further processed using the PLA probes detecting primary antibodies (Duolink In Situ PLA probe Anti-Mouse plus and Duolink In Situ PLA probe Anti-Rabbit minus) (1/5 v:v for 1 h at 37 °C). Ligation and amplification were done as indicated by the supplier (Sigma-Aldrich) and cells were mounted using the mounting medium Mowiol (30%) (Calbiochem). To detect red dots corresponding to CB_2-NMDA-Hets, samples were observed in a Zeiss LSM 880 confocal microscope (ZEISS, Germany) equipped with an apochromatic 63X oil-immersion objective, and 405-nm and 561-nm laser lines. For each field of view, a stack of two channels (one per staining) and 3 *Z*-planes with a step size of 1 μm were acquired. Andy's algorithm, a specific ImageJ macro for reproducible and high-throughput quantification of the total PLA foci dots and total nuclei, was used for data analysis [48].

Statistical analysis

The data in graphs are the mean ± SEM (at least n=5). GraphPad Prism 9 software (San Diego, CA, USA) was used for data fitting and statistical analysis. One-way ANOVA followed by post hoc Bonferroni's test were used when comparing multiple values. Experiments performed in samples from transgenic mice and age-matched controls were analyzed independently, i.e., quantitative inter-group differences were not addressed. When a pair of values were compared, Student's t test was used. Significant differences were considered when the p value was <0.05.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13195-021-00920-6.

Additional file 1: Figure S1. Signaling in HEK-293T cells expressing NMDA and GHSR1a receptors. HEK-293T cells transfected with the cDNAs for two protomers of the NMDA receptor: GluN1 (1 μg) and GluN2B (0.75 μg) and/or with the cDNA for the GHS-R1a (1 μg), were treated with selective agonists (15 μM NMDA for NMDAR and/or 100 nM Ghrelin for GHS-R1a). When indicated cells were pretreated with selective receptor antagonists (1 μM MK-801 or 1 μM YIL-781 for GHS-R1a). Panels A-C: Intracellular cAMP levels were determined by TR-FRET as described in Methods. As G_i coupling was assessed, decreases in cAMP levels were determined in cells previously treated with 0.5 μM forskolin (15 min). Values are the mean ± S.E.M. of 3 independent experiments performed in triplicates. ANOVA Summary: Panel A; F: 90.9, p<0.001, Panel B; F: 52.1, p<0.001 and Panel C; F: 49.6, p<0.001.

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Authors' contributions

Conceptualization and design: RF and GN. Transgenic animals and genotyping: CAS. Experiments: RRS, AL, JL, JBR, JSC. Data analysis: RRS, AL, and JL. Figures: RRS. Validation of results: RF and GN. Writing of first draft: RRS, CAS, RF, and GN. All authors have edited and approved the submitted version.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This study uses mice and experimental protocols that have been approved by ad hoc Ethics Committee (see the "Materials and Methods" section "$APP_{Sw/Ind}$ Transgenic Mice" for details).

Consent for publication

The authors have approved the final version and concur with submission.

Competing interests

The authors declare that they have no competing interests.

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3.2 N-Methyl-D-Aspartate Receptor Link to the MAP Kinase Pathway in Cortical and Hippocampal Neurons and Microglia Is Dependent on Calcium Sensors and Is Blocked by α-Synuclein, Tau, and Phospho-Tau in Non-transgenic and Transgenic $APP_{Sw,Ind}$ Mice.

Rafael Franco, David Aguinaga, Irene Reyes, Enric I Canela, **Jaume Lillo**, Airi Tarutani, Masato Hasegawa, Anna Del Ser-Badia, José A Del Rio, Michael R Kreutz, Carlos A Saura, Gemma Navarro.

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Els receptors de N-metil-D-aspartat (NMDARs) responen al glutamat, permetent el flux d'ions de Ca^{2+} a l'interior citoplasmàtic i la senyalització de la via de les MAPK. Tant la via de senyalització per Ca^{2+} com per MAPK juguen un paper important en la neurotransmissió i correcte funcionament neuronal. Mitjançant l'ús d'un sistema heteròleg d'expressió hem demostrat que el receptor NMDA interacciona amb els sensors de calci calmodulina, calneuron-1 i NCS1, però no ho fa amb la caldendrina. El receptor NMDA es troba present en cultius primaris de neurones i micròglia tant de còrtex com d'hipocamp. La calmodulina en micròglia i neurones i el NCS1 en neurones, són necessaris per l'activació de la via de senyalització de les MAPK induïda per NMDA. La via de les MAPK es va veure bloquejada en cultius primaris de neurones i micròglia pel tractament amb α-sinucleïna, Tau, i pTau; proteïnes estretament relacionades amb malalties neurodegeneratives. Aquest bloqueig per proteïnes patogèniques també es va observar en cultius primaris del model d'Alzheimer $APP_{Sw,Ind}$. Un marcat increment de l'expressió de complexos NMDAR-NCS1 es va observar en neurones de ratolí $APP_{Sw,Ind}$, mentre que en micròglia $APP_{Sw,Ind}$, els complexos sobreexpresats van ser NMDAR-NCS1 i NMDAR-CaM. Aquests resultats demostren que l'α-sinucleïna, la Tau, i la pTau bloquegen la senyalització de NMDA i que els sensors de calci són importants per la correcta funcionalitat de NMDAR, tant en neurones com en micròglia. El fet que l'expressió dels complexos entre el NMDAR i els sensors de calci, especialment NCS1, es vegi alterada, sembla indicar que les cèl·lules neuronals contraresten aquest efecte de l'α-sinucleïna, la Tau, i la pTau, incrementant la senyalització del NMDA.

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N-methyl-D-aspartate receptors (NMDARs) respond to glutamate to allow the influx of calcium ions and the signaling to the mitogen-activated protein kinase (MAPK) cascade. Both MAPK- and Ca^{2+}-mediated events are important for both neurotransmission and neural cell function and fate. Using a heterologous expression system, we demonstrate that NMDAR may interact with the EF-hand calcium-binding proteins calmodulin, calneuron-1, and NCS1 but not with caldendrin. NMDARs were present in primary cultures of both neurons and microglia from cortex and hippocampus. Calmodulin in microglia, and calmodulin and NCS1 in neurons, are necessary for NMDA-induced MAP kinase pathway activation. Remarkably, signaling to the MAP kinase pathway was blunted in primary cultures of cortical and hippocampal neurons and microglia from wild-type animals by proteins involved in neurodegenerative diseases: α-synuclein, Tau, and p-Tau. A similar blockade by pathogenic proteins was found using samples from the $APP_{Sw,Ind}$ transgenic Alzheimer's disease model. Interestingly, a very marked increase in NMDAR–NCS1 complexes was identified in neurons and a marked increase of both NMDAR–NCS1 and NMDAR–CaM complexes was identified in microglia from the transgenic mice. The results show that α-synuclein, Tau, and p-Tau disrupt the

signaling of NMDAR to the MAPK pathway and that calcium sensors are important for NMDAR function both in neurons and microglia. Finally, it should be noted that the expression of receptor–calcium sensor complexes, specially those involving NCS1, is altered in neural cells from $APP_{Sw,Ind}$ mouse embryos/pups.

Keywords: Alzheimer's disease, calmodulin, calneuron-1, caldendrin, NCS1, extracellular signal-regulated kinase, glutamate receptor, proximity ligation assay

INTRODUCTION

As longevity increases, neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's (PD) diseases become a challenge for health and social security systems. In consequence, there is an urgent need for interventions that either prevent neurodegeneration or delay disease progression. Whereas PD management includes successful therapeutic strategies for symptom management, they do not stop neurodegeneration. The situation in the case of AD is worse; the current anti-AD drugs, acetylcholinesterase inhibitors, and *N*-methyl-D-aspartate receptor (NMDAR) modulators are of little anti-symptomatic efficacy and do not prevent disease progression (Gauthier et al., 2016; Szeto and Lewis, 2016).

The NMDAR is one of the most important mediators of excitatory neurotransmission in the brain. It is a tetrameric protein complex formed by two GluN1 and a combination of one or two GluN2A or GluN2B subunits. Receptor subunit expression varies in different brain regions and they convey slightly different neural responses. Interestingly, cortical and hippocampal receptors contain mainly GluN2A and GluN2B subunits (Watanabe et al., 1993; Monyer et al., 1994; Laurie et al., 1997), being those subunits associated to learning and memory (Woodhall et al., 2001; Bidoret et al., 2009). A decrease in NMDAR expression and a variation in subunit composition has been reported in senescence and in AD animal models (Ułas and Cotman, 1997; Wakabayashi et al., 1999; Mishizen-Eberz et al., 2004).

Irrespective of the fact that the NMDAR is a target of current anti-AD medications (memantine), extrasynaptically located NMDA receptors have a relevant role in neurodegeneration. Activation of extrasynaptic receptors may not only regulate expression of Tau (Paterlini et al., 1998) but they also induce transcriptional inactivation of CREB (Rönicke et al., 2011; Grochowska et al., 2017) and also contribute to early synaptic dysfunction. Thus, NMDAR might be relevant targets to prevent early synaptic dysfunction and potentially delay neuronal cell death.

Neural cells express calmodulin, which is ubiquitously expressed, and some other specific calcium-binding proteins that, upon Ca^{2+} binding, participate in events related to neurotransmission and plasticity. One of them, frequenin/NCS1 was first identified in the nervous system of *Drosophila* (see Dason et al., 2012 for review) and later found to be a relevant calcium sensor in the central nervous system of mammals. NCS1 like calneuron-1 and caldendrin, contains EF hand domains that participate in Ca^{2+} binding and mediate the conformational changes that unfolds a myriad of events affecting signaling pathways and impacting on gene transcription (McCue et al., 2010; Burgoyne and Haynes, 2012). Affinity for Ca^{2+} is variable and, for instance, calcium binds with less affinity to NCS1 than to calneuron-1 (Mikhaylova et al., 2006, 2009). Despite Ca^{2+} is the ion transported across NMDAR (see Pankratov and Lalo, 2014; Paoletti et al., 2013 for review), the modulatory role of EF-hand calcium-binding proteins in NMDA receptor function is poorly understood. In this study, we wanted to assess whether NMDAR may directly interact with calcium sensors in neural cells and whether this might affect the coupling of the NMDAR to downstream effectors.

N-methyl-D-aspartate receptor-mediated calcium influx is upstream of several intracellular signaling cascades. Interestingly, NMDARs are also expressed in glial cells where their physiological role is not yet fully elucidated. The first aim of this paper was to look for potential interactions between NMDA receptors and calcium-binding proteins. We identified in both neurons and microglia that calcium-binding proteins may interact with NMDAR and we determined how these proteins affect NMDAR-mediated MAP kinase activation. The second aim was to investigate how such NMDAR signaling may be affected by α-synuclein and Tau proteins. Finally, we analyzed whether the results obtained in non-transgenic mice were similar or not to those obtained using cortical and hippocampal neurons and microglia from the $APP_{Sw,Ind}$ transgenic AD mouse model.

RESULTS

NMDAR May Interact With Calneuron-1, Calmodulin, and NCS1

The activation of ionotropic NMDAR results in Ca^{2+}-influx and the kinetics as well as amplitude of these synaptic signals are decoded by calcium sensors (Raghuram et al., 2012). Previous data suggest an interaction of NMDAR and CaM (Ehlers et al., 1996) that was here confirmed by means of bioluminescence resonance energy transfer (BRET) assays in a heterologous expression system where GluN1 fused to Rluc and the GluN2B were co-expressed for proper NMDA receptor reconstitution and functional activity (**Figure 1**). First, immunocytofluorescence assays performed in HEK-293T cells co-expressing NMDAR and CaM showed a prominent degree of co-localization. Subsequently, a saturation curve demonstrating a specific interaction was obtained in BRET assays using GluN1Rluc and CaMYFP in the presence ($BRET_{max}$ 47 ± 2 mBU and $BRET_{50}$ 31 ± 6; **Figure 1**) or absence ($BRET_{max}$ 191 ± 8 mBU and $BRET_{50}$ 3.5 ± 1; **Supplementary Figure S1A**) of GluN2. We then tested the interaction of other neuron-specific

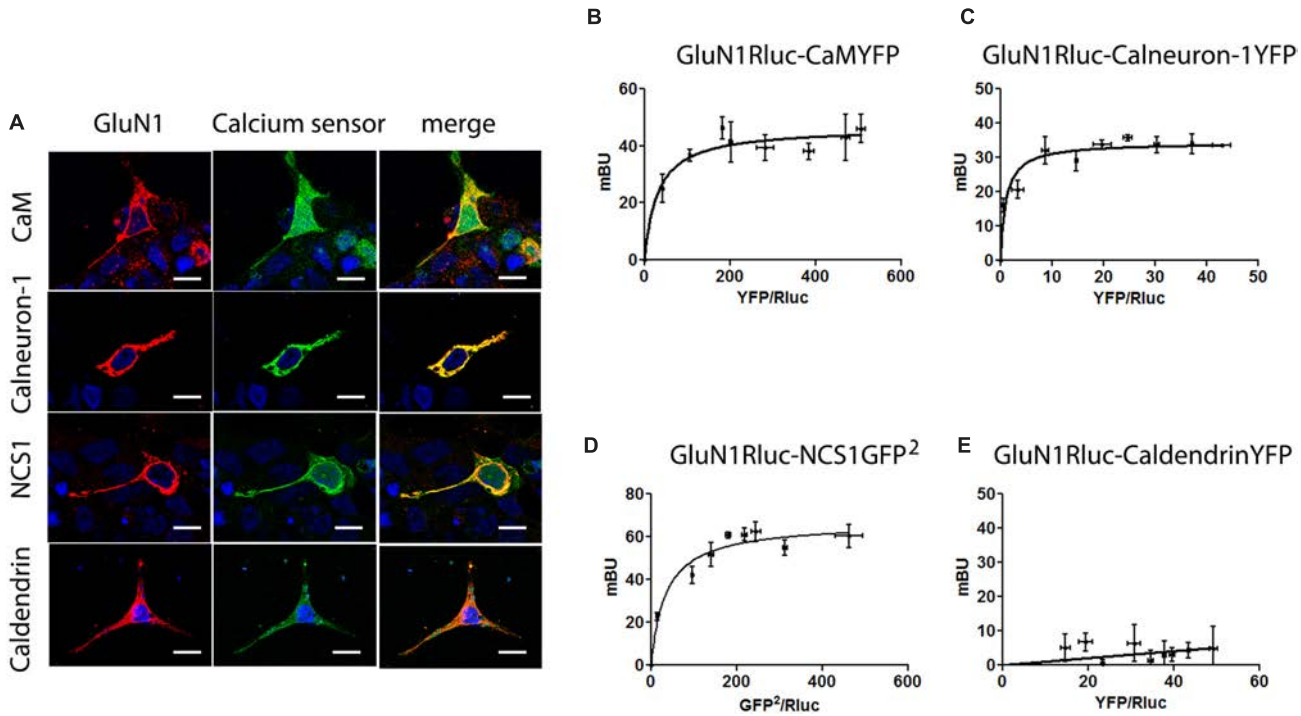

FIGURE 1 | The NMDAR receptor interacts with calmodulin, calneuron-1, and NCS1. **(A)** Confocal microscopy images of HEK-293T cells transfected with cDNAs for GluN1Rluc (0.5 μg), GluN2B (0.3 μg), and CaMYFP (0.5 μg), calneuron-1-YFP (0.75 μg), NCS1YFP (0.5 μg), or caldendrinYFP (0.75 μg). Calcium sensors fused to YFP were identified by their own fluorescence (green) and GluN1Rluc by immunoreactivity (red); co-localization is shown in yellow; scale bars: 10 μm. **(B–E)** BRET [$BRET^1$ except $BRET^2$ in **(D)**] saturation experiments in HEK-293T cells transfected with cDNAs for GluN1RLuc (0.3 μg), GluN2B (0.3 μg), and increasing amounts of cDNA for CaMYFP (0.05–0.4 μg) **(B)**, calneuron-1YFP (0.05–1 μg) **(C)**, NCS1-GFP^2 (0.05–0.6 μg) **(D)**, or caldendrinYFP (0.05–1 μg) **(E)**. Values are the mean ± SEM (n = 8).

calcium sensors by immunocytofluorescence and BRET assays. Co-localization was proven for the calcium-binding proteins NCS1 and calneuron-1 but not for caldendrin (**Figure 1A**). BRET results confirmed that NMDAR may interact with NCS1 and calneuron-1 but not with caldendrin. In fact, a saturation BRET curve was obtained using the GluN1Rluc and calneuron-1YFP in the presence ($BRET_{max}$ 34 ± 1 mBU and $BRET_{50}$ 1.0 ± 0.3; **Figure 1C**) or absence ($BRET_{max}$ 43 ± 4 mBU and $BRET_{50}$ 10 ± 3; **Supplementary Figure S1B**) of GluN2. It should be noted that BRET with GluN1Rluc and NCS1-YFP in the presence of GluN2 lead to an unspecific signal. However, in the absence of GluN2, the BRET of the GluN1Rluc and NCS1-YFP pair was saturable ($BRET_{max}$ 73 ± 7 mBU and $BRET_{50}$ 30 ± 7; **Supplementary Figure S1C**). We repeated the energy transfer experiment using $BRET^2$ (instead of regular BRET or $BRET^1$) and the GluN1Rluc and NCS1-GFP^2 pair (in the presence of GluN2). $BRET^2$ was saturable ($BRET_{max}$ 67 ± 3 mBU and $BRET_{50}$ 35 ± 7; **Figure 1D**). This result can be explained due to a better orientation between donor and acceptor when NCS1-GFP^2 was used. Finally, in HEK-293T cells expressing GluN1Rluc and caldendrinYFP, the linear unspecific signal observed in the presence (**Figure 1E**) and absence of GluN2 (**Supplementary Figure S1D**) indicates a lack of interaction between this calcium sensor and the NMDAR. In conclusion, these data demonstrate that GluN1/N2-NMDAR may interact with calneuron-1, CaM, and NCS1 but not with caldendrin.

NMDAR-Mediated ERK1/2 Phosphorylation Is Regulated by CaM, Calneuron-1, and NCS1

N-methyl-D-aspartate receptor activation leads to the activation of the mitogen-activated protein kinase (MAPK) pathway (Wang et al., 2007). In preliminary experiments, we confirmed that application of NMDA induces the phosphorylation of ERK1/2 and increases the level of intracellular calcium while it did not modify the levels of cAMP (data not shown), which were determined as a control because the NMDAR is not coupled to heterotrimeric G proteins linked to adenylate cyclase. We then tested whether co-expression of NMDAR and calcium sensors affected calcium mobilization. In transiently transfected HEK-293T cells co-expressing the receptor and the calcium-binding proteins, NMDA treatment led to a dose–response increase in the calcium signal (**Figure 2**). NMDA-induced increases in Ca^{2+} levels were obtained when co-transfecting the receptor and CaM, calneuron-1, or NCS1. Whereas the maximal effect was similar when the three sensors were heterologously expressed and when CaM was heterologously expressed, the maximal effect was lower when calneuron-1 or NCS1 were individually

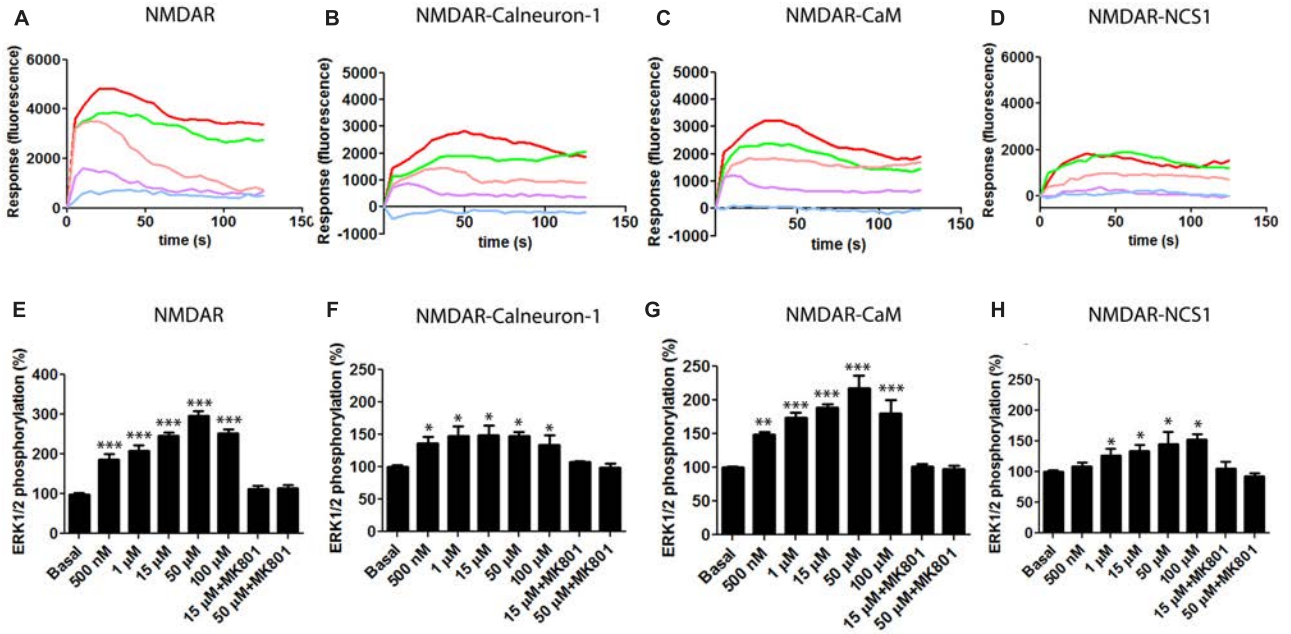

FIGURE 2 | NMDAR -mediated signaling in the presence of calcium sensors. **(A–D)** HEK-293T cells were transfected with the cDNAs for an engineered calcium sensor, 6GCaMP (0.5 μg), GluN1 (0.4 μg), GluN2B (0.25 μg), and calneuron-1 (0.5 μg) **(B)**, CaM (0.3 μg) **(C)**, NCS1 (0.3 μg) **(D)**, or all three **(A)**. Cells were treated with different concentrations of NMDA [500 nM (purple), 1 μM (pink), 15 μM (green), and 50 μM (red)] or pretreated with the NMDAR specific antagonist MK-801 (10 μM) followed by 15 μM NMDA stimulation (blue). Representative traces of intracellular Ca^{2+} responses over time are shown from five independent experiments. **(E,F)** HEK-293T cells were transfected with cDNAs for GluN1 (0.4 μg), GluN2B (0.25 μg), and calneuron-1 (0.5 μg) **(F)**, CaM (0.3 μg) **(G)**, NCS1 (0.3 μg) **(H)**, or all three **(E)**. Cells were treated with different concentrations of NMDA (from 500 nM to 100 μM) or pretreated with the NMDAR specific antagonist MK-801 (10 μM) followed by treatment with NMDA (15 or 50 μM) and ERK1/2 phosphorylation levels were measured. Values are the mean ± SEM ($n = 7$). Significant differences over basal condition were calculated by one-way ANOVA and Bonferroni *post hoc* test (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$).

expressed; interestingly, the peak signal when NCS1 was expressed occurred at lower NMDA concentrations (**Figure 2**). In all cases, pretreatment with the specific NMDAR antagonist MK-801 (10 μM) followed by 15 μM NMDA treatment, completely abolished NMDA-induced calcium signals (**Figure 2**).

We subsequently analyzed how NMDA treatment of cells leads to ERK1/2 phosphorylation in cell expressing NMDAR and the different calcium sensor proteins. HEK-293T cells expressing GluN1 and GluN2 subunits, and CaM, calneuron-1, and NCS1 responded to NMDA treatment and the effect was blocked by the pretreatment with MK-801 (10 μM) (**Figure 2E**). Again, it was observed that exogenously expressed CaM produced the highest levels of NMDA-induced ERK1/2 phosphorylation. Responses were evident but smaller in cells expressing calneuron-1 or NCS1 (**Figure 2**).

Interaction of NMDAR and Calcium Sensors in Cultured Neurons and Microglia

To demonstrate the role of calcium sensor modulation of NMDAR signaling toward the MAP kinase pathway, we moved to primary cultures from mouse brain. When cortical neurons kept for 12 days in culture were treated with increasing concentrations of NMDA (1.5–50 μM), ERK1/2 phosphorylation was obtained (**Figure 3A**). It should be noted that no segregation of synaptic and extrasynaptic NMDAR was yet evident in these cultures. We subsequently used a RNA-based silencing approach to assess the role of each calcium sensor on the NMDAR-mediated responses. When calneuron-1 expression was reduced, the signal obtained was slightly reduced and/or similar depending on the NMDA concentration (**Figure 3A**). By contrast, the effect of NMDA was totally blocked when CaM or NCS1 expressions were silenced (**Figure 3A**). Equivalent results were obtained in neurons from mouse hippocampus (**Figure 3B**). These results in cortical and hippocampal neurons indicate that CaM or NCS1 allows signaling from NMDAR activation to ERK1/2 phosphorylation.

Similar experiments were performed in primary cultures of microglia from cortex or hippocampus. Rather surprisingly, it was observed that NMDAR activation in microglia leads to significant ERK1/2 phosphorylation. In cortical and hippocampal microglia, the NMDAR signal was not blunted when siRNAs against calneuron-1 or NCS1 were used but only when CaM expression was silenced (**Figures 3C,D**). NCS1 is barely expressed in microglia while calneuron-1 (Hradsky et al., 2015) is not expressed. To obtain more information concerning the occurrence of interactions between NMDAR and calcium sensors, *in situ* proximity ligation assays (PLAs) were performed. The technique is suitable to detect complexes of two endogenous proteins, either in tissue slices or in primary cell cultures. PLA showed that cortical and hippocampal neurons display complexes of NMDAR with both CaM and NCS1 sensors

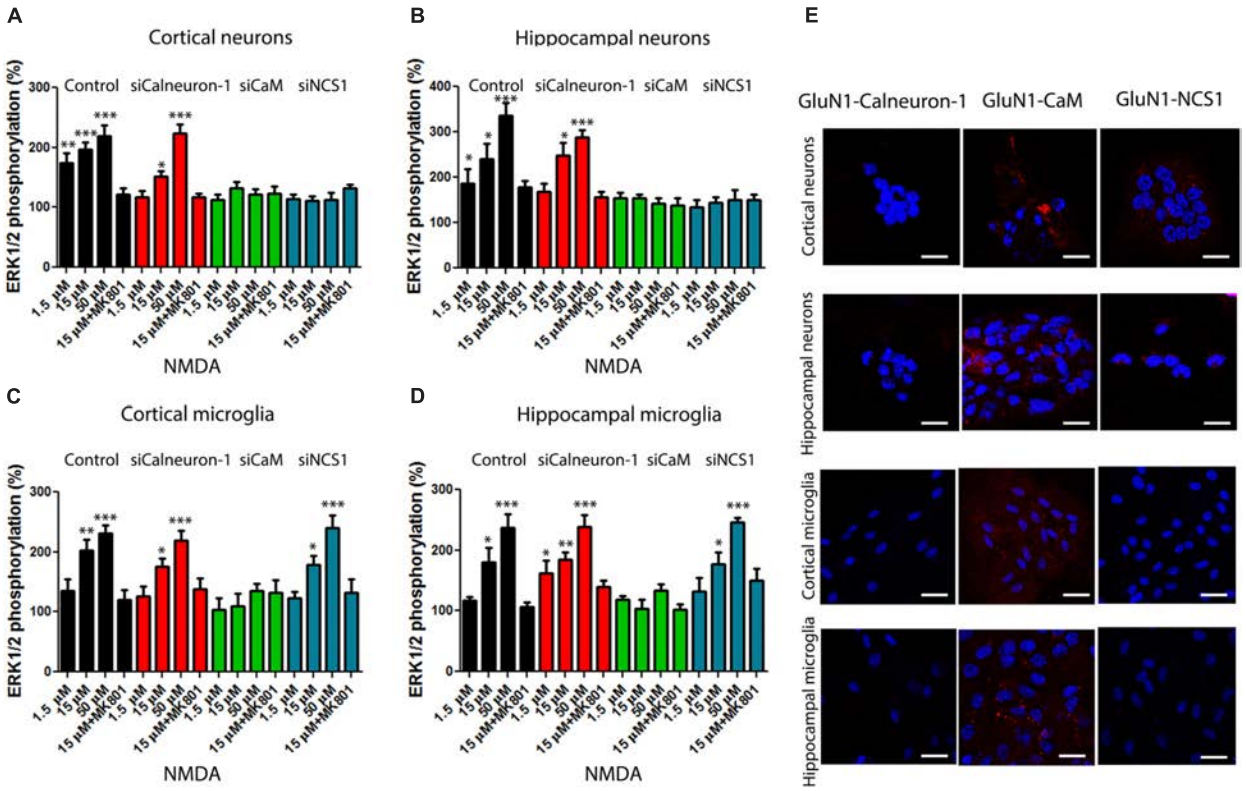

FIGURE 3 | NMDAR-mediated signaling in primary cultures of neurons and microglia from cortex and hippocampus. **(A–D)** MAPK phosphorylation levels were analyzed after stimulating with NMDA (at the indicated concentrations) primary cultures of cortical **(A)**, or hippocampal neurons **(B)**, or of cortex **(C)** or hippocampal microglia **(D)**. Experiments were performed in cells transfected or not (black bars) with siRNA to silence calneuron-1 (red bars), CaM (green bars), or NCS1 (blue bars) expression. Different concentrations of NMDA (from 50 to 1.5 μM) were used to activate the receptor and the NMDAR antagonist, MK-801 (10 μM), was used for specificity checking. Labels in *X* axis are equal in bar graphs of panels **(A–D)**. Values are the mean ± SEM ($n = 12$). Significant differences over basal condition were calculated by one-way ANOVA and Bonferroni *post hoc* test (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$). **(E)** Proximity ligation assays (PLAs) were performed in primary cultures of mice cortex or hippocampus and primary antibodies specific for NMDAR and for calneuron-1, CaM, or NCS1. Confocal microscopy images are shown (superimposed sections) in which heteromers appear as red clusters (in neurons or microglia). Scale bars = 10 μm (neurons) and 20 μm (microglia). In all cases, cell nuclei were stained with Hoechst (blue).

(**Figure 3E**). By contrast, in microglial cultures, NMDAR only interacted with CaM (**Figure 3E**). The PLA specificity was demonstrated by lack of signal when a primary antibody was omitted (**Supplementary Figure S2**). These results fit with the results of ERKs phosphorylation, namely, NMDAR interact with and its signaling to MAPK depends on CaM and NCS1 in cortical and hippocampal neurons, whereas the signaling *via* NMDAR in cortical and hippocampal microglia only depends on CaM.

α-Synuclein Fibrils Block NMDA-Induced MAPK Activation

It is well established that α-synuclein is a presynaptic protein that contributes to PD pathogenesis. First, to test the potential effect of recombinant human α-synuclein on NMDAR-mediated activation of the MAPK pathway, primary cultures of hippocampal or cortical neurons were treated with increasing concentrations of α-synuclein fibrils obtained by sonication (1–100 μg/L) (**Supplementary Figures S3D,H**). Thus, in subsequent experiments, the concentration of α-synuclein used was 10 μg/L. In acute treatment, the protein was added 2 h prior treatment with NMDA, whereas in chronic treatment, α-synuclein was preincubated for 7 days. ERK1/2 phosphorylation assays were performed in primary cultures of neurons or microglia from cortex or hippocampus, treated or not with siRNAs able to knockdown calneuron-1, CaM, or NCS1 expression. First of all, we observed that in all neurons and glia (hippocampal and cortical) cells, the acute treatment completely abolished the effect of NMDAR on ERK1/2 phosphorylation (**Figure 4**). When cultures were treated with siRNA for calneuron-1 (red), CaM (green), or NCS1 (blue), the effect was similar. Accordingly, when CaM and NCS1 in neurons or CaM in microglia are silenced thus blocking the NMDA response, α-synuclein could not display any effect. When similar experiments were undertaken under a chronic condition (10 μg/L α-synuclein for 7 days), the results were similar (**Supplementary Figure S4**). Adenosine deaminase (ADA), which binds to various cell surface receptors that are widely expressed in CNS and peripheral cells [CD26/DPPIV and adenosine receptors (Franco et al., 1988; Corset et al., 2000;

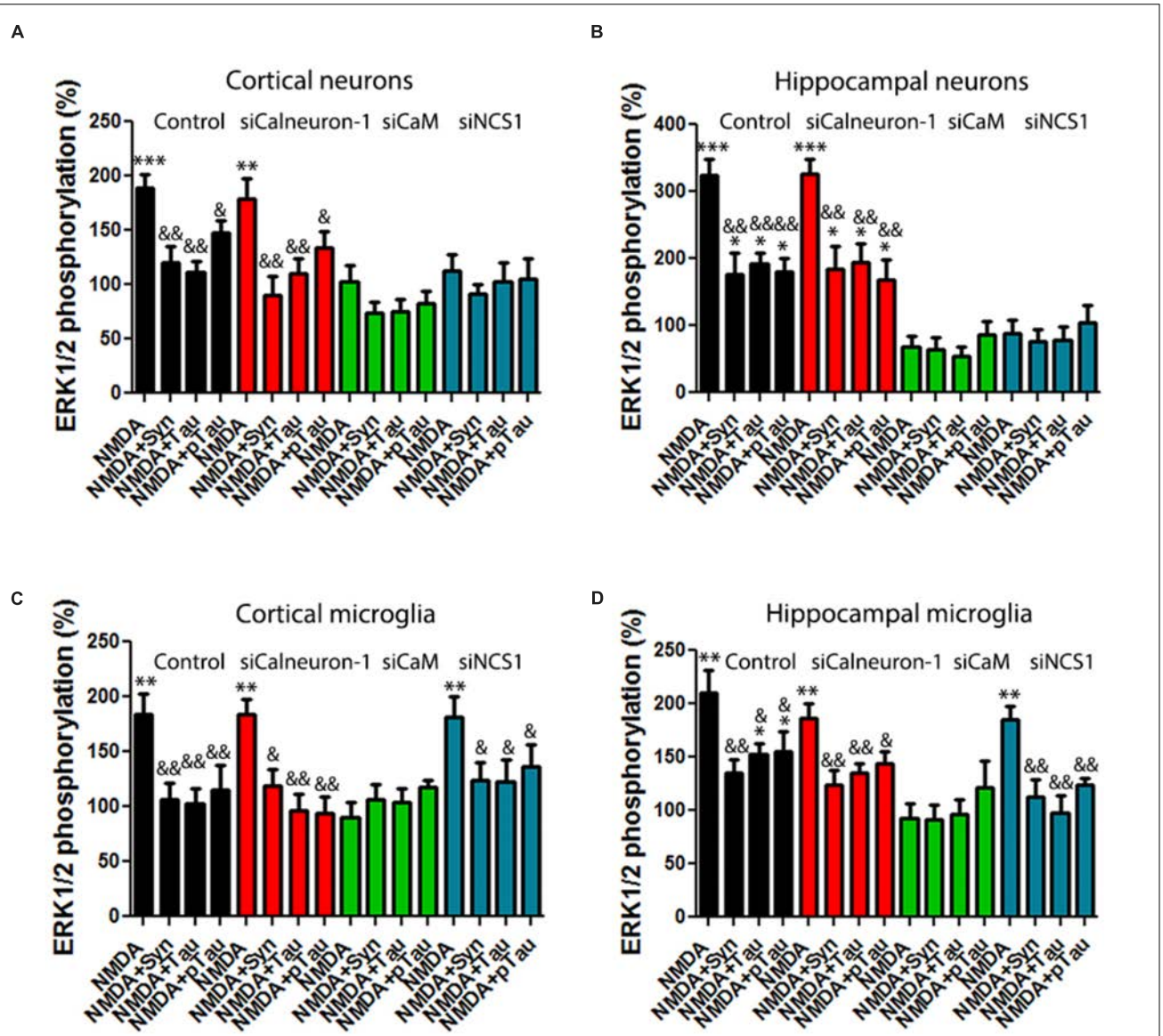

FIGURE 4 | Acute treatment with Tau, p-Tau, or α-synuclein inhibits NMDAR-mediated signaling in primary cultures. **(A–D)** MAPK phosphorylation levels were analyzed after stimulating primary cultures of cortical **(A)**, or hippocampal neurons **(B)**, or of cortical **(C)**, or hippocampal microglia **(D)**. Labels in *X* axis are equal in all bar graphs. Experiments were performed in cells transfected or not (black bars) with siRNA to silence calneuron-1 (red bars), CaM (green bars), or NCS1 (blue bars) expression. Assays were performed in cells treated with α-synuclein, Tau, or p-Tau for 2 h prior to 15 μM NMDA addition. Values are the mean ± SEM ($n = 10$). Significant differences over non-treated cells (**$p < 0.01$ and ***$p < 0.005$) or over NMDA treatment ($^{\&}p < 0.05$ and $^{\&\&}p < 0.01$) were calculated by one-way ANOVA and Bonferroni *post hoc* test.

Ruiz et al., 2000; Herrera et al., 2001; Pacheco et al., 2005)] served as a negative control; ADA did not affect the NMDAR-mediated ERK1/2 phosphorylation (**Supplementary Figure S3**). In summary, acute or chronic α-synuclein fibril treatment of primary cultures blocks engagement of MAP kinase by NMDA.

Tau and p-Tau Block NMDA-Induced MAPK Activation

Tau is a microtubule-associated protein that interacts with tubulin. Normal adult human brain Tau contains 2–3 moles phosphate/mole of Tau protein (Iqbal et al., 2010) while AD patients contain hyperphosphorylated Tau, which is an aberrant form leading to neurofibrillary tangles. Interestingly, *trans*-synaptic spreading of pathological Tau among interconnected neural circuits have been postulated to be critical in tauopathies and linked to progression of AD pathology (Medina and Avila, 2014; Goedert et al., 2017; Mudher et al., 2017). To elucidate the relevance of Tau and p-Tau effects over NMDAR function, experiments similar to those above described for α-synuclein were performed in primary cultures of cells pre-treated with either Tau of p-Tau. First, neuronal primary cultures were

treated with increasing concentrations of Tau (0.05–5 μg/L) and p-Tau (0.05–5 μg/L) (**Supplementary Figures S3B,C,F,G**). In subsequent experiments, the concentration of Tau or p-Tau was 0.5 μg/L. In acute treatment, each of these proteins was added 2 h prior treatment with NMDA, whereas in chronic treatments, cells were incubated for 7 days with Tau or p-Tau. The results showed that both p-Tau and Tau inhibited NMDAR function in acute (**Figure 4**) and also in chronic conditions (**Supplementary Figure S4**). In summary, Tau and p-Tau markedly affected receptor signaling to MAP kinases in both neurons and microglia from cortex or hippocampus. ERK1/2 phosphorylation assays were performed in primary cultures of neurons or microglia from cortex or hippocampus, treated or not with siRNAs able to knockdown calneuron-1, CaM, or NCS1 expression. When CaM and NCS1 in neurons or CaM in microglia were silenced thus blocking the NMDA response, Tau or p-Tau could not display any effect.

NMDA Receptor/Calcium Sensor Interactions in Neurons From $APP_{Sw,Ind}$ Transgenic Model

N-methyl-D-aspartate receptor function is altered in neurons affected by AD. Accordingly, we isolated primary cultures of neurons from transgenic $APP_{Sw,Ind}$ and control mice to check for occurrence of complexes (by PLA) and for the integrity of the link of NMDAR to the MAPK pathway. First of all, NMDA potentiated ERK1/2 phosphorylation in $APP_{Sw,Ind}$ transgenic mice (white bars) with respect to phosphorylation in control animals (black bars) (**Figure 5A**). Moreover, when neurons from $APP_{Sw,Ind}$ transgenic mice were transfected with siRNA to silence CaM or NCS-1 expression, the MAPK phosphorylation was completely abolished, indicating that CaM and NCS-1 proteins are (as in control animals) required for NMDAR function. By contrast, silencing of calneuron-1 had no effect over NMDA actions. PLAs developed to assess the formation of NMDAR–calcium sensor complexes in primary cultures showed that in control animals (black bars) 77% of neurons express NMDAR–CaM complexes while only 21 and 14% of neurons showed, respectively, NMDAR–NCS1 and NMDAR–calneuron-1 clusters (**Figure 5C,D**). Looking for differences in transgenic animals, the most striking result was the increase in the percentage of cells expressing NCS1–NMDAR complexes (from 21 to 68%) plus the significant increase in the number of clusters per cell (from circa 2 to 5.5; **Figure 5**). These results suggest in the AD mice model that the link NMDAR–MAPK in neurons requires CaM and NCS1 calcium sensor expression, with a more relevant role of NCS1 as the amount of NMDAR–NCS1 heteromers is altered if compared with control animals.

Finally, NMDAR function was assayed in neuronal primary cultures of $APP_{Sw,Ind}$ transgenic mice treated with Tau, p-Tau, or α-synuclein for 2 h prior to 15 μM NMDA addition. The results show that not only Tau and p-Tau proteins but also α-synuclein were able to significantly decrease NMDA-induced MAPK activation (**Figure 5**). These results indicate that neither endogenous Tau nor p-Tau can revert the higher sensitivity of NMDA action in neurons of transgenic mice.

NMDA Receptor/Calcium Sensor Interactions in Microglia From $APP_{Sw,Ind}$ Transgenic Model

To analyze the status of the NMDAR-MAP kinase link in an AD model, primary cultures of microglia from non-transgenic (control) or $APP_{Sw,Ind}$ transgenic mice were cultured and treated with NMDA (15 μM). Interestingly, the results of ERK1/2 phosphorylation indicated that NMDAR function was potentiated in microglia from the $APP_{Sw,Ind}$ transgenic mice (white bars in **Figure 6**). When cultures were transfected with siRNA to silence calneuron-1 expression, a similar result was obtained thus suggesting that calneuron-1 is not involved in the NMDA effect on MAPK activation (**Figure 6A**, microglia from control in black, from transgenic in white). By contrast, when cultures were transfected with siRNA to silence CaM expression, it was observed that the NMDAR signaling completely disappeared in microglia from both control and $APP_{Sw,Ind}$ mice, indicating that CaM interaction with NMDAR is required for receptor-mediated engagement of the MAPK pathway. In fact, these results are in agreement with the data shown in **Figure 3**. Finally, when microglia primary cultures of $APP_{Sw,Ind}$ transgenic mice and control animals were treated with siRNA to decrease NCS1 expression, the abolishment of NMDA effect was only observed in the $APP_{Sw,Ind}$-derived cells (**Figure 6A**). The results indicate that the NCS1–NMDAR interaction is relevant for receptor function in the microglia of the AD mouse model.

Finally, formation of complexes of NMDA receptors and calcium-binding proteins was detected by PLA in primary cultures of microglia from $APP_{Sw,Ind}$ and control mice. Interestingly, we observed that in control animals (black bars), only CaM formed heteromeric complexes with the NMDA receptor (35% of cells showing around three red dots/ cell containing dots) with negligible values for interactions with calneuron-1 or NCS1 (respectively, 1.85 and 4.8% of cells expressing red dots). When $APP_{Sw,Ind}$ microglia was analyzed, the number of complexes between NMDAR and CaM was markedly higher (65% of cells showing around 6 red dots/ cell containing dots), whereas the degree of interaction with calneuron-1 was negligible (1.9% of cells containing red dots). It should be also noted that complexes of NCS1 and NMDAR were also noticeable in the $APP_{Sw,Ind}$ microglia thus indicating a NMDAR-NCS1 interaction in microglia that deserves attention when addressing AD pathophysiology. While the NCS1 expression in microglia from wild-type animals is reportedly low (Averill et al., 2004; Hradsky et al., 2015; Nakamura et al., 2017), the substantial increase in NMDAR-NCS1 complexes shown in **Figures 6B,C**, suggests that expression of NCS1 in microglia from the $APP_{Sw,Ind}$ mice is significant.

DISCUSSION

The arguably two most relevant neuronal second messengers whose intracellular concentration changes upon cell surface receptor activation are cAMP and Ca^{2+}. In our efforts to

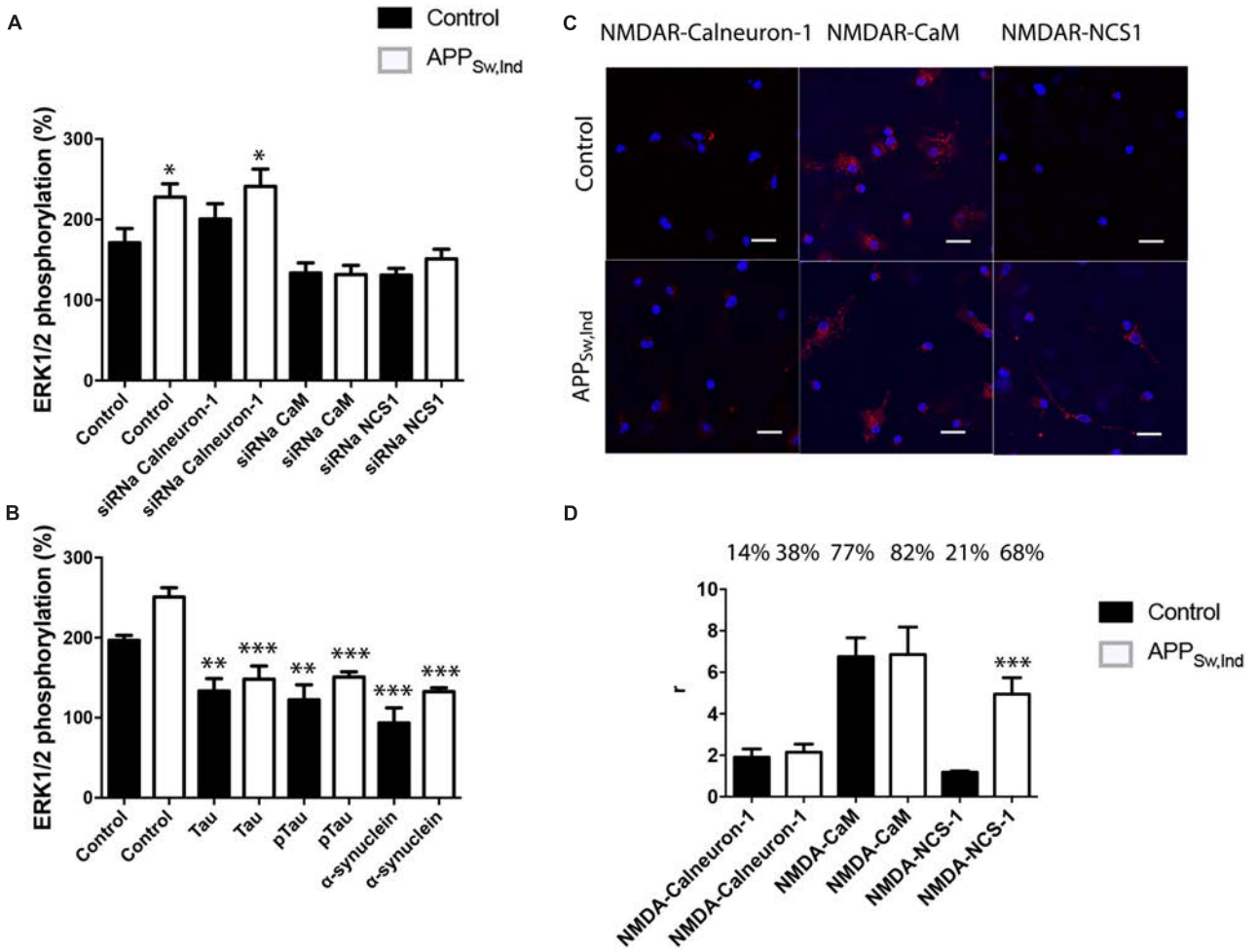

FIGURE 5 | NMDAR-mediated signaling in primary neurons from $APP_{Sw,Ind}$ mice. **(A,B)** Primary cultures from non-transgenic control (black bars) and $APP_{Sw,Ind}$ (white bars) embryos were isolated and transfected or not with siRNA for silencing calneuron-1, CaM, or NCS1. In **(A)**, neurons were treated with 15 μM NMDA, while in **(B)**, neurons were previously treated with α-synuclein, Tau, or p-Tau for 2 h prior to 15 μM NMDA addition; finally, ERK1/2 phosphorylation was measured. Values are the mean ± SEM ($n = 6$). One-way ANOVA followed by Bonferroni's multiple comparison *post hoc* test were used for statistics analysis. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus NMDA treatment; comparing only white (non-transgenic data) or only black (transgenic data) bars. **(C)** PLA performed in primary cultures from non-transgenic control (black bars) and $APP_{Sw,Ind}$ (white bars) embryos, using specific primary antibodies raised against NMDAR and calneuron-1, CaM, or NCS1. Confocal microscopy images (stacks of four consecutive planes) show heteroreceptor complexes as red clusters surrounding Hoechst-stained nuclei (blue). Scale bar: 15 μm. The bar graph **(D)** shows the number of red dots/cell and the number above bars indicate the percentage of cells presenting red dots. Values are the mean ± SEM ($n = 5$). One-way ANOVA followed by Bonferroni's multiple comparison *post hoc* test was used for statistics analysis ($^{***}p < 0.001$, versus control).

look for cAMP- and calcium-signaling cross-talk, we have shown direct interactions between calcium sensors and receptors for neurotransmitters/neuromodulators that are coupled to G proteins (GPCRs), i.e., metabotropic receptors. Interestingly, the interaction with calcium sensors in conditions of elevated Ca^{2+} levels results in marked modulation of the signaling events mediated by the cell surface GPCRs expressed in neurons (Lian et al., 2011; Mikhaylova et al., 2011; Navarro et al., 2012a,b, 2014).

In a heterologous expression system, we found that with the exception of caldendrin, all investigated calcium sensors may interact with the GluN1 subunit of NMDAR. Caldendrin is highly abundant in pyramidal neurons (Seidenbecher et al., 1998; Mikhaylova et al., 2018) and also part of the protein complex formed by the calcium sensor and an interacting partner, Jacob, that links NMDAR-mediated signaling to the nucleus (Dieterich et al., 2008) in a cytocrin fashion (Navarro et al., 2017). Our data using a heterologous expression system indicate that no direct interaction occurs with the GluN1 subunit under these conditions.

The NMDAR is one of the upstream players that impact on the MAP kinase signaling pathway. The present study suggests that the coupling of the MAPK pathway to NMDAR activation depends on the expression of calcium sensors. Interestingly, CaM is providing the link in primary cultures of cortical and hippocampal microglia, whereas CaM or NCS1 provide the link in cortical and hippocampal neurons. Among the few studies linking calcium sensors to ionotropic related neurophysiological events, those performed by Jo et al. (2008) in synapses of the

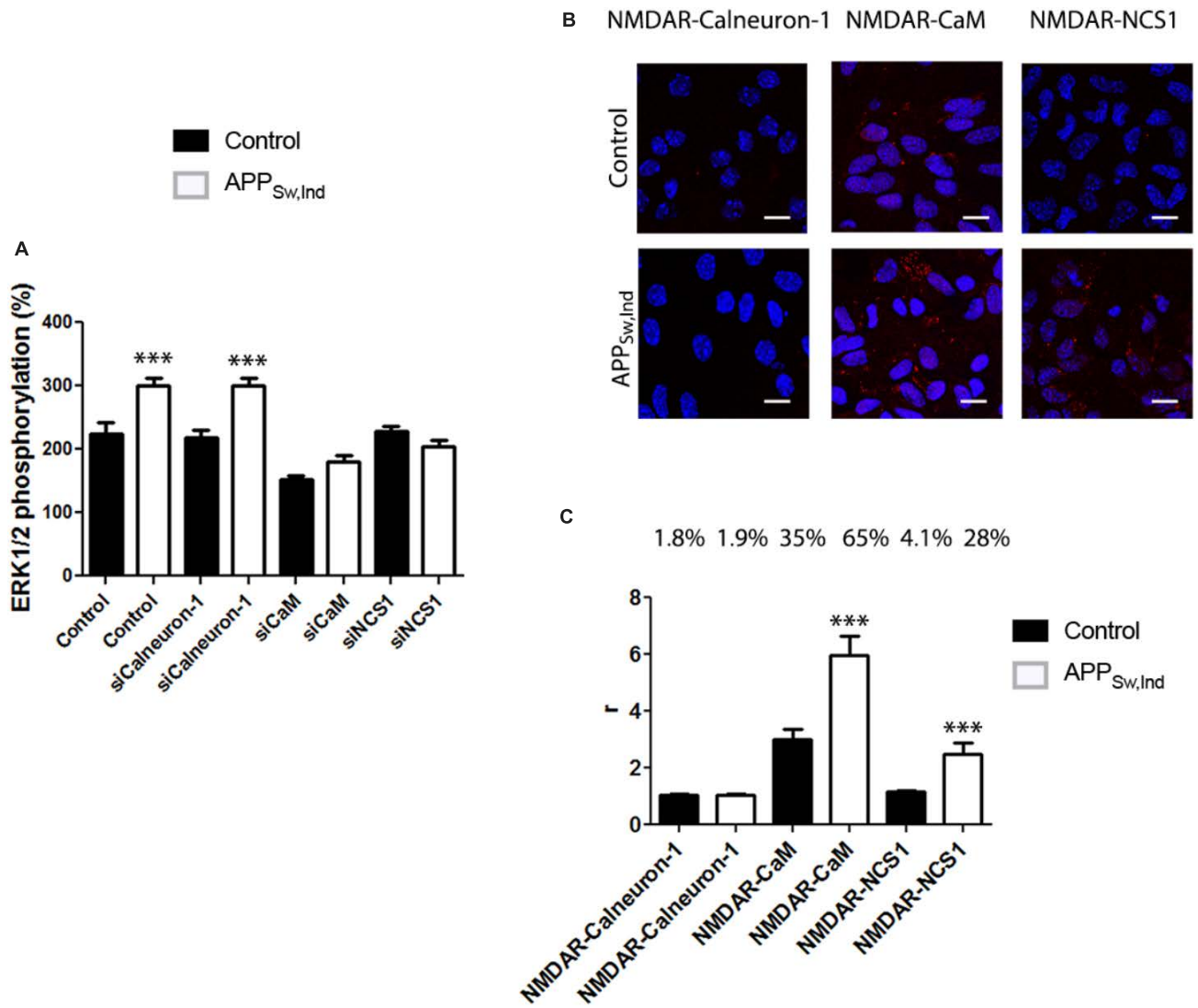

FIGURE 6 | NMDAR-mediated signaling in primary microglia from $APP_{Sw,Ind}$ mice. **(A)** Primary microglial cultures from non-transgenic control (black bars) and $APP_{Sw,Ind}$ (white bars) mice were isolated and transfected or not with siRNA for silencing calneuron-1, CaM, or NCS1. Microglia were treated with 15 μM NMDA and MAPK phosphorylation was measured. Values are the mean ± SEM ($n = 6$). One-way ANOVA followed by Bonferroni's multiple comparison *post hoc* test were used for statistics analysis (***$p < 0.001$ versus control). **(B)** Primary microglial cultures from non-transgenic control (black bars) and $APP_{Sw,Ind}$ (white bars) mice were assayed for potential interactions between NMDAR and calcium sensors. PLA was performed using primary cultures of microglia and specific primary antibodies raised against NMDAR and calneuron-1, CaM, or NCS1. Confocal microscopy images (stacks of four consecutive planes) show heteroreceptor complexes as red clusters surrounding Hoechst-stained nuclei (blue). Scale bar: 10 μm. The bar graph **(C)** shows the number of red dots/cell and the number above bars indicate the percentage of cells presenting red dots. Values are the mean ± SEM ($n = 6$). One-way ANOVA followed by Bonferroni's multiple comparison *post hoc* test was used for statistics analysis (***$p < 0.001$, comparing each condition versus control, i.e., comparing one by one white versus the corresponding black bar).

perirhinal cortex showed that long-term depression mediated by ionotropic glutamate NMDA receptors involves CaM, whereas long-term depression mediated by metabotropic G-protein-coupled glutamate receptors involves NCS1. In primary cultures of neurons, the link to MAP kinase activation was blocked by α-synuclein, Tau, and p-Tau, which are relevant players in the pathophysiology of proteinopathies such as AD and PD. The effect was similar in neurons and in microglia. Taking into account the similar blockade (both in acute and chronic treatments) by the three different proteins, and the fact that the calcium sensors modulate the coupling of NMDAR to the MAPK pathway, the blockade may result from direct interaction with NMDAR or by interference of signaling events downstream of the receptor.

Microglia, first considered as detrimental when activated due to brain damage or neurodegeneration, appear now with huge potential for neuroprotection (Franco and Fernández-Suárez, 2015). In a previous work using the $APP_{Sw,Ind}$ transgenic AD mice, we found that primary cultures of microglia from pup brain display an activated phenotype (Navarro et al., 2018).

As young $APP_{Sw,Ind}$ animals do not display any cognitive impairment, such finding could indicate that activated microglia are neuroprotective in this animal model. Accordingly, we wanted to assess possible alterations in the functioning of NMDAR in such cells. On the one hand, NMDAR are coupled to MAP kinases but with a differential finding respect to that in cells from control animals. In fact, the NMDA-induced increase of ERK1/2 phosphorylation was independent of calneuron-1 but modulated by CaM and NCS1. In parallel assays performed in cells from control animals, the effect was only inhibited by silencing CaM in microglia (or by silencing CaM and NCS-1 in neurons). Further data on assessing the complexes involving NMDA receptors confirmed a significant increase in the number of microglial cells expressing NMDAR/CaM and NMDA/NCS1 complexes and in the number of clusters per cell. In neurons, the relevant increase in both percentage of expressing cells and amount of clusters/cell concerned NMDAR/NCS1 complexes.

The results obtained in primary cultures (neurons and microglia) from transgenic mice show exacerbation of NMDA-induced MAPK activation. These results combined with the effect of pathogenic proteins leads to two possibilities, namely, (i) hyperactivation is independent of the presence of those (endogenously expressed) proteins or (ii) neural cells counteract the effect of α-synuclein fibrils, Tau, and p-Tau by increasing NMDAR signaling function. Accordingly, the molecular underpinnings and potential differences thereof in the relationship between NMDAR and calcium-binding proteins deserves further experimental effort. What it is however relevant in our findings in the AD model is the increase in the expression of NMDAR–NCS1 complexes; the consequences of such significant change (in neurons: > 3-fold in number of cells expressing complexes and >fourfold the number of complexes per cell) deserves a close scrutiny to assess its relevance in AD pathophysiology.

MATERIALS AND METHODS

Reagents

N-methyl-D-aspartate and MK-801 were purchased from Tocris Bioscience (Bristol, United Kingdom). Recombinant human α-synuclein was prepared as described (Matsuda et al., 1990) and Tau and p-Tau proteins were kindly provided by Prof. J. Avila (CBM, UAM-CSIC, Madrid, Spain). Detailed descriptions of the elaboration and processing of proteins can be found elsewhere (Pérez et al., 2002; Tarutani et al., 2016).

Expression Vectors

cDNA for the human version of the GluN1 subunit of NMDAR lacking the stop codon was obtained by PCR and subcloned to RLuc-containing vector (p*RLuc*-N1; PerkinElmer, Wellesley, MA, United States) using sense and antisense primers harboring unique restriction sites for HindIII and BamHI; the generated cDNA encodes a GluN1Rluc fusion protein. cDNA for the human version of GluN2B subunit of NMDAR was subcloned in pcDNA3.1. In functional assays, both cDNAs encoding for GluN1 and GluN2B were cotransfected. CaM gene sequence from pcDNA3 was subcloned in pEYFP-N1 vector (pEYFP: enhanced yellow variant of GFP; Clontech, Heidelberg, Germany), as previously described (Navarro et al., 2009) to generate a plasmid encoding CaM-YFP fusion protein. cDNA constructs encoding NCS1, calneuron-1, or caldendrin in pcDNA3 vectors were subcloned in pEYFP-N1 or $pGFP^2$-N1 vectors as previously described in (Navarro et al., 2012b) to generate plasmids encoding NCS1YFP, $NCS1GFP^2$, calneuron-1YFP, and caldendrinYFP fusion proteins. The cDNA for calneuron-1, caldendrin, and NCS1, cloned into pcDNA3.1, were amplified (omitting stop codons) using sense and antisense primers harboring unique HindIII and BamHI sites to clone the amplified fragments to be in frame in the pEYFP-N1 or $pGFP^2$-N1 vectors.

$APP_{Sw,Ind}$ Transgenic Mice

$APP_{Sw,Ind}$ transgenic mice (line J9; C57BL/6 background) expressing human APP695 harboring the FAD-linked Swedish (K670N/M671L) and Indiana (V717F) mutations under the PDGFβ promoter were obtained by crossing $APP_{Sw,Ind}$ to non-transgenic (control) mice (Mucke et al., 2000). Control and $APP_{Sw,Ind}$ embryos (E16.5) were genotyped individually and used for microglia cultures as described elsewhere (Navarro et al., 2018). Animal care and experimental procedures were in accordance with European and Spanish regulations (86/609/CEE; RD1201/2005). Mice were handled, as per law, by personnel with the *ad hoc* certificate (issued by the *Generalitat de Catalunya*) that allows animal handling for research purposes.

Cell Culture and Transient Transfection

HEK-293T cells were grown in in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 5% (v/v) heat inactivated fetal bovine serum (FBS) (Invitrogen, Paisley, Scotland, United Kingdom). Cells were maintained in a humid atmosphere of 5% CO_2 at 37°C. Cells were transiently transfected with the polyethylenimine (PEI, Sigma, St. Louis, MO, United States) method. To prepare mice cortex and hippocampus primary microglial cultures, brain was removed from C57/BL6 mice between 2- and 4-days-old. Microglia cells were isolated as described in (Newell et al., 2015) and plated at confluence of 40,000 cells/0.32 cm^2 and grown in DMEM medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 5% (v/v) heat inactivated FBS (Invitrogen, Paisley, Scotland, United Kingdom) for 12 days. Neuronal primary cultures were prepared from cortex and hippocampus of fetuses from C57/BL6 pregnant mice. Neurons were isolated as described in Hradsky et al. (2013) and plated at a confluence of 40,000 cells/0.32 cm^2. Striatal cells were grown in neurobasal medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 2% (v/v) B27 supplement (Gibco) in a 96-well plate for 12 days.

Silencing of constitutively expressed calcium-binding proteins was performed as described elsewhere (Navarro et al., 2014). Briefly, cortical or hippocampal cultures (neurons or microglia) growing in 96-well plates were transfected with

the Lipofectamine®2000 (Thermo Fisher) method to silence the NCS1, CaM, or calneuron-1 expression using pSuper-NCS-1 vector, pSuper-CaM vector, and calneuron-1 shRNAII (1xconstruct #2; genecopoeia). Cells were incubated for 6–8 h with the cDNA and Lipofectamine® in serum-starved medium. After 6–8 h, the medium was replaced with a complete culture medium. Validation was performed by Western blotting and the reduction in expression from control values (>75% in neurons; >85% in microglia). Rescuing assays were performed in primary cultures (already expressing endogenous CaM) by first silencing CaM using a specific siRNA and further transfection with a vector containing the CaM sequence. The results related to the NMDAR–MAPK link disappeared upon silencing but re-appeared upon "reexpression" of CaM by transfection with the specific cDNA (**Supplementary Figure S5**).

Preparation of Human α-Synuclein Fibrils

α-Synuclein fibrils were prepared by shaking purified recombinant α-synuclein as described (Masuda-Suzukake et al., 2014; Tarutani et al., 2016). Briefly, purified recombinant α-synuclein (5 mg/ml) containing 30 mM Tris–HCl (pH 7.5), 10 mM DTT, and 0.1% sodium azide were incubated for 7 days at 37°C in a horizontal shaker at 200 rpm, then ultracentrifuged at 113,000 × *g* for 20 min at 25°C. The pellets were washed with saline and ultracentrifuged as before. The resulting pellets were collected as α-synuclein fibrils and resuspended in 30 mM Tris–HCl (pH 7.5). The fibrils were fragmented using a cup horn sonicator (Sonifier® SFX, Branson) at 35% power for 180 s (total 240 s, 30 s on, 10 s off) (Tarutani et al., 2016, 2018). Before use aliquots were left at room temperature and placed in PBS 1× (pH 7.2) to a final concentration of 0.1 μg/μL. These preparations were subjected to 60 pulses of sonication (runtime 30 s: 0.5 s on, 0.5 s off in a BBR03031311digital SONIFIER sonicator). Sonicated fibril preparations were diluted in pre-warmed medium and immediately added to cells.

Bioluminescence Resonance Energy Transfer (BRET) Assays

For BRET^1, HEK-293T cells were transiently co-transfected with a constant amount of cDNA encoding for GluN1-RLuc and GluN2B in pcDNA3.1 and with increasing amounts of cDNA corresponding to calneuron-1-YFP, caldendrin-YFP, CaM-YFP, or NCS1-YFP. 48 h after transfection cells were adjusted to 20 μg of protein using a Bradford assay kit (Bio-Rad, Munich, Germany) using bovine serum albumin for standardization. To quantify protein-YFP expression, fluorescence was read in a Mithras LB 940 equipped with a high-energy xenon flash lamp, using a 30-nm bandwidth excitation filter at 485 nm. For BRET measurements, readings were collected 30 s after the addition of 5 μM coelenterazine H (Molecular Probes, Eugene, OR, United States) using a Mithras LB 940, which allows the integration of the signals detected in the short-wavelength filter at 485 nm and the long-wavelength filter at 530 nm. To quantify protein-RLuc expression, luminescence readings were performed 10 min after 5 μM coelenterazine H addition using a Mithras LB 940. For BRET^2, HEK-293T cells were transiently co-transfected with a constant amount of cDNA encoding for GluN1-RLuc and GluN2B in pcDNA3.1 and with increasing amounts of cDNA corresponding to NCS1-GFP^2. 48 h after transfection cells were adjusted to 20 μg of protein using a Bradford assay kit (Bio-Rad, Munich, Germany) using bovine serum albumin for standardization. To quantify protein-GFP^2 expression, fluorescence was read in a Fluostar Optima fluorimeter equipped with a high-energy xenon flash lamp, using a 10-nm bandwidth excitation filter at 405 nm. For BRET measurements, readings were collected 1 min after the addition of 5 μM Deep Blue C (Molecular Probes, Eugene, OR, United States) using a Mithras LB 940, which allows the integration of the signals detected in the short-wavelength filter at 405 nm and the long-wavelength filter at 510 nm. To quantify protein-RLuc expression, luminescence readings were performed 10 min after 5 μM coelenterazine H addition using a Mithras LB 940. The net BRET is defined as [(long-wavelength emission)/(short-wavelength emission)] − Cf, where Cf corresponds to [(long-wavelength emission)/(short-wavelength emission)] for the donor construct expressed alone in the same experiment. GraphPad Prism software (San Diego, CA, United States) was used to fit data. BRET is expressed as milli BRET units, mBU (net BRET × 1,000) (Canals et al., 2003, 2004; Hinz et al., 2018).

Immunocytofluorescence

HEK-293T cells were transfected with GluN1-RLuc, GluN2B, and calneuron-1-YFP, caldendrin-YFP, CaM-YFP, or NCS1-YFP were fixed in 4% paraformaldehyde for 15 min and washed twice with PBS containing 20 mM glycine before permeabilization with PBS-glycine containing 0.2% Triton X-100 (5 min incubation). HEK-293T cells were treated for 1 h with PBS containing 1% bovine serum albumin and labeled with the primary mouse anti-RLuc antibody, and subsequently treated with: Cy3 anti-rabbit [1/200; Jackson ImmunoResearch (red)] secondary antibodies for 1 h. The YFP-fusion proteins were detected by YFP own fluorescence. Samples were washed several times and mounted with 30% Mowiol (Calbiochem). Samples were observed in a Leica SP2 confocal microscope (Leica Microsystems). Scale bar: 10 μm for neurons and 20 μm for microglia cells.

Calcium Release

HEK-293T cells were co-transfected with the cDNA for the indicated receptors and 0.75 μg of GCaMP6 calcium sensor (Chen et al., 2013) using PEI protocol (Section "Cell Culture and Transient Transfection"). Forty-eight hours after transfection, cells (150,000 HEK-293T cells/well in 96-well black, clear bottom microtiter plates) were incubated with Mg^{2+}-free Locke's buffer pH 7.4 (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5.6 mM glucose, and 5 mM HEPES) supplemented with 10 μM glycine and receptor ligands were added just a few seconds before readings. Fluorescence emission intensity of GCaMP6 was recorded at 515 nm upon excitation at 488 nm on the EnSpire® Multimode Plate Reader for 335 s every 15 s and 100 flashes per well.

ERK Phosphorylation Assays

To determine ERK1/2 phosphorylation, HEK-293T cells expressing GluN1 and GluN2B subunits and calneuron-1, CaM, or NCS1, primary cultures of cortex or hippocampus microglia cells or primary cultures of cortex or hippocampus neurons were plated at a density of 40,000 cells/well in transparent Deltalab 96-well microplates and kept at the incubator between 1 and 7 days. Two to four hours before the experiment, the medium was substituted by serum-starved DMEM medium. Then, cells were pre-treated or not for 2 h or 7 days with α-synuclein, Tau, and p-Tau proteins at 37°C followed by treatment at 25°C for 10 min with vehicle or antagonists (MK-801) in serum-starved DMEM medium and stimulated for an additional 7 min with NMDA. Cells were then washed twice with cold PBS before addition of lysis buffer (20 min treatment). Ten microliters of each supernatant were placed in white ProxiPlate 384-well microplates and ERK 1/2 phosphorylation was determined using AlphaScreen®SureFire® kit (PerkinElmer) following the instructions of the supplier and using an EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA, United States).

Proximity Ligation Assays (PLAs)

Interactions between NMDAR and calcium sensors were detected using the Duolink II *in situ* PLA detection Kit (OLink; Bioscience, Uppsala, Sweden) following the instructions of the supplier. Primary cultures of neurons and microglia cells were grown on glass coverslips and were fixed in 4% paraformaldehyde for 15 min, washed with PBS containing 20 mM glycine to quench the aldehyde groups, permeabilized with the same buffer containing 0.05% Triton X-100 for 5 min, and successively washed with PBS. After 1 h incubation at 37°C with the blocking solution in a pre-heated humidity chamber, primary cultures were incubated overnight in the antibody diluent medium with a mixture of equal amounts of rat monoclonal anti-NMDAR antibody (1:200, Millipore) and a polyclonal rabbit anti-NCS1 antibody (1:100, Millipore) to detect NMDAR–NCS1 complexes, or and the rabbit polyclonal anti-calneuron-1 antibody (1:100, Abcam) to detect NMDAR-calneuron-1 complexes or and a monoclonal rabbit anti-CaM antibody (1:50, Abcam) to detect NMDAR–CaM complexes. Cells were processed using the PLA probes detecting primary antibodies (Duolink II PLA probe plus and Duolink II PLA probe minus) diluted in the antibody diluent (1:5). Ligation and amplification were done as indicated by the supplier and cells were mounted using the mounting medium with Hoechst (1/200; Sigma). Samples were observed in a Leica SP2 confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with an apochromatic 63× oil immersion objective (N.A. 1.4), and a 405-nm and a 561-nm laser lines. For each field of view, a stack of two channels (one per staining) and four to eight Z stacks with a step size of 1 μm were acquired. A quantification of cells containing one or more red spots versus total cells (blue nucleus) and, in cells containing spots, the ratio *r* (number of red spots/cell), were determined. One-way ANOVA followed by Dunnett's *post hoc* multiple comparison test was used to compare the values (% of positive cells or *r* spots/cell) obtained for each pair of receptors.

AUTHOR CONTRIBUTIONS

GN and RF conceived and designed the experiments. MK, AT, MH, and JdR provided key reagents. CS provided transgenic animals and controls. GN, DA, JdR, JL, AdS-B, IR, and EC performed the experiments and analyzed the data. RF, GN, MK, EC, and CS wrote the paper. All authors have edited the manuscript and received copy of the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2018.00273/full#supplementary-material

FIGURE S1 | The N1 subunit of NMDAR receptor may interact with calmodulin, calneuron-1 and NCS1. **(A–D)** BRET saturation experiments in HEK-293T cells transfected with cDNAs for GluN1Rluc (0.3 μg), and increasing amounts of cDNA for CaMYFP (0.05–0.4 μg) **(A)**, calneuron-1YFP (0.05–1 μg) **(B)**, NCS1YFP (0.05–0.6 μg) **(C)**, or caldendrin YFP (0.05–1 μg) **(D)**. Values are the mean ± SEM ($n = 6$).

FIGURE S2 | Proximity ligation assay (PLA) negative controls for PLAs in primary cultures of neurons and microglia. PLAs were performed in cortical (**A** neurons, **C** microglia) and hippocampal (**B** neurons, **D** microglia) cells incubated with the anti-CaM antibody but omitting the anti-NMDAR antibody. Confocal microscopy images are shown (superimposed sections) in which heteromers appear as red clusters (in neurons or microglia). Scale bars = 10 μm (neurons) and 20 μm (microglia). In all cases, cell nuclei were stained with Hoechst (blue).

FIGURE S3 | Tau, p-Tau, and α-synuclein dose–response curves in neuronal primary cultures. **(A–H)** MAPK phosphorylation levels were analyzed in primary cultures of cortical **(A–D)**, or hippocampal neurons **(E–H)**. Assays were performed in cells treated with increasing concentrations of adenosine deaminase (ADA) (5–500 μM). **(A,E)** Tau (0.05–5 μg/L) **(B,F)**, p-Tau (0.05–5 μg/L) **(C,G)**, or α-synuclein (1 μM–100 μg/L) **(D,H)** for 2 h prior to 15 μM NMDA stimulation. Values are the mean ± SEM ($n = 6$). Significant differences over NMDA treatment (control condition) ($^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.005$) were calculated by one-way ANOVA and Bonferroni *post hoc* test.

FIGURE S4 | Chronic treatment with Tau, p-Tau, or α-synuclein inhibits NMDAR-mediated signaling in primary cultures. **(A–D)** MAPK phosphorylation levels were analyzed after stimulating primary cultures of cortical **(A)**, or hippocampal neurons **(B)**, or of cortex **(C)**, or hippocampal microglia **(D)**. Experiments were performed in cells transfected or not (black bars) with siRNA to silence calneuron-1 (red bars), CaM (green bars), or NCS1 (blue bars) expression. Assays were performed in cells treated with α-synuclein, Tau or p-Tau for 7 days prior to 15 μM NMDA addition. Labels in *X* axis are equal in all bar graphs. Values are the mean ± SEM ($n = 10$). Significant differences over non-treated cells ($^{**}p < 0.01$, $^{***}p < 0.005$) or over NMDA treatment ($^{\&}p < 0.05$, $^{\&\&}p < 0.01$, and $^{\&\&\&}p < 0.001$) were calculated by one-way ANOVA and Bonferroni *post hoc* test.

FIGURE S5 | Rescue of (endogenous) CaM silencing upon transfection with the cDNA for CaM. **(A,B)** MAPK phosphorylation levels were analyzed after stimulating primary cultures of cortical neurons **(A)** or cortical microglia **(B)** with 15 μM NMDA. Experiments were performed in cells transfected or not with siRNA to silence CaM; 24 h later, cells were transfected with cDNA for CaM or with the (empty) pcDNA3.1 vector. Experiments in untransfected (left), in siRNA (center), and in siRNA plus CaM (right) cells were preformed simultaneously. Data are the mean ± SEM ($n = 5$). One-way ANOVA followed by Bonferroni's multiple comparison *post hoc* test were used for statistics analysis ($^{*}p < 0.05$, $^{**}p < 0.01$ versus NMDA treatment).

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3.3 Regulation of Expression of Cannabinoid CB_2 and Serotonin $5HT_{1A}$ Receptor Complexes by Cannabinoids in Animal Models of Hypoxia and in Oxygen/Glucose-Deprived Neurons.

Jaume Lillo, Iu Raïch, Laura Silva, David A Zafra, Alejandro Lillo, Carlos Ferreiro-Vera, Verónica Sánchez de Medina, José Martínez-Orgado, Rafael Franco, Gemma Navarro.

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El cannabidiol (CBD) és un fitocannabinoide amb un gran potencial en una de les afectacions més prevalents durant el part, la hipòxia dels nounats. El CBD és capaç d'unir-se a una gran varietat de receptors, entre ells el receptor CB_2 i el $5HT_{1A}$. Aquests dos receptors interaccionen formant complexos heteromèrics. L'objectiu d'aquest article va ser determinar si l'expressió i la funcionalitat de l'heteròmer CB_2R-$5HT_{1A}R$ es veu afectada pel CBD en un model animal d'hipòxia en nounats i en un model de deprivació d'oxigen i glucosa (GOD). Mitjançant la tècnica de BRET, es va demostrar que tant el CBD com el cannabigerol (CBG) eren capaços d'afectar l'estructura de l'heteròmer, però mentre el CBD incrementava l'afinitat de la interacció entre ambdós receptors, el CBG era incapaç de fer-ho. Tant el CBD com el CBG regulen els efectes mediats per agonistes de CB_2R i $5HT_{1A}R$. Mitjançant la tècnica de PLA vam observar que el CBD revertia la sobreexpressió de l'heteròmer CB_2R-$5HT_{1A}R$ produïda per una deprivació d'oxigen i glucosa. Quan el mateix experiment va ser realitzat en seccions de cervell d'un model animal d'hipòxia, l'administració de CBD també va resultar en una reducció dels nivells d'expressió de l'heteròmer. En conjunt, aquests resultats demostren que l'efecte neuroprotector del CBD en episodis d'hipòxia isquèmica són mediats per l'heteròmer CB_2R-$5HT_{1A}R$, confirmant el potencial del CBD en la teràpia d'hipòxia neonatal.

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Regulation of Expression of Cannabinoid CB_2 and Serotonin $5HT_{1A}$ Receptor Complexes by Cannabinoids in Animal Models of Hypoxia and in Oxygen/Glucose-Deprived Neurons

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Abstract: Background: Cannabidiol (CBD) is a phytocannabinoid with potential in one of the most prevalent syndromes occurring at birth, the hypoxia of the neonate. CBD targets a variety of proteins, cannabinoid CB_2 and serotonin $5HT_{1A}$ receptors included. These two receptors may interact to form heteromers (CB_2–$5HT_{1A}$-Hets) that are also a target of CBD. **Aims:** We aimed to assess whether the expression and function of CB_2–$5HT_{1A}$-Hets is affected by CBD in animal models of hypoxia of the neonate and in glucose- and oxygen-deprived neurons. **Methods:** We developed a quantitation of signal transduction events in a heterologous system and in glucose/oxygen-deprived neurons. The expression of receptors was assessed by immuno-cyto and -histochemistry and, also, by using the only existing technique to visualize CB_2–$5HT_{1A}$-Hets fixed cultured cells and tissue sections (in situ proximity ligation PLA assay). **Results:** CBD and cannabigerol, which were used for comparative purposes, affected the structure of the heteromer, but in a qualitatively different way; CBD but not CBG increased the affinity of the CB_2 and $5HT_{1A}$ receptor–receptor interaction. Both cannabinoids regulated the effects of CB_2 and $5HT_{1A}$ receptor agonists. CBD was able to revert the upregulation of heteromers occurring when neurons were deprived of oxygen and glucose. CBD significantly reduced the increased expression of the CB_2–$5HT_{1A}$-Het in glucose/oxygen-deprived neurons. Importantly, in brain sections of a hypoxia/ischemia animal model, administration of CBD led to a significant reduction in the expression of CB_2–$5HT_{1A}$-Hets. **Conclusions:** Benefits of CBD in the hypoxia of the neonate are mediated by acting on CB_2–$5HT_{1A}$-Hets and by reducing the aberrant expression of the receptor–receptor complex in hypoxic-ischemic conditions. These results reinforce the potential of CBD for the therapy of the hypoxia of the neonate.

Keywords: downregulation; heteromers; hypoxia; ischemia; phytocannabinoids; serotonin

1. Introduction

Cannabidiol (CBD) is one of the most studied components of *Cannabis sativa* L. The compound is approved for human use and is attracting further interest due to possible

additional health benefits in a variety of diseases/syndromes [1–8]. One important advantage over the most studied molecule in *Cannabis sativa* L., Δ^9-tetrahydrocannabinol (Δ^9-THC), is the lack of psychotropic effects. Like Δ^9-THC, CBD was supposed to act through cannabinoid receptors. Despite it may act as an agonist by binding the orthosteric center of cannabinoid CB_1 and CB_2 receptors [9–13], the compound also modulates these two receptors in an allosteric fashion [14–16]. Its mode of action seems to be multifaceted as it has, among others, agonistic properties at serotonin $5HT_{1A}$ receptors and modulates GPR55, peroxisome proliferator-activated receptors and potential cation TRPV1 channels [17–21].

CBD at 1 mg/kg single dose reduces hypoxia/ischemia-induced brain damage in newborn rats, mice, and piglets by, among other factors, diminishing excitotoxic damage, inflammation, and oxidative stress [22–25]. More recently, neuroprotection by CBD in a neonatal rat model of perinatal arterial ischemic stroke (PAIS) allows for functional recovery by reducing neuronal death and astrogliosis, as well as by decreasing apoptosis and metabolic alterations and by reducing neuroinflammation [26]. In that work, CBD was administered shortly after the end of MCAO (middle cerebral artery occlusion), and the selected dose was 5 mg/kg [26]. However, PAIS symptoms in a newborn are usually subtle and unspecific, which often determines that PAIS is not diagnosed or is diagnosed several days after delivery, when stroke is likely occurring [27]. Therefore, it is necessary to find an effective treatment despite the fact that it begins hours after the PAIS. CBD has demonstrated a broad temporal therapeutic window in adult mice models of stroke [28]. There are no data on the therapeutic window of CBD in newborn rats after PAIS. However, CBD showed a therapeutic window between 18 and 24 h in a mouse model of hypoxic-ischemic brain damage in newborns [29].

G-protein-coupled receptors may lead to protein–protein interactions resulting in heteromers, whose properties are different from those displayed by monomeric receptors [30–32]. Some of the CBD targets can form heteromers; among others, GPR55 with cannabinoid CB_1 or CB_2 receptors and the $5HT_{1A}$ receptor with the cannabinoid CB_2 receptor [25,33–36]. In addition, the pharmacological effects of CBD at cannabinoid receptors have been reported to depend on whether or not CB_1-CB_2 receptor heteromers are formed [37]. Interestingly, the benefits of CBD in preclinical models of hypoxic brain injury are mediated by CB_2 and $5HT_{1A}$ receptors, the compound being able to prevent some of the behavioral consequences of carotid artery occlusion via, among others, CB_2 and $5HT_{1A}$ receptors [38]. As the expression of heteromers formed by CB_2 and serotonin $5HT_{1A}$ receptors (CB_2-$5HT_{1A}$-Hets) increases in the brain of a model of newborn hypoxic-ischemic brain damage [35], we here addressed how CBD affects receptor pharmacology and expression of these heteroreceptor complexes in glucose-oxygen-deprived (GOD) cells. Moreover, the expression of CB_2-$5HT_{1A}$-Hets was determined in GOD neurons. For comparison, another phytocannabinoid, cannabigerol (CBG), was incorporated into the study.

2. Results

2.1. In Vivo HI-Induced Brain Damage

Rats in different groups were similar in age (8.6 ± 0.3, 8.6 ± 0.2, and 8.7 ± 0.1 d for sham, HI + vehicle, and HI + CBD, respectively; $p > 0.05$) and weight (18 ± 2, 18 ± 2, and 18.9 ± 0.6 g for sham, HI + vehicle, and HI + CBD, respectively; $p > 0.05$).

HI led to brain damage, as assessed by MRI, which was reduced by CBD treatment (volume of damage: FR3 = 19.6 ± 0.5 vs. 16.2 ± 0.4% for HI + vehicle and HI + CBD, respectively, $p < 0.05$; FR2 = 22 ± 1 vs. 14 ± 1% for HI + vehicle and HI + CBD, respectively, $p < 0.05$; FR1 = 24 ± 5 vs. 10 ± 5% for HI + vehicle and HI + CBD, respectively, $p < 0.05$).

2.2. CBD and CBG Favour CB_2–$5HT_{1A}$ Receptor Complex Formation

Due to the fact that $5HT_{1A}$ and CB_2 receptors can interact to form CB_2–$5HT_{1A}$- receptor heteromers (CB_2–$5HT_{1A}$-Hets), we first set out to assess whether CBD affects the receptor-receptor interaction. For comparison, the effect of another relevant phytocannabinoid,

cannabigerol (CBG), was also determined. Using immunocytochemical assays in HEK-293T cells that co-express the CB_2R fused to YFP and the $5HT_{1A}R$ fused to RLuc (Figure 1), it was observed that the receptors colocalize in plasma and intracellular membranes; the degree of colocalization is shown in yellow in Figure 1C.

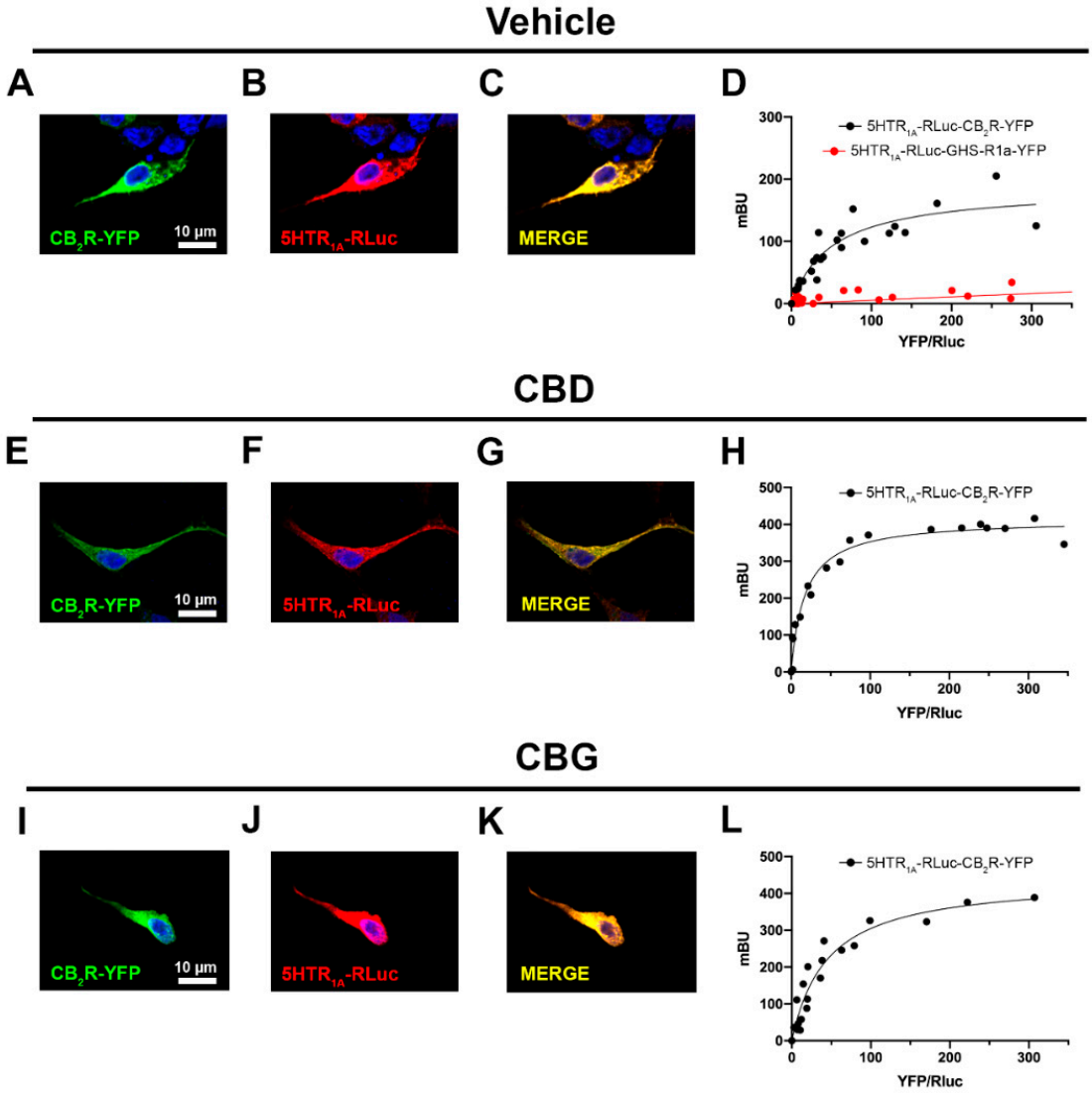

Figure 1. Effect of CBD or CBG on the $5HT_{1A}R$–CB_2R interaction. (**A**–**C**,**E**–**G**,**I**–**K**) Confocal microscope images of HEK-293T cells expressing CB_2R-YFP (0.75 µg cDNA) and $5HT_{1A}R$-RLuc (0.5 µg cDNA). Cells were pretreated with 200 µM of CBD (**A**–**C**), 200 µM of CBG (**E**–**G**), or vehicle (**I**–**K**) for 30 min. $5HT_{1A}R$-RLuc (red) was identified by immunocytochemistry using an anti-RLuc antibody. The CB_2R-YFP (green) was identified by its own fluorescence. Co-localization is shown in yellow. Cell nuclei were stained with Hoechst (blue). Scale bar: 10 µm. (**D**,**H**,**L**) Bioluminescence resonance energy transfer (BRET) assays performed in HEK-293T cells co-transfected with a constant amount of cDNA for $5HT_{1A}R$-RLuc (1.5 µg) and increasing amounts of cDNA for CB_2R-YFP (0.2 to 4 µg) or, as negative control, with a constant amount of cDNA for $5HT_{1A}R$-RLuc (0.75 µg) and increasing amounts of cDNA for GHSR-1a-YFP (0.2 to 2 µg cDNA). Transfected cells were pretreated with 200 nM of CBD (**D**), 200 nM of CBG (**H**), or vehicle (**L**) for 30 min before fluorescence emission was recorded. BRET data are expressed as the mean ± S.E.M of 8 different experiments performed in duplicates. mBU: milliBret units.

In cells pre-treated (30 min) with CBD or CBG, no significant differences were observed on receptor expression and colocalization (Figure 1). We then performed BRET experiments using HEK-293T cells expressing a constant amount of $5HT_{1A}$-RLuc and increasing amounts of CB_2R-YFP. Consistent with our previous results, a saturation BRET curve was obtained indicating interaction of the two receptors ($BRET_{max}$ = 185 ± 19 mBU; $BRET_{50}$ = 51 ± 14) to form CB_2–$5HT_{1A}$ receptor complexes (Figure 1D). Interestingly, the pre-treatment with 200 nM CBD notably increased the $BRET_{max}$ (414 ± 13 mBU) and the apparent affinity ($BRET_{50}$ = 17 ± 3), indicating that CBD increases the number of complexes formed and/or induces a structural reorganization of the CB_2-$5HT_{1A}$ receptor complex. Pre-treatment with 200 nM CBG increased the $BRET_{max}$ (440 ± 40 mBU) without significantly affecting the $BRET_{50}$ (44 ± 9) (Figure 1L). Cannabinoids did not affect receptor expression. As a negative control, HEK-293T cells expressing a constant amount of $5HT_{1A}$-RLuc and increasing amounts of GHS-R1a-YFP (Figure 1D) gave a linear signal indicating the lack of interaction between these two receptors.

2.3. CBD and CBG Blocked β-Arrestin 2 Recruitment Induced by Serotonin in Cells Expressing CB_2–$5HT_{1A}$-Hets

After showing that CBD and CBG favor the formation of the CB_2–$5HT_{1A}$ receptor complex, we questioned their effect on receptor functionality. First, β-arrestin 2 recruitment was analyzed by BRET in HEK-293T cells expressing β-arrestin 2-RLuc and either CB_2R-YFP, $5HT_{1A}$R-YFP, or CB_2R-YFP and $5HT_{1A}$R. Results from experiments in CB_2R-expressing cells showed that both CBD and CBG partially blocked the effect of the selective CB_2R agonist, JWH-133 (Figure 2A). Similarly, both phytocannabinoids partially blocked the effect of serotonin in $5HT_{1A}$R-expressing cells (Figure 2B). When results obtained in cells expressing CB_2–$5HT_{1A}$-Hets were analyzed, it was first noticed that the effect of serotonin on recruiting β-arrestin 2-RLuc to the CB_2R-YFP was marked, whereas the effect of selective CB_2R agonist was negligible (Figure 2C). In those cells expressing the CB_2–$5HT_{1A}$-Hets, both CBD and CBG completely blocked the effect induced by serotonin.

Due to the fact that both CB_2 and $5HT_{1A}$ receptors couple to G_i proteins, we performed cytosolic cAMP determination experiments after treatment with 0.5 μM forskolin in cells whose receptors were activated in the absence and presence of CBD or CBG. In cells expressing the CB_2R, the selective agonist, JWH-133, produced a significant decrease in forskolin-induced cAMP levels (Figure 2D). Interestingly, CBG (200 nM) led to a similar decrease in forskolin-induced cAMP levels. The effect of CBD was not significant, and coactivation using JWH-133 and either CBG or CBD led to values such as those obtained using JWH-133 alone (Figure 2D). In cells expressing the serotonin $5HT_{1A}$ receptor, it was CBD, but not CBG, that induced a significant decrease in the cAMP levels induced by forskolin. Coactivation using serotonin and either CBG or CBD led to values similar to those obtained using serotonin (Figure 2E). Finally, in HEK-293T cells co-expressing CB_2 and $5HT_{1A}$ receptors, both JWH-133 and serotonin produced a significant effect that was potentiated when the two compounds were added together. Interestingly, the action of serotonin, but not JWH-133, was enhanced by the two phytocannabinoids, CBD and CBG (Figure 2F). These data show that CBD and CBG differentially regulate signaling in singly transfected cells but exert a similar effect in CB_2–$5HT_{1A}$-Het-expressing cells.

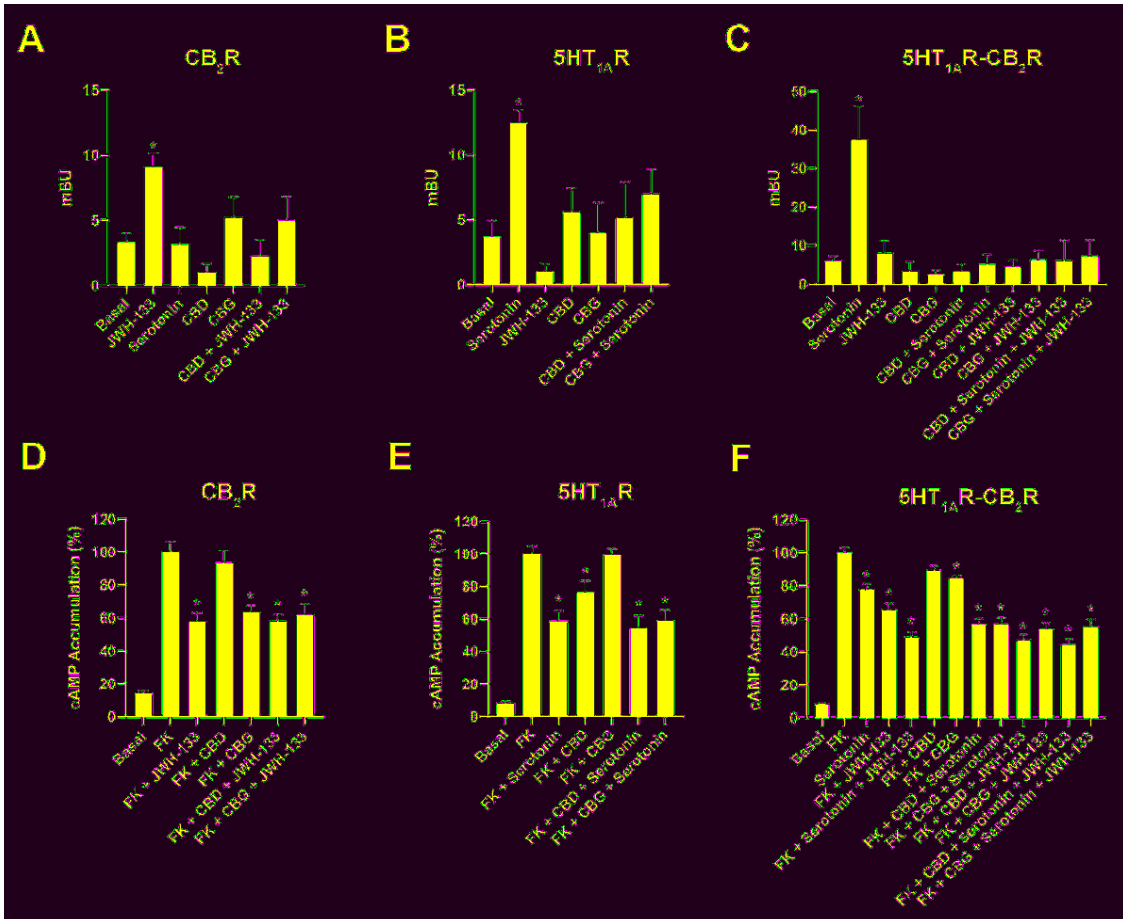

Figure 2. Effect CBD or CBG on the functionality of receptors expressed in HEK-293T cells. (**A**–**C**) β-Arrestin 2 recruitment assays were performed in HEK-293T transfected with cDNAs encoding for either CB_2R-YFP (1.5 μg) (**A**), $5HT_{1A}$R-YFP (1.5 μg) (**B**), or CB_2R (1.5 μg) and $5HT_{1A}$R-YFP (**C**). In all cases, β-arrestin 2-RLuc (1 μg cDNA) was also expressed. (**D**–**F**) Intracellular cAMP assays were performed in HEK-293T cells transfected with the cDNA encoding for either CB_2R (1.5 μg) (**D**), $5HT_{1A}$R (1.5 μg) (**E**), or both (**F**). In β-arrestin 2 recruitment and cAMP experiments, cells were pre-treated with 200 nM CBD, 200 nM CBG or vehicle and subsequently stimulated with the selective agonists, 200 nM JWH-133 -CB_2R- or 200 nM serotonin -$5HT_{1A}$R-. After the treatment with the agonists, cAMP levels after 0.5μM forskolin stimulation were detected by the LanceUltra cAMP kit and the results were expressed as a percentage with respect to levels obtained upon forskolin stimulation. β-Arrestin 2 recruitment was determined 25 min after treatment in cells expressing CB_2R (**A**) or $5HT_{1A}$R and CB_2R (**C**), or 7 min after treatment in cells expressing $5HT_{1A}$R (**B**). The values are the mean ± SEM of 10 different experiments performed in triplicate. One-way ANOVA followed by Dunnett's multiple comparison post hoc test were used for statistical analysis. * $p < 0.05$, versus basal condition in β-arrestin 2 recruitment experiments or versus 0.5 μM forskolin stimulation in cAMP assays.

2.4. CB_2–$5HT_{1A}$-Het Expression Was Upregulated in Glucose-Oxygen-Deprived (GOD) Primary Striatal Neurons

Striatal neurons seeded and cultured over 12 days were labelled using the in situ proximity ligation assay (PLA, see the Materials and Methods) with specific antibodies against CB_2 and $5HT_{1A}$ receptors. In complete medium and normoxia, approximately eight red dots were counted per every Hoechst-stained cell nucleus, indicating the expression of CB_2–$5HT_{1A}$-Hets in those neurons (Figure 3A,B). An important decrease in the receptor complex expression was observed when the same experiment was conducted in primary cultures pre-treated with CBD (approximately two red dots/cell). The effect of CBG was less marked as the number of dots per Hoechst-stained cell nucleus was around 5. Next, we

investigated the expression of CB_2–$5HT_{1A}$-Het in GOD cells. For this, the striatal neurons were maintained for 30 min in HBSS medium without glucose and subsequently placed in an anaerobic chamber for 4 h. GOD induced an important overexpression of CB_2–$5HT_{1A}$ receptor complexes (around 14 red dots/cell). Once again, pretreatment with CBD and CBG induced a significant decrease in the expression of the receptor complex, (respectively, 6 and 11 red spots/cell) (Figure 3). All together, these data indicate that CB_2–$5HT_{1A}$-Het expression is upregulated in GOD conditions and that phytocannabinoids, especially CBD, revert the effect.

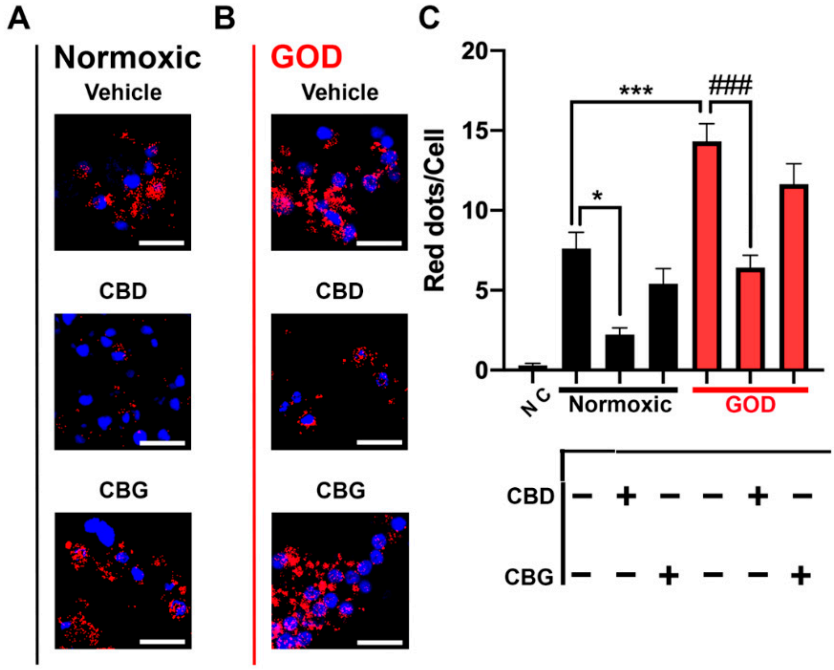

Figure 3. Effect of CBD and CBG on CB_2–$5HT_{1A}$ heteromer expression in GOD neurons. (**A**,**B**) CB_2–$5HT_{1A}$ heteromers were detected by in situ proximity ligation assay (PLA) in primary striatal neurons. Neurons were treated with either 200 nM CBD, 200 nM CBG, or vehicle for 30 min. Thereafter, cells were deprived of glucose and oxygen (GOD) (**B**) or were kept in aerated complete medium (**A**) for 4 h. Experiments were performed in samples from 6 different animals. Confocal images (stacks of 3 consecutive panels) were analyzed for assessing the number of red dots/cell. Red dots indicate expression of heteromers. Hoechst-stained nuclei appear in blue. Scale bar: 10 µm. (**C**) Quantification of the number of dots-clusters/cell was performed using the Andy's algorithm Fiji's plug-in. One-way ANOVA followed by Dunnett's multiple comparison post hoc tests were used for statistical analysis. NC: negative control; * $p < 0.05$, *** $p < 0.001$ versus normoxic vehicle, ### $p < 0.001$ versus GOD vehicle.

2.5. The CB_2–$5HT_{1A}$-Het Was Overexpressed in Brain Slices from Lesioned Animals

Once a significant increase in the expression of the CB_2–$5HT_{1A}$ receptor complex was identified in a GOD cell model, PLA experiments were performed on brain slices from injured pups. Apart from the control group, two groups of lesioned animals were generated, one treated with CBD and another treated with vehicle. Pups were first exposed to carotid electrocoagulation followed with hypoxia (10% O_2) for 112 min and treated or not with CBD. In situ PLA was first performed in cortical sections of brains taken one day after the insult. The results indicate low expression of CB_2–$5HT_{1A}$-Hets in control animals that underwent the same surgery without carotid electrocoagulation and that were not subjected to hypoxic conditions (SHAM) (Figure 4A). Upregulation of the receptor complex was induced by hypoxia (around six red dots/cell) and CBD was able to revert such upregulation (one red dot/cell) (Figure 4A). The expression days later after the insult was markedly decreased, showing about three and four red dots/cell in cerebral cortex sections

taken, respectively, 7 and 30 days after the lesion. Once again, CBD administration led to stronger downregulation in heteroreceptor complex expression (Figure 4D,F). Cortices treated with secondary antibodies in the absence of primary antibodies showed no PLA red spots/clusters, demonstrating the specificity of the technique (Figure 4B,D,F, NC bar). Taken together, these results demonstrate an upregulation of the CB_2–$5HT_{1A}$-Het induced by the hypoxic insult and a significant reversal upon CBD administration.

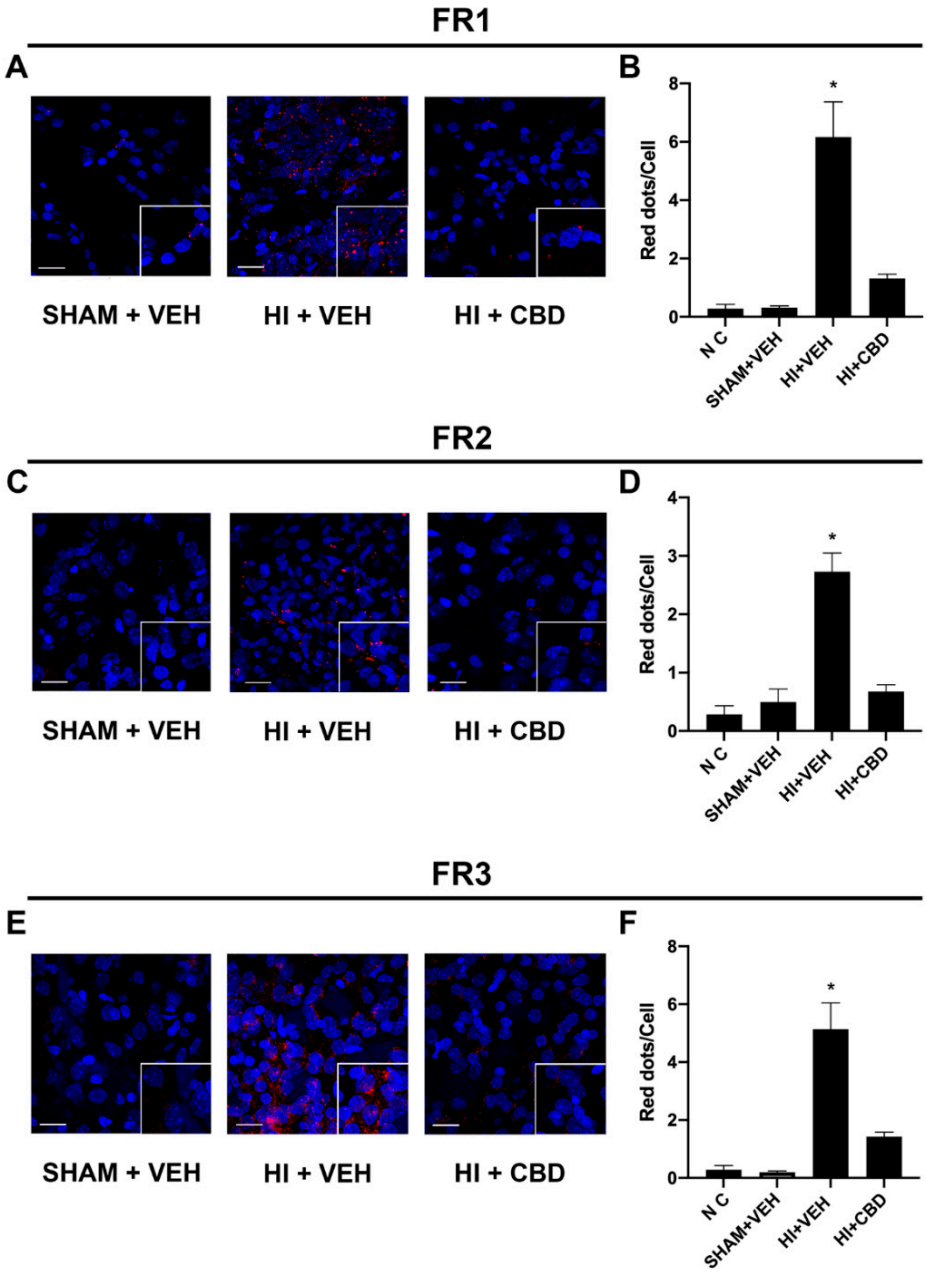

Figure 4. CB_2-$5HT_{1A}$ heteromer expression in brain slices from hypoxia-induced Wistar rats. (**A**,**C**,**E**) CB_2–$5HT_{1A}$-Hets expression in brain slices from hypoxia-induced Wistar rats (HI + VEH), hypoxia-induced Wistar rats treated with CBD (HI + CBD), and control Wistar rats (SHAM + VEH), detected by PLA. Rat brains were dissected 1 (FR3), 7 (FR2), or 30 (FR1) days after the insult, as described in the Materials and Methods. Red dots indicate expression of heteromers. Hoechst-stained nuclei appear in blue. (**B**,**D**,**F**) Quantification of the CB_2–$5HT_{1A}$Hets was conducted by detecting the number of red dots/cell using the Andy's algorithm Fiji's plug-in. Samples from 6 different animals were processed and analyzed. The NC negative control bar in panels B, D, and F refers to counts in cells treated only with secondary antibodies. Scale bar: 20 µm. * $p < 0.05$ versus SHAM+VEH.

2.6. CBD Abolished CB_2–$5HT_{1A}$-Het Functionality in GOD Striatal and Cortical Neurons

Finally, we addressed the effect of CBD or CBG pretreatment on the pharmacology displayed by receptors in striatal and cortical GOD neurons. In striatal neurons, G_i coupling was observed upon receptor activation using serotonin or JWH-133. This FK-induced lowering effect of cAMP levels after receptor activation was blocked by both CBD and CBG (Figure 5).

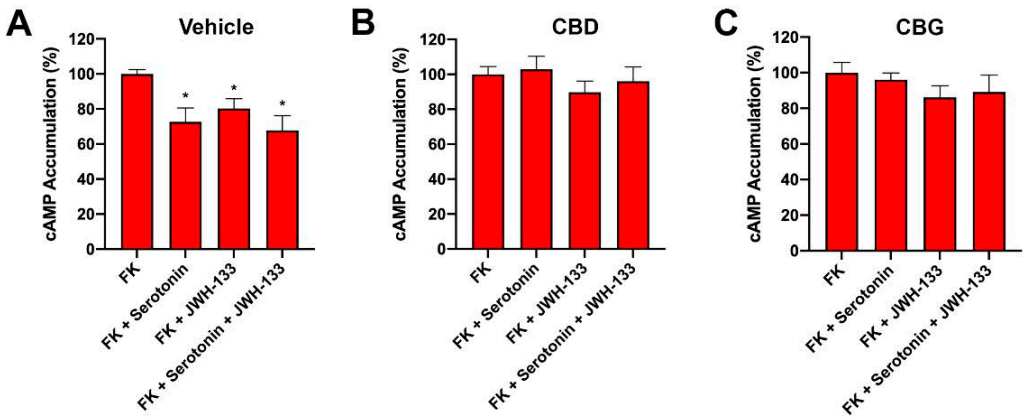

Figure 5. Effect CBD or CBG on the receptor functionality in GOD primary striatal neurons. Mouse primary striatal neurons were treated with vehicle (**A**), 200 nM of CBD (**B**), or 200 nM of CBG (**C**) for 30 min prior GOD for 4 h. Then, neurons were treated with selective agonists, 200 nM JWH-133, or 200 nM serotonin. cAMP levels were expressed as a percentage versus 0.5 µM forskolin treatment. Values are the mean ± S.E.M. of 8 different experiments performed in triplicate. One-way ANOVA followed by Dunnett's multiple comparison post hoc tests were used for statistical analysis. * $p < 0.05$ versus forskolin treatment.

In similar experiments performed on primary cortical neurons, CBG blocked the cannabinoid-receptor- and serotonin-receptor-mediated effect. In the case of CBD, the effect was less noticeable, significantly blocking the effect induced by serotonin but not that exerted by JWH-133 (Figure 6).

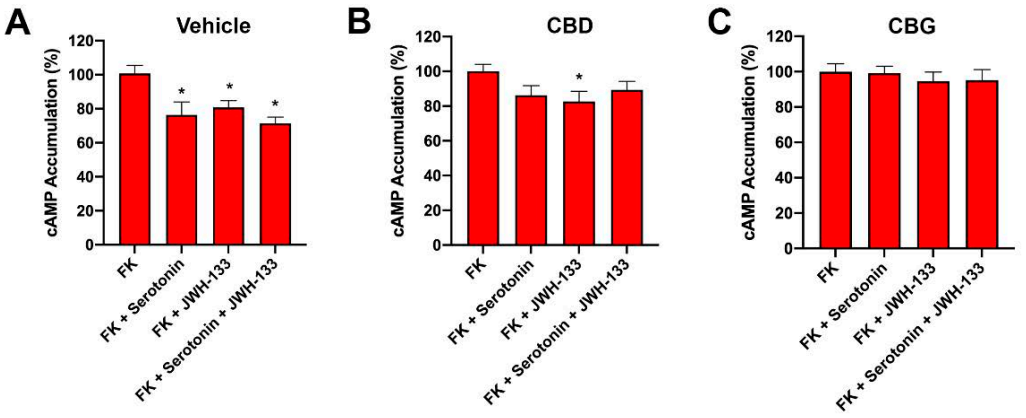

Figure 6. Effect CBD or CBG on the receptor functionality in GOD primary cortical neurons. Mouse primary striatal neurons were treated with vehicle (**A**), 200 nM of CBD (**B**), or 200 nM of CBG (**C**) for 30 min prior GOD for 4 h. Then, neurons were treated with selective agonists, 200 nM JWH-133 or 200 nM serotonin. cAMP levels were expressed as a percentage versus 0.5 µM forskolin treatment. Values are the mean ± S.E.M. of 8 different experiments performed in triplicate. One-way ANOVA followed by Dunnett's multiple comparison post hoc tests were used for statistical analysis. * $p < 0.05$ versus forskolin treatment.

3. Discussion

CBD has long been considered a neuroprotective molecule. In a previous study, it was shown in the middle cerebral artery occlusion model that CBD reduces the size of the infarcted brain area and the effect is partially blocked by WAY100135, a selective $5HT_{1A}$ receptor antagonist [39]. Hypoxia in the newborn can have negative consequences on the development of the nervous system. On the one hand, the sooner oxygenation is restored, the better the clinical outcome. On the other hand, it is necessary to limit the anatomical and cellular damage in the organ most susceptible to lack of oxygen, the brain. On the basis of experiments with a surrogate model of the disease, namely, the newborn piglet subjected to hypoxia-ischemia, CBD was proposed, several years ago, as an attractive drug to limit brain damage [22]. CBD is a phytocannabinoid that may interact with cannabinoid receptors; in both CB_1 and CB_2 receptors, the compound can enter into the orthosteric center to be a low-potency agonist and, also, it can interact with non-orthosteric sites to act as an allosteric modulator at nanomolar concentrations [14,15]. In addition, it is known that, at micromolar concentrations, CBD activates serotonin $5HT_{1A}$ receptors [18]; both CB_2 and $5HT_{1A}$ receptors are mediators of the neuroprotection provided by CBD in an animal model of neonatal hypoxia-ischemia [25].

It has been previously shown that CB_2 and $5HT_{1A}$ receptors may interact to form macromolecular complexes. The expression of such CB_2–$5HT_{1A}$-Hets is increased in the pig model of newborn hypoxic-ischemic brain damage. In addition, CB_2–$5HT_{1A}$-Het expression is tightly regulated in postnatal brain development stages; expression is relatively high at birth and declines rapidly with development of the nervous system [35]. In the rodent model used here, the increased expression of the heteromer, previously shown in the injured pig model, was reproduced and, consequently, one of the most relevant findings of this work is the significant reduction in the expression of CB_2–$5HT_{1A}$-Hets in CBD-treated lesioned rats. Interestingly, the previously reported upregulation of CB_2–$5HT_{1A}$-Hets in GOD primary neurons [35] was reversed by treating these cortical primary neurons with CBD (Figure 4).

In this study, the phytocannabinoid CBG was used in parallel with CBD because it has been suggested that the different binding modes of the cannabinoid to the CB_2R result in different output signals. A previous report addressed how the different CBD and CBG-type phytocannabinoids behave with respect to the functionality of cannabinoid CB_1 and CB_2 receptor. The results showed that it is the binding mode that makes the functional response vary from phytocannabinoid to phytocannabinoid [11]. It is tempting to speculate that differential benefits of phytocannabinoids in terms of therapeutic potential could depend on the binding mode, i.e., on how each molecule interacts with the orthosteric site and with exosites in the CB_2R. This is particularly relevant when it comes to cannabinoid receptors since (i) orthosteric sites have room accommodate different structures differently, (ii) orthosteric sites are not open to the extracellular medium, (iii) entry to the orthosteric center occurs through the lipid bilayer of the membrane, and (iv) the entrance is constituted by a narrow vestibule in which part of the chemical structure can be trapped [11,40–43]. Our results on comparing CBD and CBG effects are consistent with differences in binding modes that may be further modulated due to allosteric modulations resulting from the interaction of the $5HT_{1A}$ receptor with the CB_2 receptor. However, the differences found in cells expressing only one of the receptors were markedly reduced in cells expressing CB_2–$5HT_{1A}$-Hets. It is also true that the effect of CBG on the regulation of CB_2–$5HT_{1A}$-Het expression in primary cortical neurons was much weaker than that exerted by CBD.

The similar effect of CBD and CBG on primary GOD neurons opens the way to the hypothesis that in a hypoxia-ischemia environment, serotonin is harmful. Given that both phytocannabinoids blocked the effect of serotonin and there is consensus on the benefits of CBD in models of neonatal hypoxia, suppression of $5HT_{1A}$ receptor-mediated signaling may be beneficial. This hypothesis would fit with the need to reduce the expression of CB_2–$5HT_{1A}$-Hets shortly after birth for proper brain development. It would be good to assess the potential of $5HT_{1A}$ receptor antagonists in GOD neurons or hypoxia-ischemia

models. At present, this possibility is hampered by the fact that most of the antagonists of $5HT_{1A}$ receptor, e.g., alprenolol, may also interact with adrenergic receptors [44,45]. To our knowledge, there are no studies on the direct effect of more selective $5HT_{1A}$ receptor antagonists, e.g., spiroxatrine or WAY100135, in models of stroke or hypoxia-ischemia. There is, however, a report showing benefits of antagonizing the $5HT_{1A}$ using WAY100135 in a rodent model of intestinal ischemia-reperfusion [46].

4. Materials and Methods

4.1. Reagents

JWH-133 and serotonin were purchased from Tocris Bioscience (Bristol, UK). Coelenterazine H was purchased from Molecular Probes (Oregon, USA), and forskolin was purchased from Sigma-Aldrich (Missouri, US). Purified (>95% pure) cannabinoids were provided by Phytoplant Research S.L.U, Córdoba, Spain. Preparations of CBD were purified from the Cannabis variety GOYA (CPVO 20180113) and CBG, obtained from the variety AIDA (CPVO/20160167) following a direct crystallization method (Nadal, 2016; patent US9765000 B2 and WO2016116628A1), which allows compounds with a purity > 98% to be obtained.

4.2. HI Brain Damage Induction

Experimental procedures in rats were conducted in accordance with European and Spanish regulations (2010/63/EU and RD 53/2013) and approved by the Institutional Review Board of Hospital Clínico San Carlos-IdISSC (Madrid, Spain, protocol code ProEx 165/19, date of approval 25 February 2019). HI brain damage protocol is elsewhere described (Pazos et al., 2012). In brief, 7- to 10-day-old (P7–P10) Wistar rats were anesthetized with sevoflurane (5% induction, 1% maintenance). Exposed left carotid artery was electrocoagulated and, after recovery (a 3 h), pups were placed for 112 min into 500 mL jars in a water bath (37 °C) in 10% O_2. Control animals undertook the same surgical procedure but skipping electrocoagulation and hypoxia (SHAM). Ten minutes after the end of hypoxia, HI pups were randomly treated with s.c. injection of vehicle (HI + VEH, n = 27) or CBD (HI + CBD, n = 29). CBD was injected at a dosage of 1 mg/kg in 0.1 mL final volume. Then, rats were returned to the dam. On the day of the sacrifice, a T2WI MRI scan of the brains was carried out in the MRI Unit of the *Instituto Pluridisciplinar* (*Universidad Complutense de Madrid*, Madrid, Spain) on a BIOSPEC BMT 47/40 (Bruker-Medical, Ettlingen, Germany) operating at 4.7 T to determine the volume of damage, as described in detail elsewhere [24–26]. The rats were sacrificed 1 (FR3), 7 (FR2), or 30 (FR1) days after challenge, and the brains were removed and processed as described below.

4.3. Brain Sampling

Rats under deep anesthesia (i.p. injection of diazepam/ketamine) were sacrificed. Perfusion was performed transcardially with saline solution and 4% paraformaldehyde. Brains were removed to be embedded in paraffin. Coronal sections (30 µm thick) using a cryostat LEICA CM3050 S (Leica Microsystems, Wetzlar, Germany) were obtained for the immunohistochemical/PLA assays.

4.4. Cell Culture and Transfection

Human embryonic kidney HEK-293T (lot 612968) cells were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA). Each frozen aliquot was thawed, and the cells it contained were passaged 18 times before a new aliquot was taken. Culture medium was Dulbecco's modified Eagle's medium (DMEM) (Gibco, Waltham, MA, USA) supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, MEM non-essential amino acid solution (1/100), and 5% (*v/v*) heat-inactivated fetal bovine serum (FBS) (all supplements were from Invitrogen, Paisley, Scotland, UK). Cultures were kept in 5% CO_2 humid atmosphere (37 °C). Cells were transiently transfected using the PEI (PolyEthylenImine, Sigma-Aldrich, St. Louis, MO, USA) method as previously

described [47,48]. At 4 h after transfection, growth medium was replaced by complete medium. Experiments were carried out 48 h later.

4.5. Neuronal Primary Cultures

Neurons from the brain of fetuses (gestational age: 17 days) of pregnant CD1 mice (14–18 weeks old) were isolated as described elsewhere [49]. No ethical approval is needed for this protocol as long as no distinction is made between male/female sex of fetuses. Cells were plated at a confluence of 40,000 cells/0.32 cm^2. After trypsinization, cell suspension was repeated pipetted up and down followed by passage through a 100 µm pore mesh. Centrifugation (7 min, 200 × *g*) led to a pellet of cells that were resuspended in complete DMEM and seeded in 6-well plates at a density of 3.5 × 10^5 cells/mL. Then, 24 h later, the medium was replaced by neurobasal medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, and 2% (*v/v*) B27 medium (Gibco). Neurons were cultured for 12 days before assays. The use of NeuN allowed us to know that >90% cells in the culture were neurons.

4.6. Expression Vectors

The human cDNAs for the CB_2 and $5HT_{1A}$ receptors cloned in pcDNA3.1 were amplified using sense and antisense primers that were designed to eliminate stop codons. The primers harbored either unique EcoRI and BamHI sites to clone CB_2 and GHS-R1a receptors were subcloned to a pEYFP-containing vector to be in frame with a yellow fluorescent protein (pEYFP-N1; Clontech, Heidelberg, Germany). Primers harboring unique KpnI and BamHI and sites for β-arrestin 2 and $5HT_{1A}$R were subcloned to the pRLuc-N1 vector (PerkinElmer, Wellesley, MA, USA) to obtain a plasmid containing the sequence of a fusion protein with the Renilla luciferase protein (RLuc). A similar procedure was used to have fusion proteins with pEYFP. The generated constructs were CB_2R-YFP, $5HT_{1A}$R-YFP, $5HT_{1A}$R-RLuc, GHS-R1a-YFP, and β-arrestin 2-RLuc.

4.7. Glucose-Oxygen Deprivation (GOD)

Twenty-four hours prior to assay performance, cell medium was exchanged by glucose-free HBSS medium and treated with 200 nM CBD, 200 nM CBG, or vehicle to subsequently establish normoxic conditions (37 °C and 5% CO_2 atmosphere). Conditions were maintained for 30 min prior to placing cells in an anaerobic chamber (AnaeroPack Rectangular Jar 2.5 L; Thermo Scientific, Waltham, MA, USA) for 4 h with an anaerobic atmosphere-generator bag (AnaeroGen 2.5 L; Thermo Scientific, Waltham, MA, USA).

4.8. Immunofluorescence

HEK-293T cells transfected with cDNAs for CB_2R-YFP and $5HT_{1A}$R-RLuc were fixed in 4% paraformaldehyde for 15 min and then washed twice with PBS containing 20 mM glycine. A 0.2% Triton X-100 solution in the same buffer was used for permeabilization (5 min incubation). After 1 h in blocking solution (PBS containing 1% bovine serum albumin), cells were incubated with a mouse anti-RLuc antibody (1/100; MAB4400, Millipore, Burlington, MA, USA) and a secondary Cy3-conjugated anti-mouse IgG (1/200; 715-166-150; Jackson Immuno Research). After washing, samples were treated with mounting media (30% Mowiol; Calbiochem, San Diego, CA, USA). Nuclei were stained with Hoechst (1/100). A Zeiss 880 confocal microscope (Leica Microsystems, Wetzlar, Germany) was used for obtaining images.

4.9. Bioluminescence Resonance Energy Transfer (BRET) Assay

HEK-293T cells growing in 6-well plates were transiently co-transfected with two of the plasmids described in Section 2.6. Then, 48 h post-transfection, cells were washed twice with 0.1% glucose (*w/v*) in HBSS (137 mM NaCl, 5 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.26 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.5 mM $MgCl_2$, and 10 mM HEPES; pH 7.4). After detachment by gentle pipetting, the cells were resuspended in the same buffer. Protein

concentration was determined using a Bradford assay kit (Bio-Rad, Munich, Germany) and bovine serum albumin dilutions as standards. YFP-fluorescence was determined in 96-well black plates with a transparent bottom (Porvair, Leatherhead, UK) using a FluoStar Optima fluorimeter (BMG Labtechnologies, Offenburg, Germany) equipped with a high-energy xenon flash lamp, reading at 530 nm. Data are given as the fluorescence (20 μg protein in each sample) minus the fluorescence of cells only expressing protein-RLuc. BRET measurements were made using white 96-well plates (Porvair); in each well, a suspension (20 μg protein) of cells treated or not with cannabinoids was placed. Pretreatments were 30 min using 200 nM CBD, 200 nM CBG, or vehicle. Recordings began after the addition of 5 μM coelenterazine H (Molecular Probes, Eugene, OR). The BRET reader was a Mithras LB 940 (Berthold, Bad Wildbad, Germany), allowing integration of signals detected on the long wavelength filter at 530 nm (520–540 nm) and on the short wavelength filter at 485 nm (475–495 nm). To quantify receptor-RLuc expression, luminescence readings were collected 10 min after 5 μM coelenterazine H addition. The net BRET is defined as [(long-wavelength emission)/(short-wavelength emission)]-Cf, where Cf corresponds to [(long-wavelength emission)/(short-wavelength emission)] for the RLuc construct expressed alone in the same experiment. The BRET curves were fitted by non-linear regression. BRET values are given as milli BRET units (mBU: 1000× net BRET).

4.10. β-Arrestin 2 Recruitment

β-Arrestin 2 recruitment was determined as previously described [14] in cells transfected with one or more of the plasmids described in Section 2.6. Cells (20 μg protein) were distributed in 96-well white plates with a white bottom (Corning 3600) and incubated with compounds (see figure legends) for 10 min before the addition of 5 μM coelenterazine H. Then, 1 min after coelenterazine H addition, BRET was determined in a Mithras LB 940. To quantify protein-RLuc expression, luminescence was measured 10 min after the addition of 5 μM coelenterazine H.

4.11. cAMP Determination

The ad hoc LanceUltra kit (PerkinElmer, Waltham, MA, USA) was used for cAMP determination using homogenous assays. Transfected HEK-293T cells or primary neurons were seeded in 6-well plates. Two hours before initiating the experiment, culture medium was substituted by non-supplemented DMEM medium. After detachment, cells were re-suspended in non-supplemented medium containing 50 μM zardaverine. Cells were pretreated (30 min) with 200 nM CBD, 200 nM CBG, or vehicle and, 5 min later, stimulated with selective agonists. Forskolin (0.5 μM) or vehicle were then added for a period of 15 min. Finally, the reaction was stopped by the addition of the Eu-cAMP tracer and the ULight-cAMP monoclonal antibody prepared in the "cAMP detection buffer" of the LanceUltra kit. All steps were performed in 384-well microplates at 25 °C. Then, 60 min later, homogeneous time-resolved fluorescence energy transfer (HTRF) measures were obtained in a PHERAstar Flagship microplate reader equipped with an HTRF optical module (BMGLab technologies, Offenburg, Germany).

4.12. Proximity Ligation Assay (PLA)

Physical interaction was detected using the Duolink in situ PLA detection kit (Duolink, St. Louis, MO, USA) following the instructions of the supplier. Cells placed on glass coverslips or fixed brain sections were washed with PBS containing 20 mM glycine to quench the aldehyde groups; 0.05% Triton X-100 in the same buffer (20 min) was used for permeabilization. After 1 h at 37 °C with blocking solution, primary cultures were incubated overnight with a mixture of equal amounts of mouse anti-CB_2R (1/100; sc-293188, Santa Cruz Technologies, Dallas, TX, USA) and rabbit anti-$5HT_{1A}R$ (1/100, ab85615, Abcam, Cambridge, UK) antibodies to detect CB_2R–$5HT_{1A}R$ complexes. Neurons were processed using the PLA probes that detect primary antibodies (Duolink II PLA probe plus and Duolink II PLA probe minus) diluted in the antibody diluent solution (1:5). Ligation and

amplification were performed as indicated by the supplier. Hoechst (1/100; Sigma-Aldrich) was used to stain nuclei. For negative control, cells were treated with secondary antibodies in the absence of primary antibodies. A Zeiss 880 confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with an apochromatic 63× oil immersion objective (N.A. 1.4) and 405 and 561 nm laser lines was used for getting images. In each observation, data corresponding to a stack of two channels (one per staining) and to four Z stacks with a step size of 1 µm were acquired. Data analysis was performed using the Andy's algorithm Fiji's plug-in. One-way ANOVA followed by Dunnett's multiple comparison post hoc tests were used for statistical analysis.

4.13. Data Handling and Statistical Analysis

Data were analyzed blindly. Data are presented as the mean ± SEM. Statistical analysis was performed with SPSS 18.0 software. The test of Kolmogorov–Smirnov with the correction of Lilliefors was used to evaluate normal distribution and the test of Levene to evaluate the homogeneity of variance. Significance was analyzed by one-way ANOVA, followed by Bonferroni's multiple comparison post hoc test. Significance was considered when $p < 0.05$.

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Institutional Review Board Statement: Experimental procedures in rats were conducted in accordance with European and Spanish regulations (2010/63/EU and RD 53/2013). They were approved by the Institutional Review Board of Hospital Clínico San Carlos-IdISSC (Madrid, Spain, protocol code ProEx 165/19, date of approval 25 February 2019).

Data Availability Statement: Data that may be eventually missing can be obtained from the corresponding author upon reasonable request.

Conflicts of Interest: J.L., I.R., L.S., D.A.Z., A.L., J.M.-O., R.F. and G.N. declare no conflict of interests. C.F.-V. and V.S.d.M. declare that they work for Phytoplant Research SL, a research company that does not directly sell any product but has provided the purified phytocannabinoids obtained from *Cannabis sativa* L. that have been used in this research (web page: https://www.phytoplantresearch.com/; accessed on 20 August 2022).

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3.4 Cannabidiol skews microglia towards a neuroprotective phenotype in a model of neonatal hypoxia-ischemia.

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La hipòxia neonatal és una de les principals causes de mortalitat infantil i pot resultar en seqüeles de per vida a nivell del Sistema Nerviós Central. Els beneficis que el cannabidiol (CBD) ofereix, són el resultat de la seva interacció amb diferents receptors com el CB_2, el $5HT_{1A}$ o el PPARγ. En episodis d'hipòxia, la micròglia es troba activada en pro del seu fenotip neuroprotector M2. La possibilitat de polaritzar la micròglia cap a una funció neuroprotectora comporta unes implicacions terapèutiques molt importants per poder combatre malalties que causen mort neuronal com és el cas de l'ictus isquèmic. Per poder explorar aquesta línia d'investigació cal definir els marcadors en micròglia, que estan relacionats amb el fenotip neuroprotector M2 i també els que estan relacionats amb el fenotip proinflamatori M1. Els resultats d'aquest article demostren que el CBD, és capaç de protegir tant cultius primaris de neurones com de micròglia, dels efectes de mort cel·lular produïts per una deprivació d'oxigen i glucosa. A més, l'expressió de marcadors glials en un model animal d'hipòxia tractat amb CBD o vehicle, demostra que el cannabinoide és capaç de polaritzar la micròglia activada cap al seu fenotip neuroprotector en detriment del fenotip proinflamatori. L'estudi d'altres marcadors, tant d'oligodendròcits com d'astròcits, indica que el CBD és capaç de revertir l'activació d'aquestes cèl·lules en un model d'ictus.

Cannabidiol skews microglia towards a neuroprotective phenotype in a model of neonatal hypoxia-ischemia

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Summary
Neonatal hypoxia-ischemia is a severe condition that can result in death and/or lifelong central nervous system sequelae. Benefits of cannabidiol (CBD) result from neuroprotection asssumed to be mainly due to targeting a variety of neuronal receptors. The results of this article first show that CBD protects both primary neurons and primary glia against death induced by glucose and oxygen deprivation. Second, the expression of glial markers in an animal model of neonatal hypoxia-ischemia treated with vehicle or CBD shows that the cannabinoid was able to skew microglia towards a neuroprotective phenotype. Of interest was the expression of markers of neuroprotection in the microglia of lesioned animals. The microglia-mediated neuroprotection mechanism provided by CBD appears to be the increased expression of neuroprotective markers and the marked reduction in the release of proinflammatory factors from microglia activated by hypoxic-ischemic damage.

Keywords
Cannabinoids, stroke, glucose-oxigen deprivation, G protein-coupled receptors, neuroprotective microglia, microglial actiation, hypoxia of the neonate.

Introduction

Neonatal hypoxic-ischemic brain damage (HIBD) is a condition that can lead to death or serious sequelae, including epilepsy, autism spectrum disorder, motor deficits, cognitive deficits, and language learning difficulties (Martínez-Orgado et al., 2021). The most effective therapeutic intervention consists of the rapid restoration of oxygen supply and the limitation of the damage caused to the neural cells by the lack of oxygen. The only treatment approved so far in this regard is therapeutic hypothermia, but it is not beneficial for near 40% of severly asphyxiated newborns (Martínez-Orgado et al., 2021). Several years ago cannabidiol (CBD) was identified as a promising natural drug to decrease the consequences of the hypoxia of of HIBD in neonates (Martínez-Orgado et al., 2021). CBD at relatively low doses reduces brain damage in animals, from rats to piglets, undergoing hypoxia/ischemia. The benefits range from reducing inflammation to decreasing oxidative stress (Alvarez et al., 2008; Lafuente et al., 2011; Pazos et al., 2012, 2013). In a previous study, it was shown in the middle cerebral artery occlusion model in adult rats that CBD reduces the size of the infarcted brain area and the effect is partially blocked by WAY100135, a selective $5HT_{1A}$ receptor antagonist (Mishima et al., 2005). Similarly, WAY100135 abolishes CBD neuroprotection in asphyxiated newborn pigs (Pazos et al., 2013). CBD may also interact with a variety of receptors to mediate its beneficial effects. As a relevant example CBD allosterically regulates the functionality of cannabinoid CB_1 and CB_2 receptors (Laprairie et al., 2015; Martínez-Pinilla et al., 2017). Proteins mediated CBD actions may be expressed in different types of neurons and in glia, in particular in activated microglia.

It is know well established that, like peripheral macrophages, activated microglia may lead to different phenotypes. Although microglia can be activated in a variety of ways and display a variety of phenotypes, the M0/M1/M2 nomenclature is instrumental to assess mechanisms of microglial polarization. M0 means resting cells, M1 activated proinflammatory cells and M2 repairing and neuroprotective cells (Franco and Fernández-Suárez, 2015). M2 cells were firstly described in macrophages as undergoing an “alternative activation pathway” (Loke et al., 2000). Since then, eforts have been made to find drugs leading to skew macrophages or microglia towards the M2 phenotype. As for microglia, only in the last year has the focus been on an enormous variety of possibilities ranging from natural polyunsaturated fatty acids (Sanjay et al., 2022), natural polyphenols that regulate the LR4/NF-κB pathway (R. Li et al., 2022), drugs that modulate the JAK/STAT, HMGB1, MAPK or PPAR-γ pathways (Y. F. Li et al., 2022), SGLT2 Na^+/glucose transporter inhibitors (Pawlos et al., 2021) to agents that regulate the ionotropic P2X4 receptor (Castillo et al., 2022). In addition, autophagy-dependent cell reprogramming (Zubova et al., 2022), exosomes (Wan et al., 2022; Xin et al., 2021) or long non-coding RNAs (Gao et al., 2022) are novel proposals to afford microglia-mediated neuroprotection. CBD, which is a safe compound already approved for human use (resgistered as Epidiolex™) (Britch

et al., 2021; O'Connell et al., 2017; Wise, 2018), is able to interact and modulate the functionality of several receptors that are expressed in activated microglia. The aim of this paper was to check neuroprotective potential of CBD using primary cultures subjected to glucose-oxygen deprivation and to assess the variation in the expression of M1 and M2 markers in the activated microglia present in the brain of a neonate model of hypoxia-ischemia when animals are administered with 1 mg/mL CBD.

Results

Effect of CBD on microglial activation due to HIBD

To advance CBD into the therapy of HIBD we aimed at obtaining robust data to identify the main mechanism by which CBD provides anti-HIBD benefits. Consequently, the effect of CBD on activated microglia in P7-P10 rats exposed to electrocoagulation of the left common carotid artery and, after a 3-h recovery period, to a 10% O_2 atmosphere (112 min) was analyzed. These animals constituted the newborn hypoxic-ischemic brain damage (HI) group. CBD (1 mg/mL) or vehicle was injected 10 minutes after hypoxia. The control group underwent similar surgical procedures without carotid electrocoagulation or hypoxia (SHAM). Using antibodies against specific activation markers, microglial cells were stained in brain sections taken from brains isolated one (PT1), seven (PT7), or 30 (PT30) days after injection of CBD or vehicle.

Regarding Iba1 immunoreactivity, which corresponds to activated microglia, there was a modest expression in samples from the SHAM animals. A marked immunoreactivity was observed one day post-treatment (PT1) in the HI group (Figure 1A). This result is consistent with a quick microglial activation after the brain damage. Similar results were obtained seven or thirty days after treatment, indicating that the activation is chronic in nature (Figure 1B-C). Animals injected with CBD showed similar levels of microglial activation as vehicle-treated animals (Figure 1).

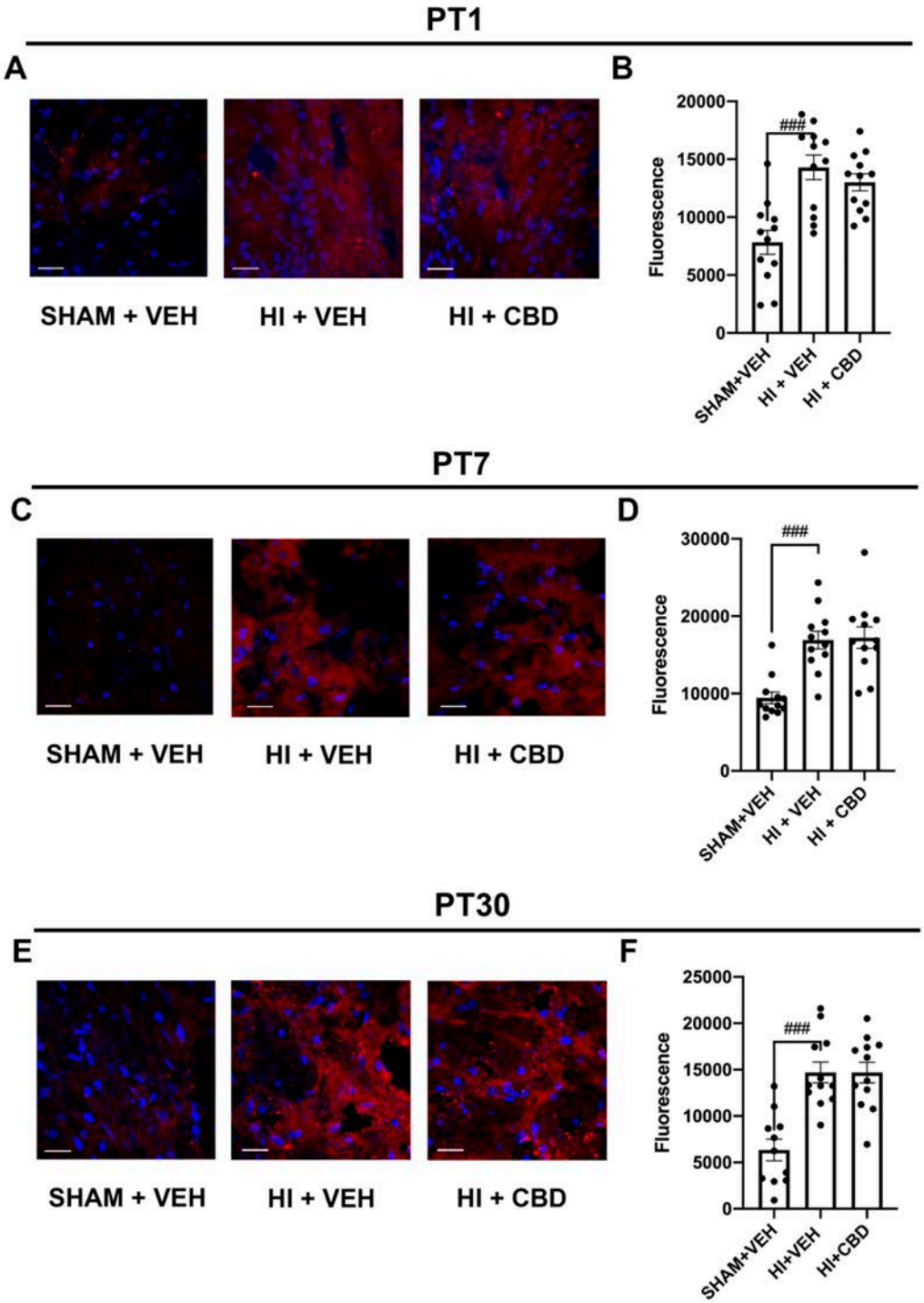

Figure 1: **Iba1 immunoreactivity.** Images were taken in brain sections from newborn hypoxic-ischemic brain damage (HI) animals treated with vehicle (HI + VEH) or CBD (HI + CBD). Images taken in brain sections from SHAM animals are also shown. Sections were taken from brains dissected one (PT1), seven (PT7) or 30 (PT30) days after the interventions (see Methods). Confocal microscopy images (stacks of 3 consecutive panels) show immunoreactivity (red) in preparations treated with Hoechst, which stains nuclei (blue). Panels **B, D, F**: Quantification of red fluorescence using the Fiji plugin of Andy's algorithm. Samples from 5 different animals were processed and analyzed. One-way ANOVA followed by Dunnett's multiple comparison post-hoc test were used for statistical analysis. ###p <0.001 versus SHAM+VEH condition. Scale bar: 20 μm.

CBD induces microglial polarization towards a neuroprotective phenotype

To characterize post-HIBD activated microglia we first used an antibody against a microglia proinflammatory phenotype, the inducible nitric oxide synthase (iNOS). In all samples from SHAM animals the immunoreactivity was negligible if compared with that of samples from HI animals (Figure 2). In lesioned animals the increase in iNOS labeling was seemingly progressive upon time. These results indicate that there is a proinflammatory response that persists even at day 30. Importantly, CBD treatment markedly reduced the proinflammatory response; the beneficial effect of the phytocannabinoid was evident in samples taken one day, seven days or 30 days after the damage (Figure 2).

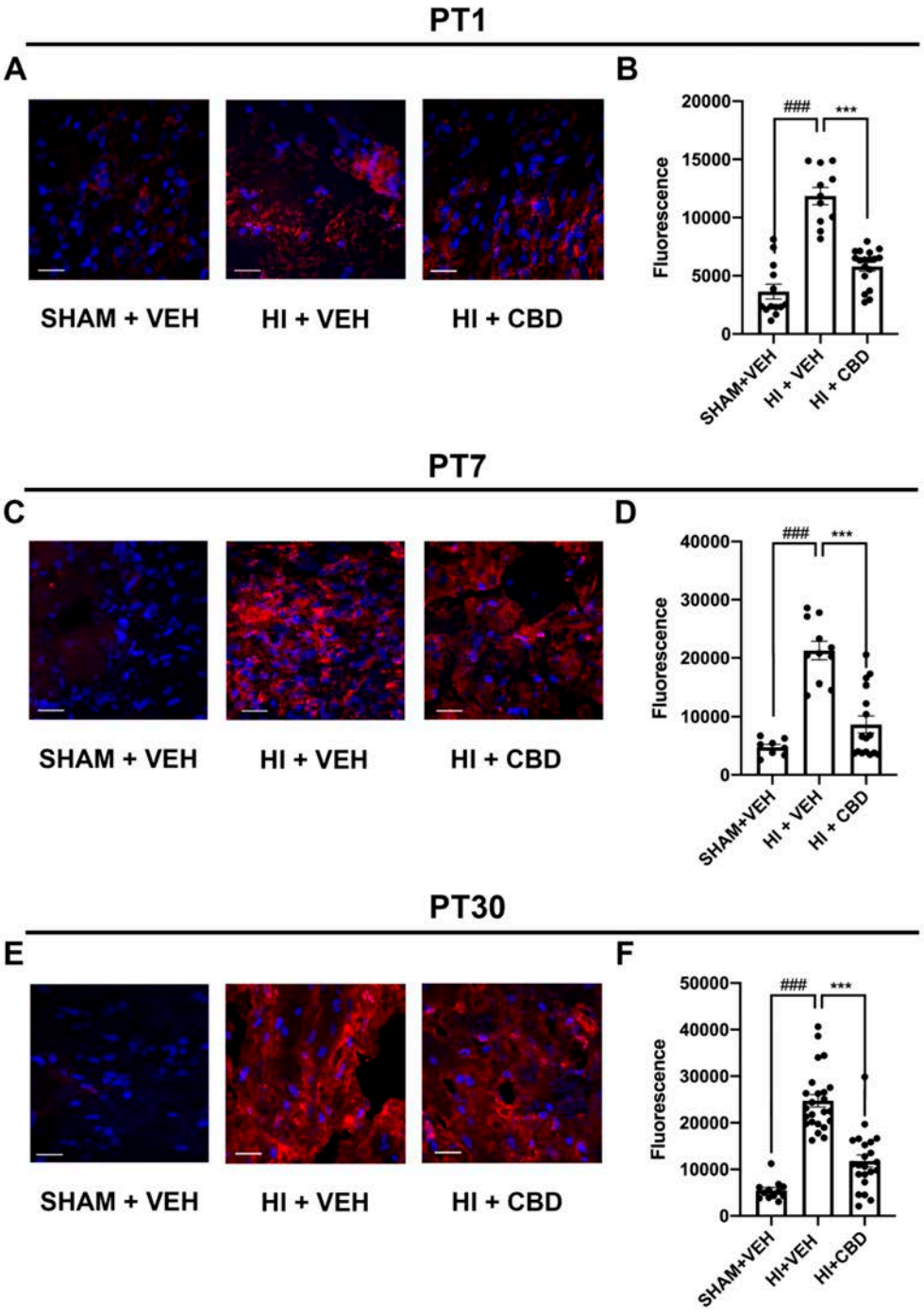

Figure 2: **iNOS immunoreactivity.** Images were taken in brain sections from newborn hypoxic-ischemic brain damage (HI) animals treated with vehicle (HI + VEH) or CBD (HI + CBD). Images taken in brain sections from SHAM animals are also shown. Sections were taken from brains dissected one (PT1), seven (PT7) or 30 (PT30) days after the interventions (see Methods). Confocal microscopy images (stacks of 3 consecutive panels) show immunoreactivity (red) in preparations treated with Hoechst, which stains nuclei (blue). Panels **B, D, F**: Quantification of red fluorescence using the Fiji plugin of Andy's algorithm. Samples from 5 different animals were processed and analyzed. One-way ANOVA followed by Dunnett's multiple comparison post-hoc test were used for statistical analysis. ###p <0.001 versus SHAM+VEH condition; ***p< 0.001 versus HI+VEH. Scale bar: 20 μm.

To assess the appearance of alternative activation-skewed microglia, antibodies against a marker for the neuroprotective phenotype, Arginase I, were used. The expression of the enzyme in samples from SHAM animals was very low. In contrast, immunoreactivity was significant in all samples from newborn HIBD animals (Figure 3). This finding suggest that the neuroprotective marker is quickly expressed after the damage. Importantly, CBD treatment significantly increased Arginase I expression compared to vehicle-treated animals, approximately two-fold. The power of CBD to increase Arginase I expression was evident in samples taken one day, seven days, or 30 days after the injury (Figure 3).

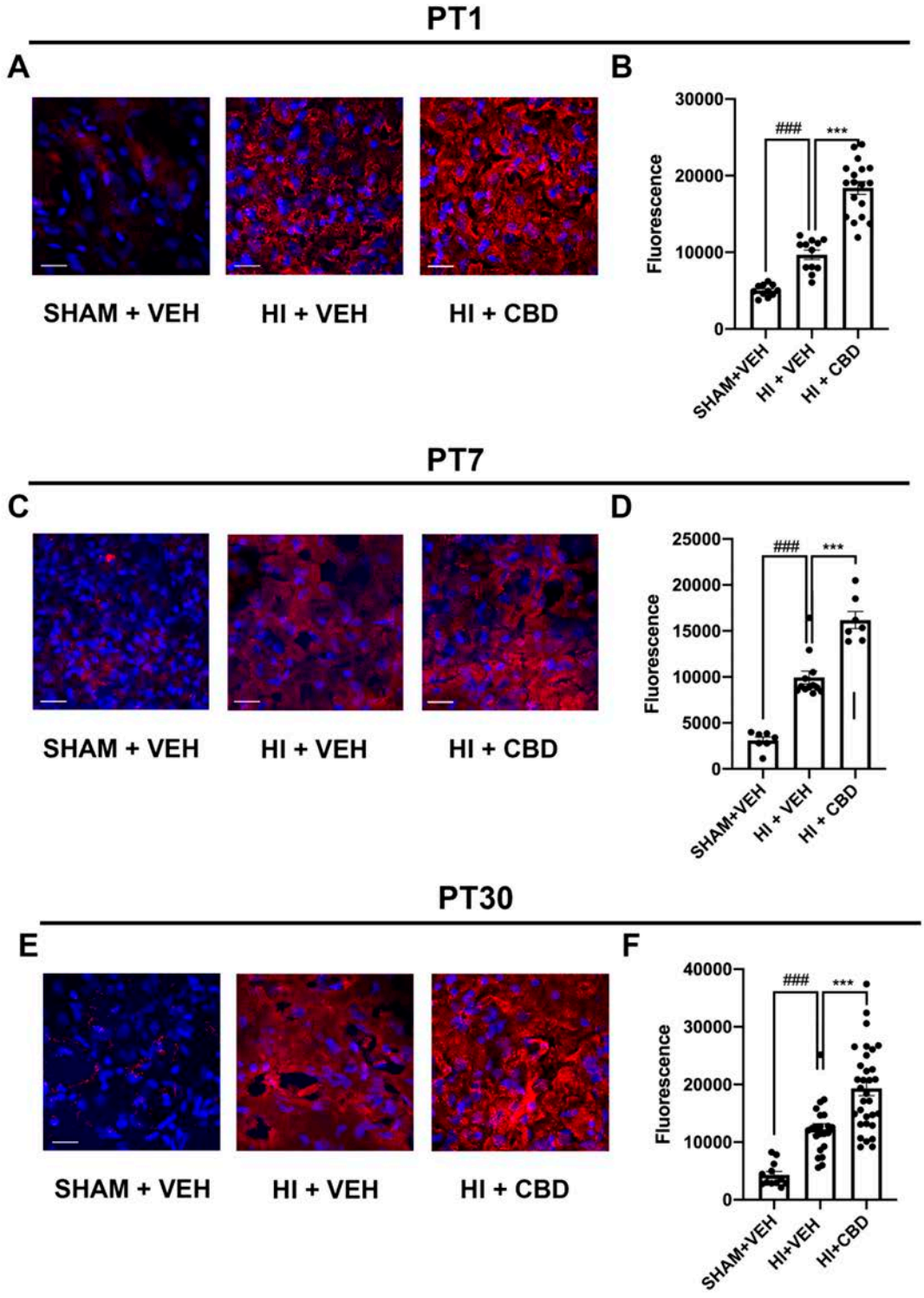

Figure 3: **Arginase I immunoreactivity.** Images were taken in brain sections from newborn hypoxic-ischemic brain damage (HI) animals treated with vehicle (HI + VEH) or CBD (HI + CBD). Images taken in brain sections from SHAM animals are also shown. Sections were taken from brains dissected one (PT1), seven (PT7) or 30 (PT30) days after the interventions (see Methods). Confocal microscopy images (stacks of 3 consecutive panels) show immunoreactivity (red) in preparations treated with Hoechst, which stains nuclei (blue). Panels **B, D, F**: Quantification of red fluorescence using the Fiji plugin of Andy's algorithm. Samples from 5 different animals were processed and analyzed. One-way ANOVA followed by Dunnett's multiple comparison post-hoc test

were used for statistical analysis. #p<0.05, ###p <0.001 versus SHAM+VEH condition; ***p< 0.001 versus HI+VEH. Scale bar: 20 μm.

Post-HIBD CBD treatment decreases the immunolabeling of GFAP and of the marker of immature oligodendrocytes

To identify the amount of reactive astroglia, antibodies against the glial fibrillary acidic protein (GFAP) were used. After quantification with Andy's algorithm Fiji's plug-in, a significant increase in GFAP immunoreactivity was observed in sections from lesioned animals. GFAP label in animals treated with CBD was similar to that obtained in non-lesioned animals (Figure 4). The results indicate that CBD treatment decrease damage induced activated astroglia to levels observed from SHAM animals.

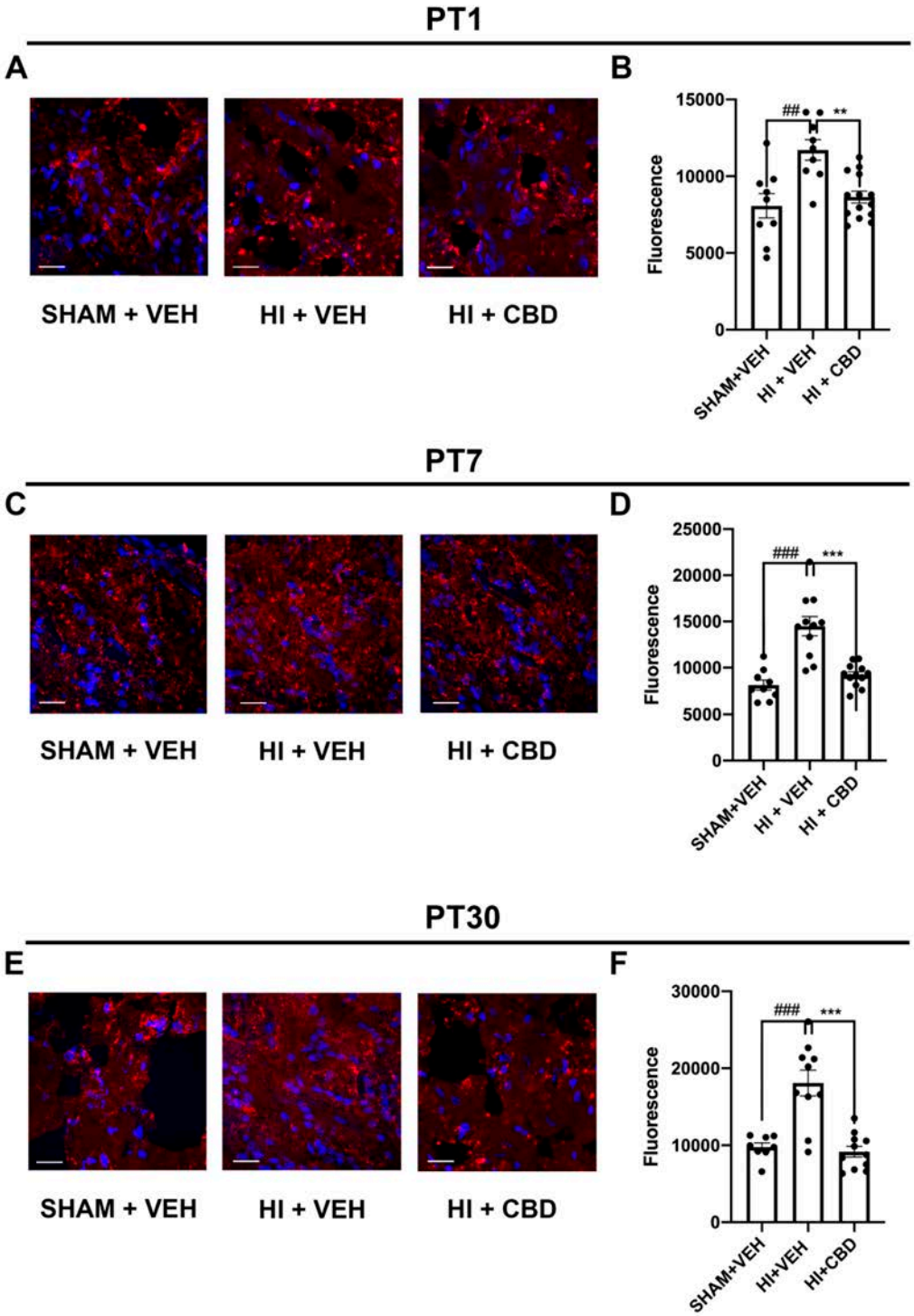

Figure 4: **GFAP immunoreactivity.** Images were taken in brain sections from newborn hypoxic-ischemic brain damage (HI) animals treated with vehicle (HI + VEH) or CBD (HI + CBD). Images taken in brain sections from SHAM animals are also shown. Sections were taken from brains dissected one (PT1), seven (PT7) or 30 (PT30) days after the interventions (see Methods). Confocal microscopy images (stacks of 3 consecutive panels) show immunoreactivity (red) in preparations treated with Hoechst, which stains nuclei (blue). Panels **B, D, F**: Quantification of red fluorescence using the Fiji plugin of Andy's algorithm. Samples from 5 different animals were processed and analyzed. One-way ANOVA followed by Dunnett's multiple comparison post-hoc test were used for

statistical analysis. ##p< 0.01, ###p <0.001 versus SHAM+VEH condition; ** p<0.01, ***p< 0.001 versus HI+VEH. Scale bar: 20 μm.

SOX-10 was selected as a marker of immature oligodendrocytes. The low SOX-10 immunoreactivity, increased upon the lesion and the increase was similar at PT1, PT7 and PT30. The treatment with CBD reverted the increase in all cases (Figure 5).

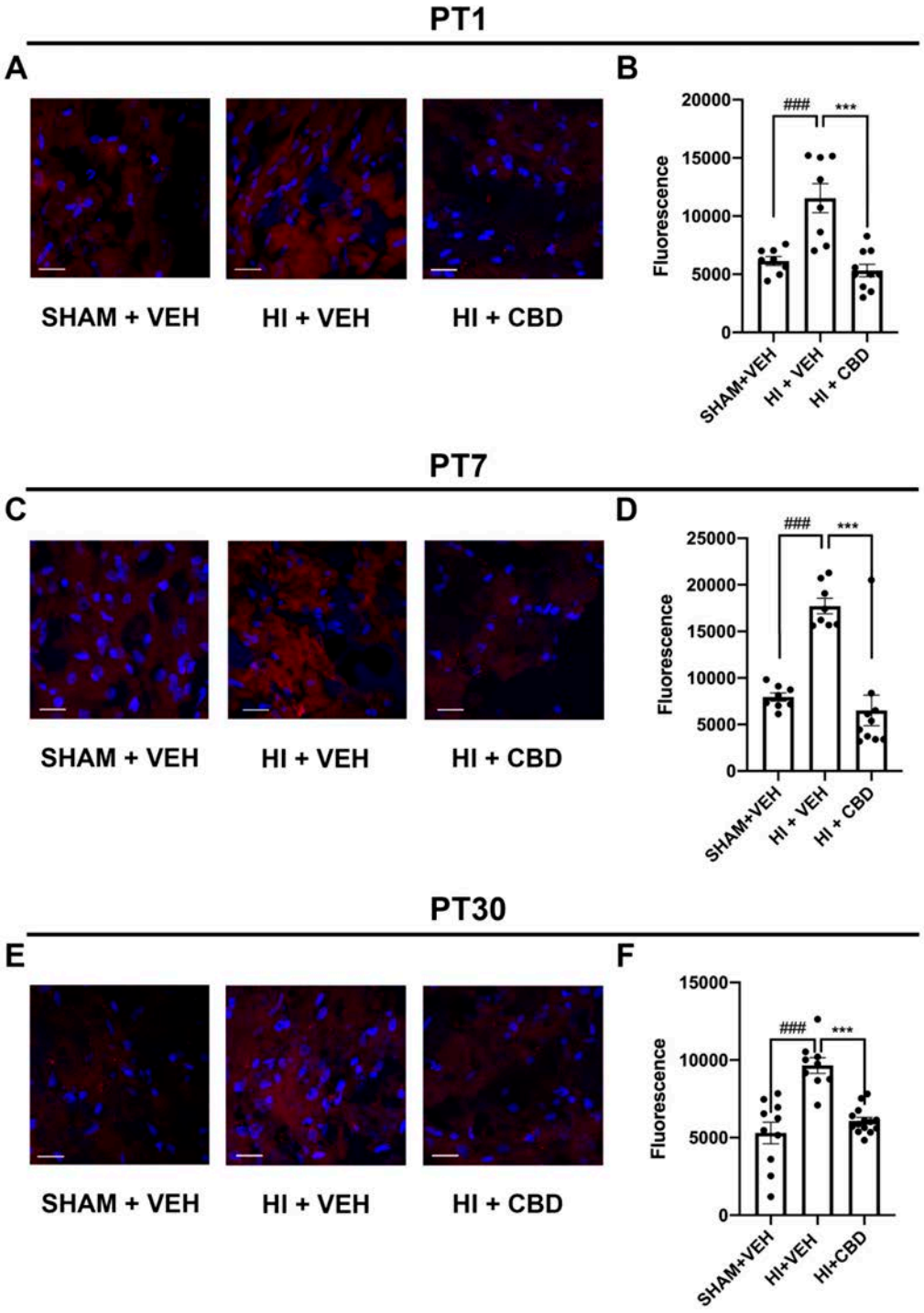

Figure 5: **SOX10 immunoreactivity.** Images were taken in brain sections from newborn hypoxic-ischemic brain damage (HI) animals treated with vehicle (HI + VEH) or CBD (HI

+ CBD). Images taken in brain sections from SHAM animals are also shown. Sections were taken from brains dissected one (PT1), seven (PT2) or 30 (PT30) days after the interventions (see Methods). Confocal microscopy images (stacks of 3 consecutive panels) show immunoreactivity (red) in preparations treated with Hoechst, which stains nuclei (blue). Panels **B, D, F**: Quantification of red fluorescence using the Fiji plugin of Andy's algorithm. Samples from 5 different animals were processed and analyzed. One-way ANOVA followed by Dunnett's multiple comparison post-hoc test were used for statistical analysis. ###p <0.001 versus SHAM+VEH condition; ***p< 0.001 versus HI+VEH. Scale bar: 20 μm.

CBD prevents neuronal death after glucose and oxygen deprivation

The neuroprotective effect of CBD was evaluated by analyzing cell death in primary cells under 4 h of glucose and oxygen deprivation. As expected, due to the lack of toxic effects, CBD (200 nM) was found to induce no effect on primary cells under normoxic conditions in medium containing glucose (Figure 6A). 4 h GOD induced >40 % neuronal death in either cortical, striatal or hippocampal neurons (Figure 6A-C). Interestingly, CBD treatment in medium containing glucose partially reverted the cell death observed under oxygen and glucose deprived neurons (Figure 6). In primary glial cells, GOD induced a >30% reduction in cell number, an effect that was also partially reverted by CBD pretreatment. This result indicates the potent effect of CBD in preventing neuronal and glial cell death after GOD.

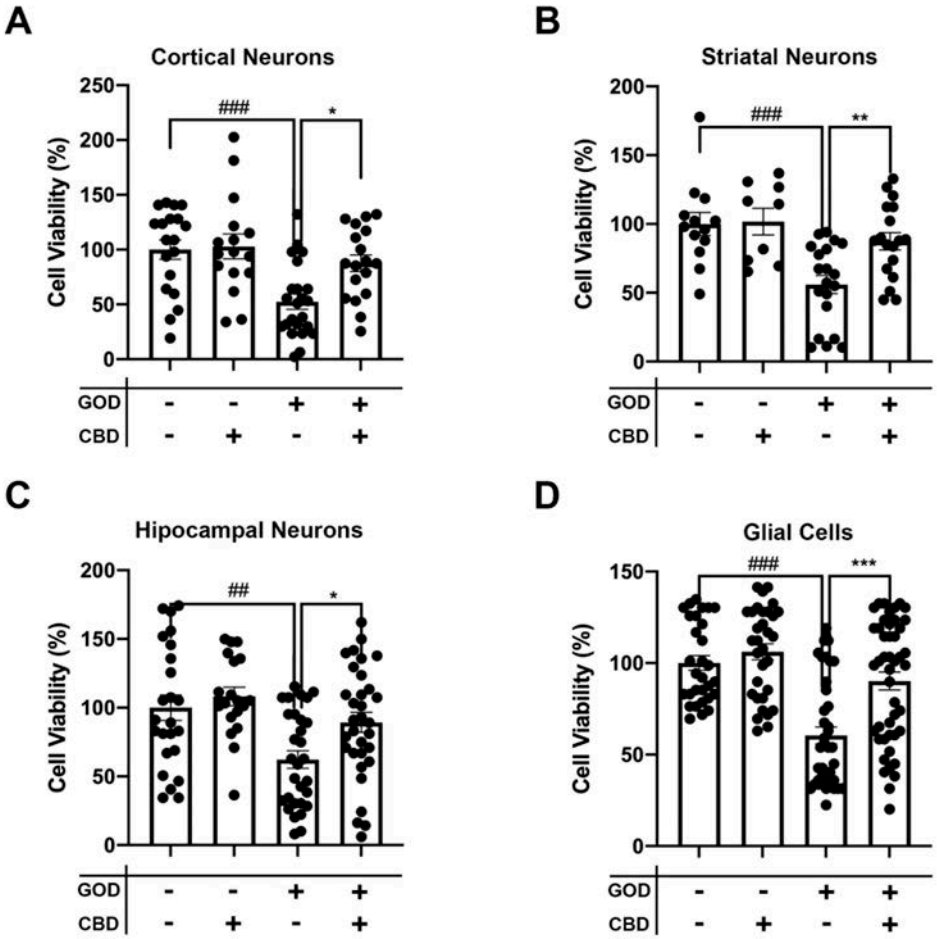

Figure 6: **Effect of CBD on primary cultures of neurons and glia viability after GOD.** Cell viability was determined in GOD primary cortical neurons (**A**), GOD primary striatal neurons (**B**), GOD primary hippocampal neurons (**C**) and primary glial cells (**D**) from CD1 mice. Cells treated with 200 nM CBD or vehicle for 30 min underwent a glucose and oxygen deprivation (GOD) or were maintained at normoxia in glucose-containing supplemented medium (See Methods for details). 20 h after treatment, cells were stained with Trypan Blue and sampled in a Neubauer chamber. The unstained (viable) and stained (nonviable) cells were counted separately, and the percentage of viability was calculated as the total number of viable cells/total number of cells x 100. Experiments were performed in samples from 6 different animals. One-way ANOVA followed by Dunnett's multiple comparison post-hoc test were used for statistical analysis. #p <0.05, ###p <0.001 versus GOD^--CBD^- condition; #p <0.05, ##p <0.005, ###p <0.001; *p <0.05, **p <0.01 versus GOD^+-CBD^- condition.

Discussion

Recent evidence suggests that microglia are key in protecting neurons from death as a result of acute or chronic damaging events occurring in the CNS. In neonatal HIBD, the microglia are activated and it is obvious that, in the case of patient survival and limited damage, the microglia have evolved towards their neuroprotective/resolving phenotype. The possibility of skew microglia towards a neuroprotective function has great therapeutic potential to combat a variety of

diseases characterized by neuronal death. There are two main issues to advance into finding microglia-centered interventions to prevent neuronal death. One is related to the most convenient target(s) in microglia and the second is to better define the neuroprotective markers in microglia that are associated to the neuroprotective phenotype. In this article, we have exploited an acute model of CNS damage in which i) glia are activated and ii) CBD causes damage reversal. The question to be answered was to what extent CBD exerts its beneficial effects by acting on the glia, with special emphasis on the possibility that the compound is biasing the microglia towards the so-called M2 phenotype in which Arginase I is one of the main markers.

Previous experiments in a piglet model of diffuse HIBD and a rat model of focal ischemia demonstrate the post-insult increase in microglia population (Barata et al., 2019; Ceprián et al., 2017). In these conditions, CBD does not reduce significantly $Iba1^+$ cell population, but reduces the proportion of ameboid microglia, consistent with a proinflammatory phenotype. The animal model of HIBD used here shows that there is a marked activation of microglia with a marked upregulation of iNOS, a proinflammatory marker. Importantly, iNOS upregulation was persistent and detectable even after 30 days post-injury. Also noteworthy was to discover that the treatment with CBD markedly reduced iNOS upregulation 1, 7 and 30 days after treatment. Somewhat unexpectedly, Arginase I, which is considered a marker of M2 microglia (Franco and Fernández-Suárez, 2015), was upregulated right after the lesion. However, the benefits of CBD correlated with a marked increase in Arginase I expression as early as 1 day after treatment but was maintained at 7 and 30 days after treatment. Taken together, these results question the hypothesis of pure proinflammatory or pure neuroprotective markers (Amici et al., 2017; Kanazawa et al., 2017). They also question the vision of a continuum of phenotypes that goes from the most proinflammatory to the most neuroprotective, going through various intermediate states (Devanney et al., 2020; Mesquida-Veny et al., 2021). The results reported here are more consistent with a time-dependent shift in the balance of the expression of molecules in microglia. It is likely that the physiological progression from proinflammatory action right after lesion to the resolving neuroinflammation may occur only in spcific setups but not in acute lesions (e.g. hypoxia of the neonate or stroke) or in the chronic scenarios underlying neurodegenreative diseases. Consequently, to be successful in finding ways to skew microglial phenotypes, further research on time-dependent expression changes of markers in physiological and pathological settings is needed.

The increase of GFAP immmunoreactivity after the lesion is probably refelceting an increased expression of the marker due to astrogial activation, an effect widely described after HIBD in animal models of HIBD (Martínez-Orgado et al., 2021). Such activation was also moderated by pretreatment with CBD. In contrast, the findings regarding SOX-10 activation are counterintuitive. SOX-10 is described as a transcription factor that in oligondendroglia promotes myelin gene

expression and allows oligodendrocyte survival. Therefore, the increase in SOX-10 expression is a well-known feature appearing shortly after HIBD, representing a proliferative reaction to restore HIBD-induced myelin damage (Back, 2014). However, the persistence of increased levels of SOX-10 several days after HIBD is linked to the accumulation of immature oligodendrocytes due to HIBD-induced impairment of the maturational process from pre-oligodendrocyte towards mature myelinating oligodendrocytes (Back, 2014). As a consequence, the population of mature myelinating oligodendrocytes is reduced 30 days after HIBD in brain of rats submitted to HIBD in the neonatal period (Ceprián et al., 2019). Such reduction is not observed when newborn rats are treated with CBD, leading to the restore of myelination [Ceprian et al, 2019]. Thus, the effects of CBD on SOX-10 expression were reflecting the protective effects of CBD on olygodendroglial maturational processes. Besides, SOX proteins, SOX-10 included, may be expressed in astrocytes (Klum et al., 2018) and SOX-10 may in vitro convert astrocytes into oligodendrocytes (Khanghahi et al., 2018). It is then possible that the increase in GFAP and SOX-10 after injury is due to de novo gliogenesis and conversion of astrocytes to oligodendrocytes.

Material and Methods

Reagents

CBD was purchased from Cerilliant (Texas, USA). The antibodies used were the following: Monoclonal mouse anti-Arginase I (ref. 610708, BD Bioscience, California, CA, USA), monoclonal mouse anti-iNOS (MA5-17139, Invitrogen, Waltham, MA, USA), polyclonal goat anti-GFAP (PA5-18598, Invitrogen, Waltham, MA, USA), polyclonal goat anti-Iba1 (ab107159, Abcam, Cambridge, UK) and monoclonal mouse anti-SOX10 (ab216020, Abcam, Cambridge, UK).

HI Brain Damage Induction

Experimental procedures in rats were conducted in accordance with European and Spanish regulations (2010/63/EU and RD 53/2013) and approved by the Institutional Review Board of Hospital Clínico San Carlos-IdISSC (Madrid, Spain, protocol code ProEx 165/19, date of approval 25 February 2019). Hypoxic-ischemic (HI) brain damage protocol is elsewhere described (Pazos et al., 2012). In brief, 7- to 10-day-old (P7–P10) Wistar rats, anesthetized with sevoflurane (5% induction, 1% maintenance), were exposed to left carotid artery electrocoagulation; upon 3 h recovery, pups were placed for 112 min into 500 mL jars kept at 37 °C under 10% O_2 atmosphere. Control animals (SHAM) underwent the same interventions but skipping electrocoagulation and hypoxia. Ten minutes after the end of hypoxia, HI pups were treated with i.p. injection of vehicle (HI + VEH, n = 27) or CBD (HI + CBD, n = 29).

CBD was injected at a dosage of 1 mg/kg in 0.1 mL final volume. Then, rats were returned to the dam. On the day of the sacrifice, a T2WI MRI scan of the brains was carried out in the MRI Unit of the Instituto Pluridisciplinar (Universidad Complutense de Madrid, Madrid, Spain) on a BIOSPEC BMT 47/40 (Bruker-Medical, Ettlingen, Germany) operating at 4.7 T to determine the volume of damage, as described in detail elsewhere [24–26]. The rats were sacrificed 1 (PT1), 7 (PT7), or 30 (PT30) days after challenge, and the brains were removed and processed as described below.

Brain dissection

The rats were sacrificed under deep anesthesia (diazepam/ketamine i.p. injection) and transcardially perfused with 4% paraformaldehyde in saline. Each harvested brain was embedded in paraffin to obtain coronal sections (30 µm thick) using a LEICA CM3050 S cryostat (Leica Microsystems, Wetzlar, Germany).

Neuronal primary cultures

To prepare primary neurons, brains from fetuses of pregnant CD1mice were removed (gestational age: 17 days). Neurons were isolated as described in Hradsky et al. (Hradsky et al., 2011) and cells were seeded at a density of 40,000 cell/0.32 cm^2. Briefly, after removal of the meninges, samples were dissected and digested with 0.25% trypsin (20 min at 37ºC). The effect of trypsin was stopped by adding an equal volume of culture medium (supplemented DMEM). A single-cell suspension was obtained by repeated pipetting followed by passage through a 100 µm-pore mesh. Pelleted (7 min, 200×g) cells were resuspended in supplemented DMEM and seeded at a density of 3.5×10^5cells/mL in 6-well plates. After 24 h medium was replaced by neurobasal medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin and 2 % (v/v) B27 medium (GIBCO, Waltham, MA, USA). Primary neurons were assayed after 12 days in culture. Using NeuN as a marker, the percentage of neurons in the culture was >90%.

Glucose-Oxygen deprivation (GOD).

Primary cells in glucose-free HBSS medium were placed for 4 h (37ºC) in a 2.5 L Oxoid jar containing an anaerobic atmosphere-generator bag (AnaeroGen; Thermo Scientific, Waltham, Massachusetts, U.S). Cells were then treated with 200 nM CBD or vehicle for 30 min. Thereafter, the medium was substituted by in Neurobasal medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 2% (v/v) B27 supplement (GIBCO) and kept at 37ºC in 5% CO_2 humid atmosphere for 20 h before assessing cell death using Trypan Blue. Control cells received the same vehicle or CBD treatment, but were always

maintained in glucose-containing medium and grown in a humidified 5% CO_2 atmosphere.

Immunohistochemistry

Sections from fixed brains were treated for 1 h with blocking solution (PBS containing 1% bovine serum albumin) and treated ON ant 4ºC, with one of the following primary antibodies: monoclonal mouse anti-Arginase I (1/100, ref. 610708, BD Bioscience, California, CA, USA), monoclonal mouse anti-iNOS (1/200, MA5-17139, Invitrogen, Waltham, MA, USA), polyclonal goat anti-GFAP (1/100, PA5-18598, Invitrogen, Waltham, MA, USA), polyclonal goat anti-Iba1 (1/100, ab107159, Abcam, Cambridge, UK) or monoclonal mouse anti-SOX10 (1/200, ab216020, Abcam, Cambridge, UK). Then, sections were incubated at RT for 2 h with the following secondary antibodies: Cy3-conjugated anti-mouse IgG (1/200, 715-166-150, Jackson ImmunoResearch, Pennsylvania, PA, USA) or Cy3-conjugated anti-goat IgG (1/200, 705-167-003, Jackson ImmunoResearch, Pennsylvania, PA, USA). Stained sections were washed several times with PBS-Gly 20 mM and mounted with 30% Mowiol (Calbiochem, San Diego, CA, USA). Nuclei were stained with Hoechst (1/100). Samples were observed under a Zeiss 880 confocal microscope (Leica Microsystems, Wetzlar, Germany)

Viability assay

Cells were scrapped from the plate and resuspended in neurobasal medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin and 2 % (v/v) B27 (GIBCO). Trypan blue staining was performed mixing 1 part of 0.4% trypan blue and 1 part of cell suspension in a plastic tube. After ~3 min of incubation at room temperature, 10 µl of the mixture was sampled in a Neubauer chamber and counted with a Countess II FL (Life Technologies, California, CA, USA). The unstained (viable) and stained (nonviable) cells were counted separately, and the percentage of viability was calculated as: total number of viable cells/total number of cells x 100.

Data Handling and Statistical Analysis

Data concerning animal sections were analyzed blindly. Data are presented as the mean ± SEM. Statistical analysis was performed with SPSS 18.0 software. The test of Kolmogorov–Smirnov with the correction of Lilliefors was used to evaluate normal distribution and the test of Levene to evaluate the homogeneity of variance. Significance was analyzed by one-way ANOVA, followed by the Dunnett's multiple comparison post-hoc test.

Declarations

Ethical Approval

Animal handling, sacrifice, and further experiments were conducted according to the guidelines set in Directive 2010/63/EU of the European Parliament and the Council of the European Union that is enforced in Spain by National and Regional organisms; the 3R rule (replace, refine, reduce) for animal experimentation was also taken into account. The specific bioethical protocol was approved by the Institutional Review Board of Hospital Clínico San Carlos-IdISSC (Madrid, Spain, protocol code ProEx 165/19, date of approval 25 February 2019).

Competing interests

J.L., I.R. L.S., D.A.Z., A.L., J.M-O, G.N. and R.F. declare no conflict of interest other than having received purified phytocannabinoids from Phytoplant Research SL, without any specific indication on the research to be performed with the compounds. C.F.-V. and V.S.d.M. declare that they work for Phytoplant Research SL, a research company that does not directly sell any product but has provided the purified phytocannabinoids obtained from *Cannabis sativa* L. that have been used in this research (web page: https://www.phytoplantresearch.com/; accessed on 15 November 2022).

Author contributions

R.F., G.N. and J.M-O. designed and supervised the project. L.S. did the lesion, administered CBD and removed the brain before further processing. J.L. and D.A.Z. sectioned brain tissue and performed immunohistochemical assays. J.L., I.R. and A.L. did animal manipulation, tissue dissection and preparation of primary cultures. J.L. and A.L. performed cell death assays. C.F-V. and V.S.d.M. provided the necessary reagents to carry out the project. R.F., G.N. and J.L. wrote the first draft. All authors have edited the manuscript and have approved the submitted version.

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Data availability

Raw data may be provided by corresponding authors upon reasonable request.

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3.5 Cannabidiol decreases pTau and Aβ axonal transport and improves spatial memory in 5xFAD mice model of Alzheimer's disease.

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Article en vies de redacció i revisió per ser enviat a la revista *Nature Communications*

La malaltia de l'Alzheimer és el tipus de demència més comú arreu del món, representant dos terços del total de malalts de més de 65 anys. L'Alzheimer afecta a més de 50 milions de persones, i com que un dels factors de risc més importants de la malaltia és l'edat, s'espera que aquesta xifra arribi a duplicar-se en les següents dècades a causa de l'increment de l'esperança de vida a nivell global. El Cannabidiol (CBD), el segon fitocannabinoide més abundant de la planta del cànnabis, forma part del Sistema Cannabinoide i ha demostrat capacitat per induir la neurogènesi i reduir la toxicitat induïda pel pèptid β-amiloide i altres símptomes de demència en models d'Alzheimer tant en estudis preclínics com clínics. L'objectiu principal d'aquest article va ser investigar els possibles efectes beneficiosos del CBD en la malaltia de l'Alzheimer. Emprant ratolins 5xFAD, model animal per la malaltia d'Alzheimer, vam observar que el CBD és capaç de polaritzar la micròglia activada cap al seu fenotip neuroprotector en detriment del fenotip proinflamatori i revertir l'increment d'expressió dels receptors CB_1 i CB_2 observat en ratolins 5xFAD. A més, en cultius primaris de neurona es va observar que el tractament amb CBD reduïa el transport neurona-neurona de les proteïnes associades amb l'Alzheimer Aβ, Tau i pTau i restaurava la pèrdua de neurites induïda pel tractament amb Aβ, Tau i pTau. Finalment, l'administració de CBD en ratolins 5xFAD va ser capaç de revertir les pèrdues de memòria a curt i llarg termini associades a l'Alzheimer.
D'aquesta manera, en aquest article s'ha demostrat l'ampli espectre dels beneficis del CBD, des d'un nivell molecular fins a un nivell de comportament, bassant-se en la reducció de la formació tant de plaques amiloides com de cabdells neurofibril·lars.

Cannabidiol decreases pTau and AB axonal transport and improves spatial memory in 5xFAD mice model of Alzheimer's disease.

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Abstract

Alzheimer's disease is the most common type of dementia worldwide, accounting for two-thirds of all patients over 65 years of age. Alzheimer's affects more than 50 million people, and since one of the most important risk factors for the disease is age, this number is expected to double in the coming decades due to increase in life expectancy. Cannabidiol (CBD), the second most abundant phytocannabinoid in the cannabis plant, is part of the Cannabinoid System and has demonstrated the ability to induce neurogenesis and reduce β-amyloid peptide-induced toxicity and other dementia symptoms in Alzheimer's models both in preclinical and clinical studies. The main objective of this article was to investigate the possible beneficial effects of CBD in Alzheimer's disease. Using 5xFAD mice, an animal model for Alzheimer's disease, we observed that CBD is able to polarize activated microglia towards their neuroprotective phenotype to the detriment of the proinflammatory phenotype and reverse the increased expression of CB_1 and CB_2 receptors observed in 5xFAD mice. Furthermore, in primary neuronal cultures, CBD treatment was observed to reduce the neuron-neuron transport of the Alzheimer-associated proteins Aβ, Tau and pTau and to restore neurite loss induced by Aβ, Tau and pTau treatment. Finally, the administration of CBD in 5xFAD mice was able to reverse the short- and long-term memory losses associated with Alzheimer's.
Thus, this paper has demonstrated the broad spectrum of benefits of CBD, from a molecular to a behavioral level, based on the reduction of both amyloid plaque and Neurofibrillary tangles.

Key words: CBD, cannabinoid system, Alzheimer, neurites.

Introduction

Alzheimer's disease (AD) is nowadays the most common dementia worldwide, with two thirds of cases of people aged more than 65. It is estimated that more than fifty million people suffer it[1]. One of the main risk factors of AD is age, thus, this data will not stop rising in the coming years due to the global aging of the world population, doubling the number of cases in the following decades.

Exist two different AD subtypes, familial and sporadic. At molecular level, both express plaques formed by parenchymal deposition of amyloid-β (Aβ) and intraneuronal accumulation of hyperphosphorylated Tau protein[2,3,4]. These aberrant protein aggregates lead to inflammation and oxidative stress, but also microglia activation, astrogliosis, characteristic losses of neurons, neuropil, and synaptic elements, provoking a progressive impairment of cognitive and behavioral functions including comprehension, language, memory, attention, reasoning, and judgment. Nowadays, this pathology has no cure, only treatments to counteract the symptomatology. In this sense, have been approved four available drugs, an antagonist of the N-methyl-D-aspartate (NMDA) receptor (memantine) and three are acetylcholinesterase inhibitors (rivistagmine, donepezil and galantamine)[5].

The Endocannabinoid System (ECS) plays an essential role in cognitive function and brain memory in different aspects. The cannabinoid system is characterized by retrograde signaling, that is to say, ligands can be synthesized through components of the plasma membrane of the postsynaptic neuron in response to different stimuli, be released into the synaptic space and bind to cannabinoid receptors that are expressed on the presynaptic neuron, thus regulating the release of other neurotransmitters. It should be noted that there are also cannabinoid receptors in post-synaptic neurons with an important pathophysiological implication. The Cannabinoid System is made up of three main components, the CB_1 and CB_2 cannabinoid receptors, the CB_1 receptor being the most expressed membrane receptor at CNS level and the CB_2 receptor having a strong involvement in the immune system, the cannabinoid compounds and the enzymes of synthesis and degradation of these. Cannabinoids are divided into endocannabinoids, being anandamide and 2-AG (*2*-araquidonilglicerol) the most studied with greater involvement at a physiological level, synthetic cannabinoids and phytocannabinoids, which are those compounds extracted directly from the *Cannabis sativa* plant.

Cannabis sativa contain more than 500 distinct compounds and 120 have been classified as phytocannabinoids showing different structures and pharmacological properties[6]. In 1940 were isolated the first compounds: cannabinol and cannabidiol (CBD)[7], however, the CBD structure was not described since 1963. Then, in 1964, the most abundant and active phytocannabinoid, Δ^9-Tetrahydrocannabinol (Δ^9-THC), the main responsible of the psychoactive effects. Cannabinoids are involved in synaptic responsiveness and plasticity[8]. Specifically, low doses of Δ^9-THC have demonstrated inducing neurogenesis and reducing Aβ toxicity and plaque deposition in rodents hippocampus and other dementia symptoms in both pre-clinical and clinical studies[9].

Cannabidiol is the second most abundant phytocannabinoid; it comprises up to 40% of the total compounds extract. It can bind both cannabinoid receptors, acting as an allosteric modulator at nanomolar concentrations on both receptors[10,11] and binding in the orthosteric site of CB_1R at micromolar concentrations. It shows a bias functionality[12] with anti-oxidant[13], anti-neuroinflammatory[14], anxiolytic[15], antipsychotic and neuroprotective properties. Moreover, multiple basic and clinic assays have demonstrated its potential to combat different pathological conditions such as schizophrenia, social phobia, post-traumatic stress, depression, bipolar disorder, sleep disorders, epilepsy, substance abuse and dependence and neurodegenerative diseases as Parkinson[16]. Specifically, different studies demonstrate the capacity of CBD to decrease neuroinflammation and reactive gliosis as well as to induce neurogenesis, preventing the development of cognitive deficits in AD rodent models[17]. Meanwhile, it is important to point out that a part from cannabinoid receptors, CBD induce some of its effects by activating a wide spectrum of other receptors such as some orphan receptors plus serotonin ($5\text{-}HT_{1A}$), PPARs, vanilloid receptors, adenosine receptors, N-methyl-D-aspartate (NMDA) receptor, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors opioid receptors, and dopamine receptors[18,19].

Hence, the main objective of the study consists in investigating the possible beneficial effects of cannabidiol in the neurodegenerative disease of Alzheimer. Focusing our attention in the decrease of the symptomatology propagation, glia activation and neuronal death. The interest on CBD relays on the lack of psychoactive effects and its beneficial effects in different pathophysiology conditions convert it in a powerful compound with multiple benefits for AD.

Results

CBD injection polarizes microglia to a neuroprotective M2 phenotype in cortical and hippocampal sections.

To test CBD involvement in microglia polarization, the 5xFAD mice model of Alzheimer disease (AD) was injected with one daily dose of CBD (10 mg/Kg) for 4 weeks. AD is a multifactorial pathology that mainly affects cortical and hippocampal areas. In this sense, cortical and hippocampal brain sections of the AD mice model 5xFAD treated or not with CBD and control mice were analyzed by immunohistochemistry. First, activated microglia was detected by Iba1 primary antibody and a secondary Cy3 conjugated anti-rabbit antibody in cortical sections. As observed in Figure 1A, 5xFAD mice showed a significant increase in activated microglia in comparison with control mice, that was not affected in that animals treated with CBD. However, when analyzing Arginase I staining, a marker of the M2 neuroprotective phenotype, it was observed a significant increase in 5xFAD animals compared to controls that was more potent in that animals injected with CBD (Fig 1B). As opposed, it was analyzed the inducible nitric oxide synthase (iNOS) against a microglia M1 proinflammatory phenotype. The signal in AD mice model triplicated the fluorescence of control mice (Figure 1C) while CBD injections reduced this effect.

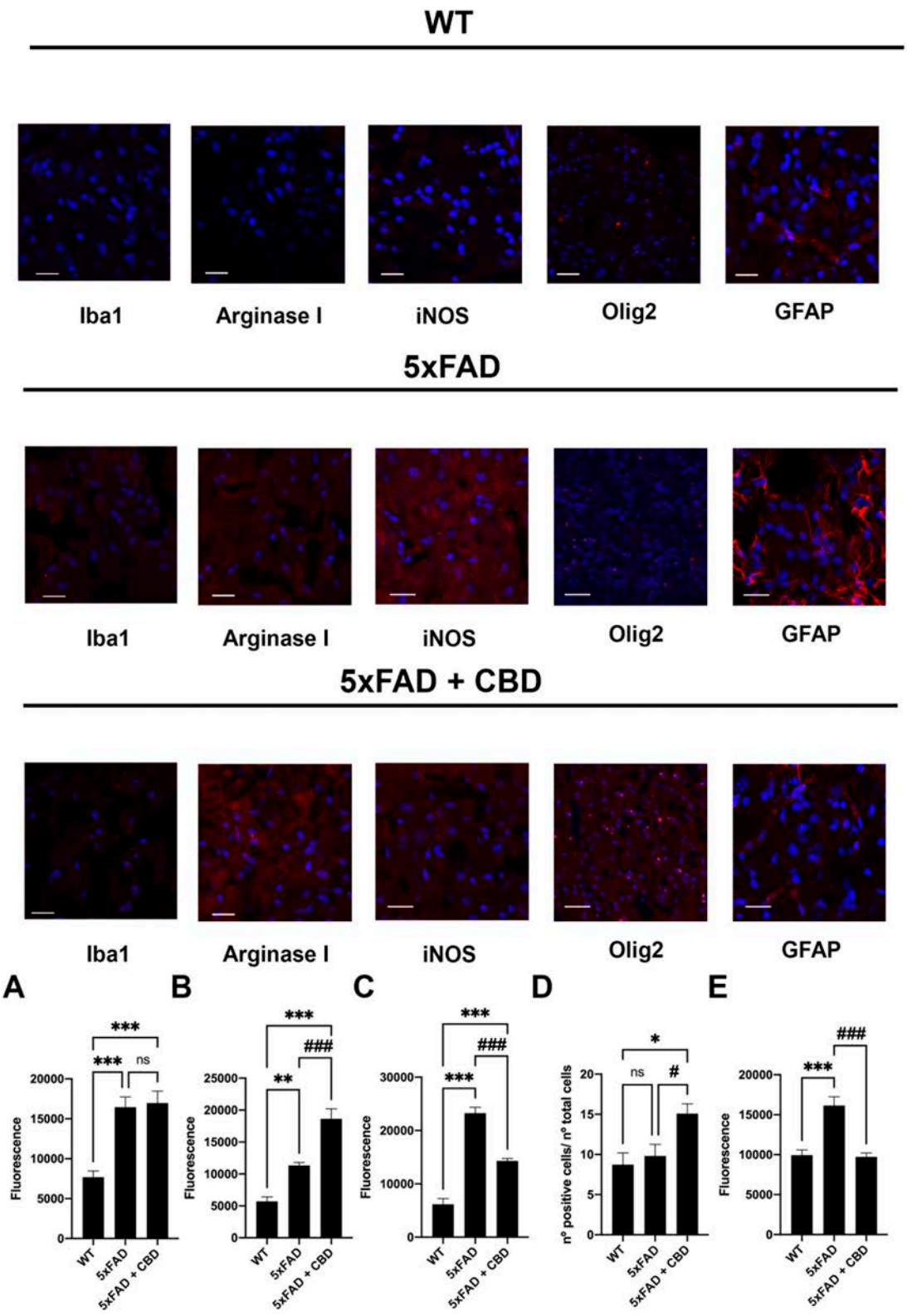

Figure 1**: Immunohistochemistry in cortical sections from 5xFAD mice injected with CBD. (A-D**). Cortical sections from 5xFAD mice (5xFAD), 5xFAD mice treated with i.p. injection of pure CBD (5xFAD + CBD) and control Wild Type mice (WT), were analyzed by immunohistochemistry using specific anti-Iba1, anti-Arginase I, anti-iNOS, anti-Olig2 and anti-GFAP antibodies. Confocal microscopy images (stacks of 3 consecutive panels) show protein immunoreactivity in red over Hoechst-stained nuclei

(blue). Quantification of the protein immunoreactivity was done using the Andy's algorithm Fiji's plug-in. Samples from 5 different animals were processed and analyzed. One-way ANOVA followed by Dunnett's multiple comparison post-hoc test were used for statistical analysis. *p <0.05, **p <0.005, ***p< 0.001 versus WT; #p <0.05, ##p <0.005, ###p <0.001 versus 5xFAD. Scale bar: 20 μm.

These results indicate that CBD injections polarize microglia to a M2 neuroprotective phenotype in the AD mice model 5xFAD, showing a neuroprotective role. Afterwards, it was assessed the expression of oligodendrocyte precursor cells that differentiate in mature oligodendrocytes providing myelin to neurons by detecting the oligodendrocyte transcription factor 2 (Olig2). It was detected a similar Olig2 immunoreactivity when comparing 5xFAD animals and controls. But CBD treated animals showed a significant increase in the number of oligodendrocyte precursor cells (Fig 1D). Finally, it was identified the amount of reactive astroglia by the antibody against the glial fibrillary acidic protein (GFAP). After quantification with Andy's algorithm Fiji's plug-in, it was observed a significant increase in GFAP immunoreactivity in 5xFAD animals compared to control mice. However, this effect was completely reverted in animals treated with CBD (Fig 1E). These results demonstrate that CBD treatment favors oligodendrocyte precursor cells expression while decreases activated astroglia cells in the AD mice model 5xFAD. When the same experiments were performed in hippocampal neurons it were obtained similar results (Figure 2). The only difference was observed in Olig2 immunoreactivity where CBD treatment showed no increase in comparison to 5xFAD or control mice.

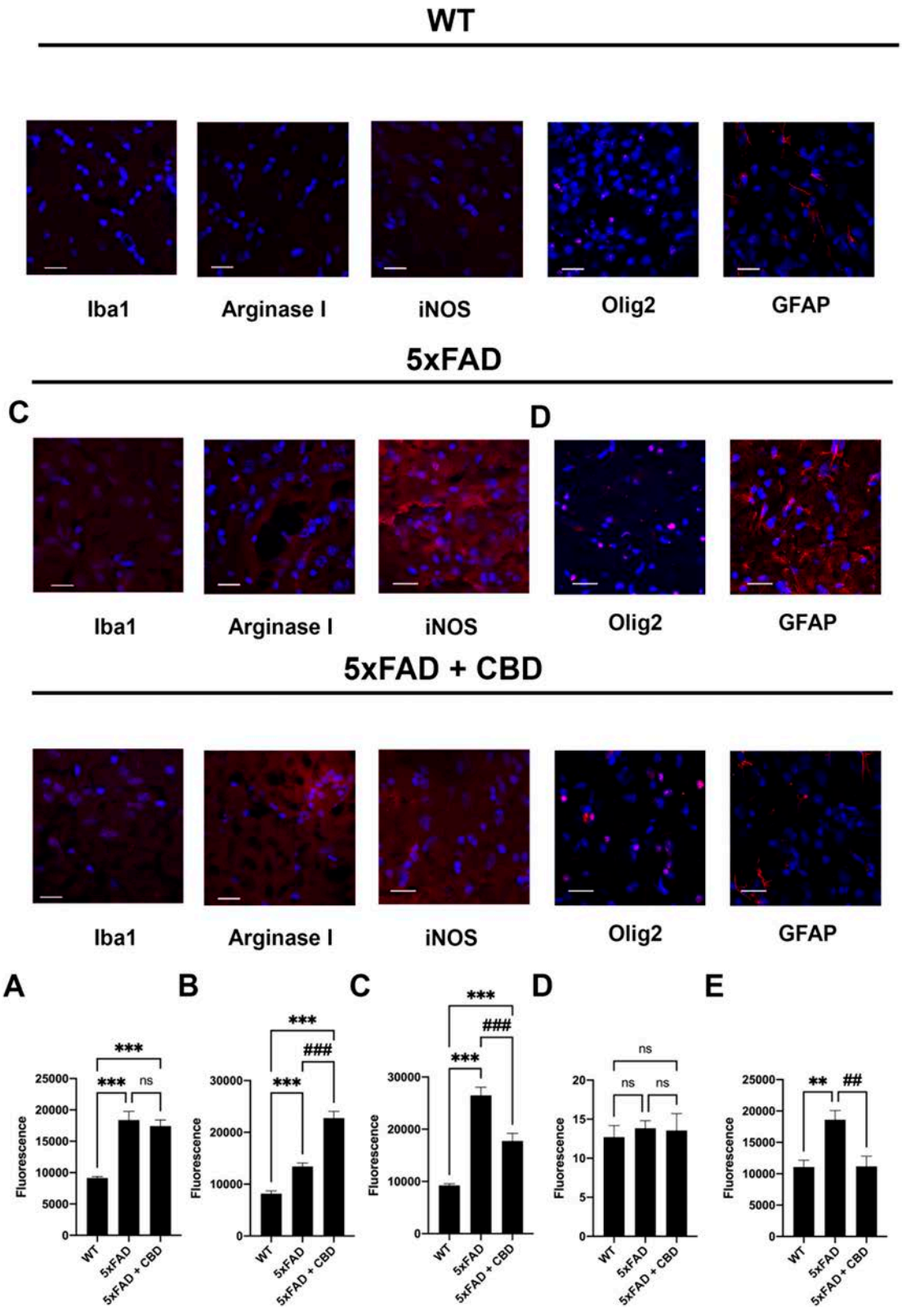

Figure 2**: Immunohistochemistry in hippocampal sections from 5xFAD mice injected with CBD. (A-D**). Hippocampal sections from 5xFAD mice (5xFAD), 5xFAD mice treated with i.p. injection of pure CBD (5xFAD + CBD) and control Wild Type mice (WT) were analyzed by immunohistochemistry using specific anti-Iba1, anti-Arginase I, anti-iNOS, anti-Olig2 and anti-GFAP antibodies. Confocal microscopy images (stacks of 3 consecutive panels) show protein immunoreactivity in red over Hoechst-stained nuclei

(blue). Quantification of the protein immunoreactivity was done using the Andy's algorithm Fiji's plug-in. Samples from 5 different animals were processed and analyzed. One-way ANOVA followed by Dunnett's multiple comparison post-hoc test was used for statistical analysis. *p <0.05, **p <0.005, ***p< 0.001 versus WT; #p <0.05, ##p <0.005, ###p <0.001 versus 5xFAD. Scale bar: 20 μm.

CBD treatment decreases amyloid beta peptide (Aß), Tau and pTau axonal transport in neuronal primary cultures.

One of the mechanisms that could slow the progression of Alzheimer's disease is the decrease in the transport of abnormal aggregates of pTau and beta-amyloid proteins. Different authors have purposed the involvement of cannabinoids in altering Tau axonal transport. In this sense, to demonstrate CBD potential in affecting protein axonal transport it were prepared microfluidic devices of two different chambers. As tauopathies extend further to the entorhinal-hippocampal connection; the first chamber was cultured with cortical neurons and treated with Aß (500 nM), Tau (1 μM), pTau (1 μM) or α-synuclein (4μM) as control and the second chamber with non-treated hippocampal neurons. The day after, cortical neurons were treated with CBD or vehicle for 24 h. By immunocytochemistry assay it was demonstrated that Aß1-42 aggregates were transported from cortical to hippocampal neurons (Fig 3A). Interestingly, neurons treated with CBD showed a strong decrease in Aß1-42 axonal transport. Although the deposits of Aß forming plaques are one of the most important features of Alzheimer's disease, this pathology also expresses tangles of twisted fibers of Tau protein. Then, it was analyzed axonal transport of Tau protein. First, it was assessed that Tau aggregates moved from cortical neurons in chamber 1 to hippocampal neurons in chamber 2 in the microfluidic device. Furthermore, CBD treatment significantly reduced the spread of Tau from cortical to hippocampal cells (Fig 3B). In AD, Tau protein is highly phosphorylated at several residues, being specifically Ser202 and Thr205 between the most common ones. Then, it was also investigated pTau axonal transport. Results indicated that pTau protein is less transported than Tau protein, while CBD treatment induced an important decrease of pTau spread from cortical to hippocampal neurons (Fig 3C). α-synuclein is another protein that forms aggregates in Parkinson's disease. Finally, as control, α-synuclein axonal transport was also investigated. Confocal images showed low levels of α-synuclein transport from cortical to hippocampal neurons that were even lower in that neurons treated with CBD (Fig 3D).

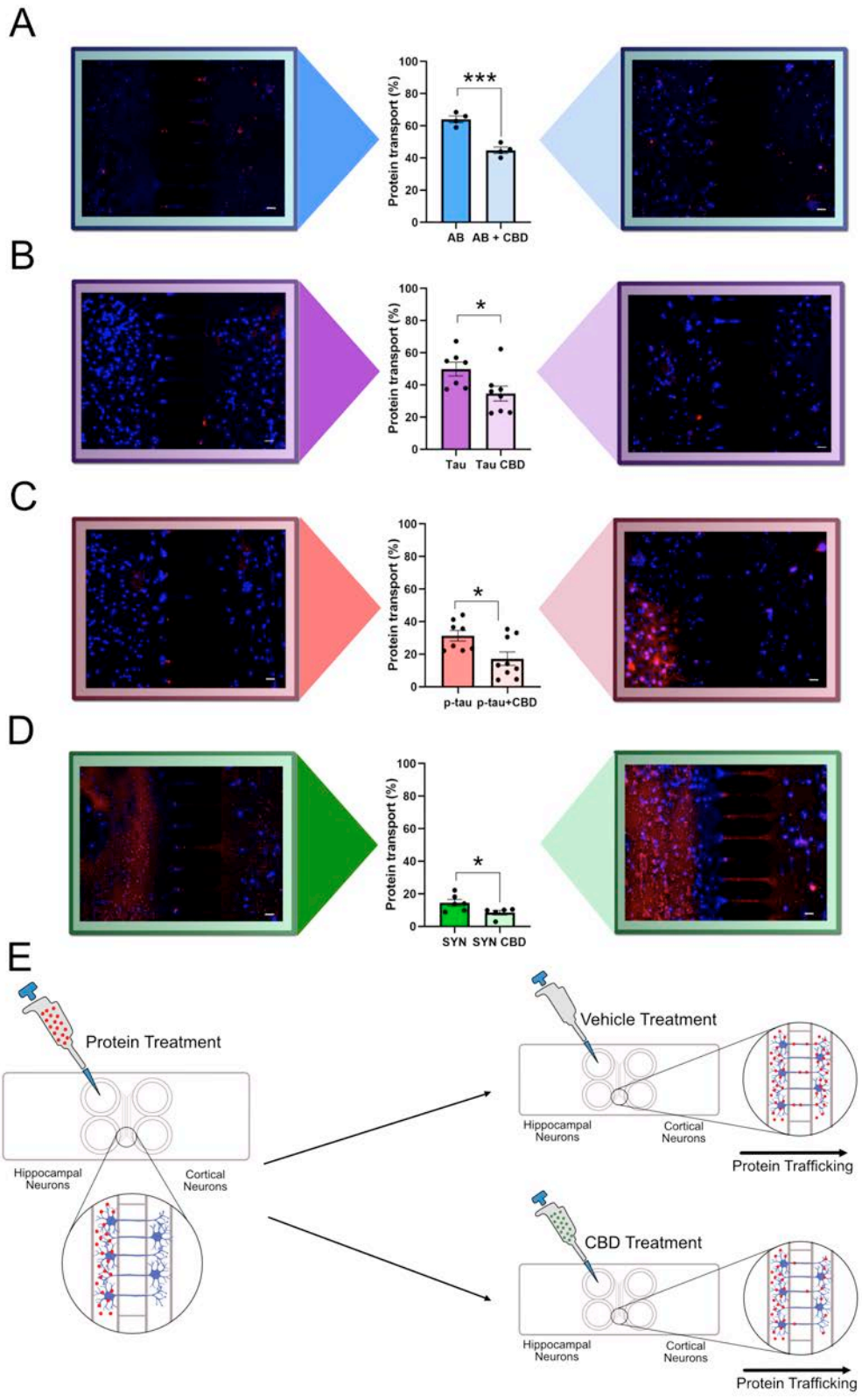

Figure 3. **Detection of amyloid beta peptide (Aß), α-synuclein, Tau and pTau axonal transport in mice cortical and hippocampal neurons**. Both, mice cortical and hippocampal neurons were grown in the microfluidic device (**A-D**). On DIV 10 and once the axons fully crossed the microgrooves (150 µm distance), Aß (500 nM) (**A**), Tau (1 µM) (**B**), pTau (1 µM) (**C**) or α-synuclein (4µM) (**D**) proteins were added into the top left

well of each device for 24 h. On DIV 11, neurons were treated with CBD (200 nM) or vehicle. Neurons were labeled with a rabbit anti-Aß antibody (1/100, ab201060), rabbit anti-tau antibody (1/100, abcam ab32057), rabbit anti-ptau (S396) antibody (1/100, abcam ab109390) or mouse anti-α-synuclein antibody (1/100, ab1903) and subsequently marked with a Cy3 anti-rabbit (1/200, Jackson InmunoResearch) secondary antibody (red). Following 2 h of incubation, cells were washed and subsequently imaged using confocal microscope with 25X (yellow squares) and 40X (green squares) objectives (Zeiss LSM 880). Bar graph shows quantification of the amount of fluorescence in the microfluidic channel opposite to the treated channel in comparison with total fluorescence. Values are the mean ± S.E.M. of 5 independent experiments performed in triplicates. One-way ANOVA followed by Bonferroni's multiple comparison post hoc test were used for statistical analysis *p <0.05, **p <0.005, ***p< 0.001 versus control condition). (**E**) A schematic representation of the microfluidic device and the location of the treatment, allowing migration through the axons in the microchannels.

CBD stimulation increases neuronal viability after Aß treatment

In AD, neuronal death is correlated with memory failure, one of the main symptoms of the pathology. To go deep inside in CBD effect over neuronal death, primary cultures of cortical neurons were seeded in 6 well plates at 50 % confluency for 12 days. After, neurons were treated with Aß1-42 for 48 h and then stimulated with CBD or vehicle for 24 h more. Results showed that Aß1-42 treatment induced around 45 % of neuronal death that was practically reverted in cells treated with CBD (Fig 4A). A similar experiment was performed in primary cultures of cortical neurons treated with NMDA for 2 days. NMDA toxicity induced around 40 % of neuronal death. CBD stimulation counteracted this effect to similar values as cells treated with vehicle (Fig 4A). The same experiment was performed in primary cultures of hippocampal neurons. Again, CBD stimulation recovered neuronal survival from Aß1-42 and NMDA toxicity to similar values as neurons treated with vehicle (Fig 4D).
All together, these data show an important neuroprotective effect of CBD in primary cultures of neurons under Aß1-42 peptide or NMDA toxicity.

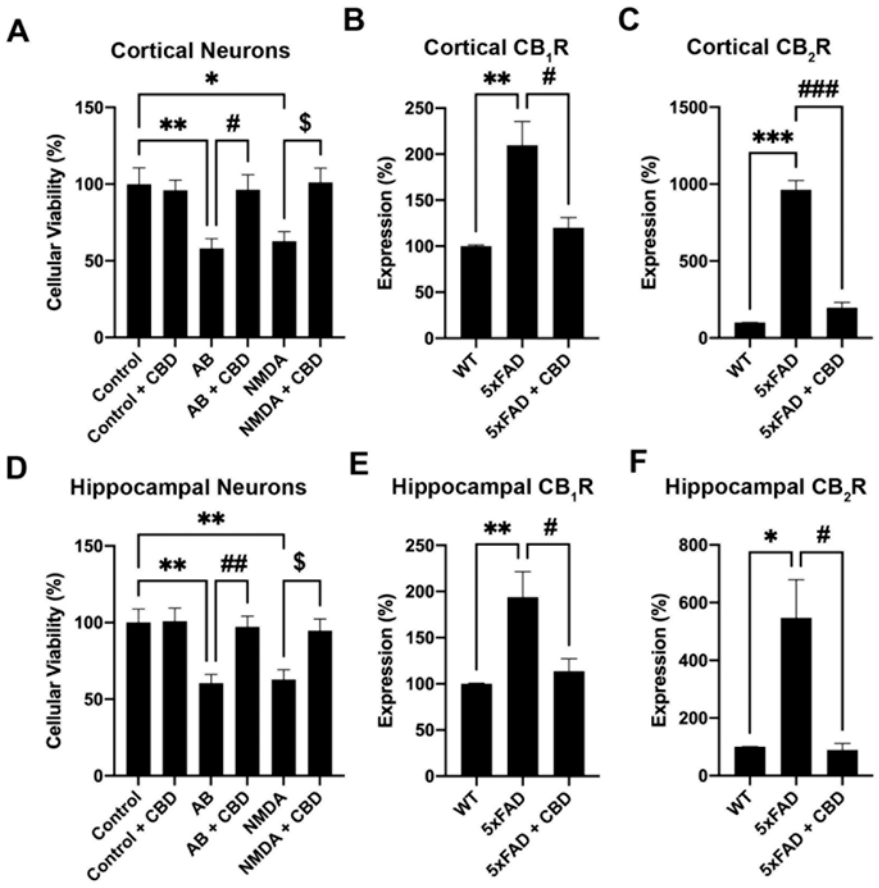

Figure 4: **Expression of CB_2R and CB_1R in cortical and hippocampal neurons after CBD injection. (A,D)**. Cellular viability determination was performed in primary cultures of cortical neurons (**A**) and hippocampal neurons (**B**) from C57BL/6J mice. Cells were treated with NMDA 30 µM, Aß 500 nM or vehicle for 48 hours. After, cells underwent a CBD treatment of 200 nM for 24 h more. Cells were stained with Trypan Blue and sampled in a Neubauer chamber. The unstained (viable) and stained (nonviable) cells were counted separately, and the percentage of viability was calculated as the total number of viable cells/total number of cells x 100. Experiments were performed in samples from 6 different animals. One-way ANOVA followed by Dunnett's multiple comparison post-hoc test were used for statistical analysis. *p <0.05, ***p <0.001 versus Control; #p <0.05, ##p <0.005, ###p <0.001 versus Aß condition; $p <0.05, $$p <0.005, $$$p <0.001 versus NMDA condition. **(B,C,E,F)** Quantification of CB_2R and CB_1R mRNA extracted from the hippocampus and cortex of 5xFAD mice (5xFAD), 5xFAD mice treated with i.p. injection of pure CBD (5xFAD + CBD) and control Wild Type mice (WT) was performed by qPCR analysis and normalized with the b-actin housekeeping gene. Values are the mean ± S.E.M. of 5 independent experiments. One-way ANOVA followed by Bonferroni's multiple comparison post hoc test were used for statistical analysis *p <0.05, **p <0.005, ***p< 0.001 versus Control; #p <0.05, ##p <0.005, ###p <0.001 versus 5xFAD).

CBD injection reduces cannabinoid CB_1 and CB_2 receptors expression in 5xFAD mice

Cannabinoid CB_2 receptor show low expression levels in hippocampal and cortical neurons. However, it has been observed an important increase in

receptor expression in neuroinflammation processes[20]. To evaluate the expression levels of cannabinoid receptors in 5xFAD mice it was performed a quantitative PCR with the mRNA obtained from the dissection of the cortex and the hippocampus. In Fig 4B,E can be observed double expression of cannabinoid CB_1 receptor in 5xFAD animals compared to control mice (C57BL/6) both in cortex and hippocampus. As expected, the analysis of CB_2R expression showed a stronger increase with eight and five times more expression in 5xFAD cortex and hippocampus respect to control mice respectively (Fig 4C,F). When the quantitative PCR was performed in animals one daily injected with CBD (10 mg/Kg) for 4 weeks, it was observed that cannabinoid CB_1 receptor expression returned to similar levels as control mice in both tissues, cortex and hippocampus (Fig 4B,E) while cannabinoid CB_2 receptor expression showed similar levels as control mice in hippocampus while doubled expression in cortex (Fig 4C,F).

CBD treatment leads to spatial memory improvement in 5xFAD mice

To investigate any potential role of cannabidiol in cognitive improvement, it was evaluated the spatial memory in the AD mice model 5xFAD treated or not with daily injections of CBD (10mg/Kg). The exploration time during the familiarization phase of the NORT task was unchanged by CBD treatment. The evaluation of the short-term memory was done with the novel object recognition test (NORT) 2 h after the first trial-familiarization. Results revealed that 5xFAD animals exhibited a significant cognitive deficit (Fig 5A) in comparison with control mice (C57BL/6). However, CBD treatment strongly reduced the loss of short-term memory (Fig 5A).

Then, it was analyzed the long-term memory with the novel object recognition test (NORT) 24 h after the first trial-familiarization. Again, it was observed an important deficit in cognition that was practically reverted in that animals daily injected with CBD (Fig 5B).

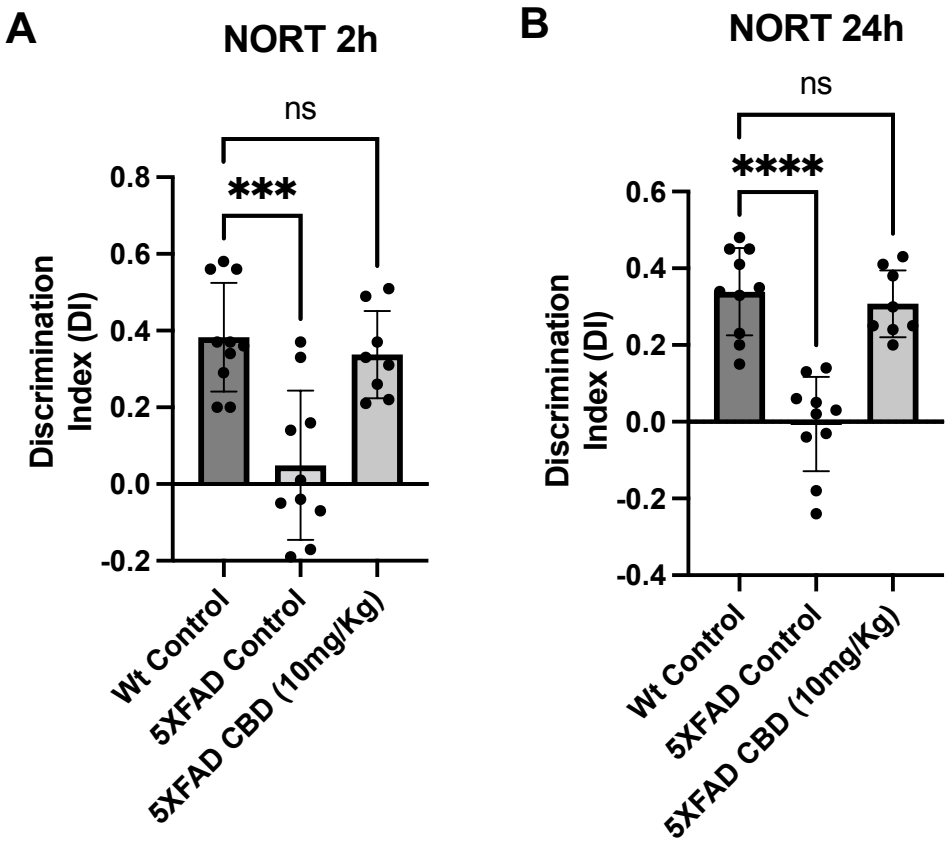

Figure 5. **Novel object recognition test performed in 5xFAD mice** (**A-B).** A novel object recognition test (NORT) was performed 2h (Short-term memory) or 24h (Long term memory) after the first trial-familiarization. Experiments were performed in samples from 28 different animals (10 WT animals, 10 5xFAD animals and 8 5xFAD animals treated with CBD (10 mg/Kg)). One-way ANOVA followed by Bonferroni's multiple comparison post hoc test were used for statistical analysis. *p <0.05, **p <0.005, ***p< 0.001 versus Control.

CBD treatment improves neurite patterning in neuronal primary cultures treated with Aß

Another characteristic neurophatological change of Alzheimer's disease is the loss and alteration of synaptic elements that evolve in parallel with amyloid plaques and neurofibrillary tangles accumulation. In this sense, we first evaluated the Aß effect on neurite pattering in neuronal primary cultures. To detect neurite pattering it was performed an immunocytochemistry assay with anti-Nectin over green stained neurons with an anti-F-actin antibody fused to an Alexa 488 fluorophore. Data obtained demonstrates an important loose in neurite formation after 48 h of Aß1-42 treatment (around 55%). Then, to evaluate the CBD potential, the experiment was repeated treating neuronal primary cultures with Aß and after with CBD for 24 h. It was observed that cannabidiol partially recovered the neurite pattern loss induced by Aß1-42 (Fig 6A, C). Similar experiment was performed with Tau and pTau proteins. Both induced a significant decrease in neurite formation that were partially recovered by CBD treatment. As control, it was also tested the α-synuclein effect. Contrary to Aß1-42, Tau and pTau proteins, the neurite loss induced by α-synuclein was not recovered by CBD treatment (Fig 6A, C). These results point a different molecular mechanism by which CBD induces neuroprotection in AD than in Parkinson's disease.

The obtained data presents CBD as a potential target to combat not only molecular deficits in AD but also cognitive impairment.

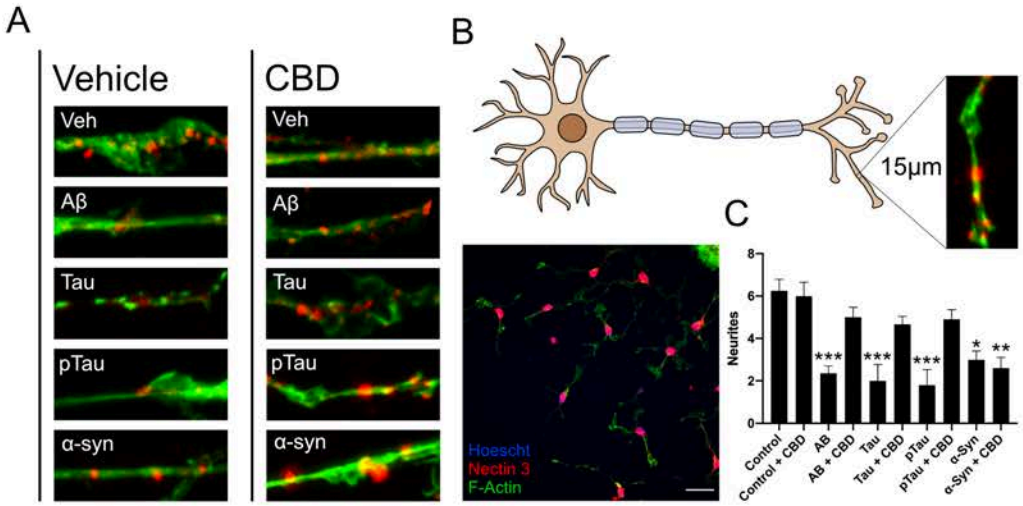

Figure 6. **Effect of CBD over neurite patterning**. **(A)** On DIV 10, neurons were treated with Aß (500 nM), Tau (1 µM), pTau (1 µM) or α-synuclein (4µM) for 24 h. On DIV 11, neurons were treated with CBD (200 nM) or vehicle. Neurite patterning was detected by immunocytochemistry using an anti-Nectin 3 antibody (Abcam, 1/1000). Neurons were detected with the anti-F-actin antibody fused to an Alexa 488 fluorophore (ThermoFisher,

1/400). Cell nuclei were stained with Hoechst (blue). Scale bar: 25 μm. **(C)** Quantification was performed over segments of 15 μm. Each red dot represents a neurite formation. Values are the mean ± S.E.M. of 9 independent experiments performed in triplicates. One-way ANOVA followed by Bonferroni's multiple comparison post hoc test were used for statistical analysis *p <0.05, **p <0.005, ***p <0.001 versus control condition. (**B**) A schematic representation of the section length used to quantify the neurite formation.

Discussion.

Alzheimer's disease (AD) is the most common dementia worldwide with around 60 % of the total cases. It is characterized by the production and accumulation of amyloid-β plaques and Tau hyperphosphorylated tangles, producing neuroinflammation and oxidative stress and consequently neuronal death and behavioral and cognitive deficits including apathy, disinhibition, memory loss, and motor function deregulations[17].

Cannabidiol is the second most abundant fitocannabinoid in Cannabis sativa plant and is investigated as a potential multifunctional treatment for AD due to its average of beneficial actions. Other cannabinoids such as (delta)^9-THC have also shown neuroprotection in AD, however CBD lack of psychoactive effects is still a huge challenge. On the one hand, it has been purposed that CBD show neuroprotection and antioxidant properties on β-amyloid peptide-induced toxicity in cultured rat PC12 cells[13] and also modulates microglial cell function in vitro[21,22]. More specifically, it has been described that cannabinoids prevent β-amyloid (Aβ)-induced microglial activation. In this sense, we moved to analyze microglia phenotype in the AD mice model 5xFAD that express human PSEN1 and APP transgenes with a total of five mutations linked to Alzheimer's disease: the M146L and L286V mutations in PSEN and the Swedish (K670N/M671L), Florida (I716V), and London (V717I) mutations in APP. By immunohistochemistry assays it was demonstrated that CBD treatment shows a neuroprotective role in the AD mice model 5xFAD in cortical and hippocampal neurons by i) polarizing microglia to a neuroprotective M1 phenotype ii) decreasing the activated astroglia cells and iii) increasing the expression of oligodendrocyte precursor cells expression in cortex.

CBD can bind both CB_1 and CB_2 cannabinoid receptors, however different data point CB_2R as the responsible for microglia skew to a neuroprotective phenotype. JWH-133, a CB_2R selective agonist, attenuates microglial activation and downregulates the concentrations of pro-inflammatory mediators in pneumococcal infection in vitro and in vivo[23]. Moreover, in the $APP_{Sw/Ind}$ AD mice model, CB_2R activation favors the M2 microglia phenotype[24,25]. Consequently, the induced effect of CBD over microglia polarization seems to be induced trough CB_2R activation.

The role of plaques and tangles in AD is still unknown. Different studies purpose a close relationship between plaques formation and Tau phosphorylation. It has been demonstrated that Aβ-pE(3) and pTau Ser202/Thr205 levels strongly correlate in murine and human tissues, suggesting that Aβ-pE(3) could amplify Tau phosphorylation[26]. Somehow, experts believe that Aβ and pTau aggregates could contribute to block neuronal cells communication and induce cell death,

causing memory failure, personality changes and other AD symptoms. On the other hand, Sativex administration, a cannabis extract used to combat neuropathic pain, spasticity, and other symptoms of multiple sclerosis, has shown an important reduction on phosphorylated Tau, GSK3 expression, and levels of Aβ oligomers in transgenic mice model and human[21,27]. In this regard, CBD has shown an important inhibition of β-amyloid-induced Tau protein hyperphosphorylation and nitric oxide production[28,29].

One possibility to stop the AD progression would consist in reducing the anterograde Aβ and pTau axonal transport, by reducing the spread of aberrant proteins between different neuronal regions and thus decreasing the neuroinflammation and neurodegeneration patterns. After analyzing Aß1-42, Tau and pTau axonal transport from cortical to hippocampal neurons in microfluidic devices of two chambers it was observed that all, Aß, Tau and pTau proteins were spread from one neuron to another. Moreover, treatment with CBD for 48 hours significantly decreased Aß1-42, Tau and pTau axonal transport, being the effect over Aß1-42 the stronger decrease.

Results obtained show that CBD recovers neuronal survival after both, Aß1-42 peptide and NMDA induced toxicity in primary cortical neurons. These data agree with that published in primary neuronal cultures, indicating a strong antioxidant effect of CBD against glutamate toxicity[30]. In this context, it has also been observed by transmission electron microscopy that CBD treatment up-regulated the autophagy pathway in hippocampal neurons of APP/PS1 mice model of AD[31].

It is well accepted that cannabinoid receptor CB_2 is upregulated in proinflammatory processes in the periphery, such as the inflammatory bowel disease[32] and also in the central nervous being upregulated in the hippocampus and entorhinal cortex of AD patients in neuritic plaque-associated microglia[33,24]. In the same line, we have detected an upregulation of not only CB_2R, but also CB_1R at lower levels in 5xFAD mice. Interestingly, both cannabinoid receptors expression returned to similar expression levels as control mice after CBD daily injections for one month.

Other well established effects of CBD consist in its capacity to decrease AD associated gene expression, including genes coding for Aß production such as the beta- and gamma-secretase or for proteins responsible of Tau phosphorylation[34]. Likewise, in the hippocampus of Aβ-induced neuroinflammation mice, CBD avoids the expression of proinflammatory glial peptides[34]. In parallel, it has been show the involvement of cannabidiol in promoting neurogenesis[17]. To go deep inside in CBD induced effects, we further analyzed neurite formation in neuronal primary cultures treated with Aß1-42, Tau and pTau proteins in the presence or in the absence of CBD. In the analyzed data it has been explained the capacity of CBD to recover neuronal plasticity. In this sense, it has been observed that CBD treatment can partially decrease the neurite formation loss induced by Aß and also Tau and pTau proteins treatment.

It has been demonstrated that hemiparkinsonian female in the estrus phase and male show different answers in the nociceptive tests after different doses of CBD therapy. Then, CBD therapy seem effective for parkinsonism-induced

nociception[35]. In this line, as control, it was tested if the characteristic proteins aggregates of Parkinson disease formed by α-synuclein could alter neuronal plasticity. It was observed an important decrease in neurite formation that contrary to Aß1-42, Tau and pTau proteins was not recovered by CBD treatment.

Finally, it was evaluated the capacity of CBD to combat cognitive impairment in AD. Most studies point the involvement of CBD in preventing cognition deficit in neurodegenerative diseases. Some examples are the improvement of social recognition memory in the double transgenic APP × PS1 mouse model for AD[36], the beneficial effects observed in neuropsychiatric symptoms in AD patients after CBD consumption[37,38], the preserved memory in AßPP/PS1 transgenic mice when (delta)9-THC, cannabidiol (CBD) or both are chronically administrated in botanical extracts, the attenuation of contextual conditioned fear in rats induced by cannabidiol[39] or the prevention of the development of a social recognition deficit in AD transgenic mice[36]. The afforded data is huge, however there continue being some controversy, as the improvement in spatial memory detected in 14-month-old female TAU58/2 transgenic mice[40] was not observed in 4-month-old male TAU58/2 transgenic mice[41]. In our hands, the NORT test presents CBD as a potential target to combat cognitive impairment by demonstrating that daily CBD injections can revert short- and long-term memory deficits in AD mice model 5xFAD.

Therefore, in this article we have demonstrated a wide range of beneficial effects induced by CBD ranging from the molecular to the behavioral level and which may be based on the reduction of the formation of beta-amyloid plaques and the prevention of phosphorilated Tau tangles[34]. More studies should be required to underlay CBD mechanisms and translate the preclinical studies into clinical assays.

Material and Methods

Reagents

CBD was purchased from Cerilliant (Texas, US). The antibodies used were the following: Monoclonal mouse anti-Arginase I (ref. 610708, BD Bioscience), monoclonal mouse anti-iNOS (MA5-17139, Invitrogen), polyclonal goat anti-GFAP (PA5-18598, Invitrogen), polyclonal goat anti-Iba1 (ab107159, Abcam), monoclonal mouse anti-SOX10 (ab216020, Abcam), polyclonal rabbit anti-Nectin 3 (ab63931, Abcam) and polyclonal rabbit anti-F-actin antibody fused to an Alexa 488 fluorophore (A12379, ThermoFisher). N-methyl-D-aspartate (NMDA) was purchased from Tocris Bioscience (Bristol, United Kingdom). α-synuclein was prepared as described[42] and Tau and pTau proteins were kindly provided by Prof. J. Avila (CBM, UAM-CSIC, Madrid, Spain). Detailed descriptions of the elaboration and processing of proteins can be found elsewhere[43].

Animals

Wild-type (Wt) and 5xFAD (n = 28) male mice of 4-month-old were used to perform cognitive and molecular studies. We divided these mice randomly into three groups: Wt Control (n = 10), 5xFAD Control (n = 10), and 5xFAD treated with the phytocannabinoid cannabidiol (CBD) (5xFAD CBD (10 mg/Kg); n = 8). The sample size for the intervention was chosen following previous studies in our laboratory and using one of the available interactive tools (http://www.biomath.info/ power/index.html). Experimental groups received either one dose of vehicle (2% w/v, Tween 80 (Fischer, USA) or one daily dose of 10 mg/Kg/day of CBD dissolved in 2% Tween 80 via oral gavage for 4 weeks. Animals had free access to food and water and were kept under standard temperature conditions (22 ± 2°C) and 12h:12h light-dark cycles (300 lux/0 lux). After the treatment period, cognitive tests were performed in the animals.

Studies and procedures involving mouse behavior test, brain dissection and extractions followed the ARRIVE and standard ethical guidelines (European Communities Council Directive 2010/63/EU and Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research, National Research Council 2003) and were approved by Bioethical Committees from the University of Barcelona and the Government of Catalonia. All efforts were made to minimize the number of animals used and their suffering.

Novel object recognition test

The protocol employed was a modification of Ennanceur and Delacour (1988). The experimental apparatus used for this test was a 90-degree, two-arm, 25-cm-long, 20-cm-high maze of black polyvinyl chloride. Light intensity in the middle of the field was 30 lux. Briefly, first mouse was individually habituated to the apparatus for 10 min per day for 3 days. On day 4, the mice were allowed to freely explore two identical objects (A and A or B and B) placed at the end of each arm for 10 min acquisition trial (First trial-familiarization). The mouse was then removed from the apparatus and returned to its home cage. Then, a 10-min retention trial (second trial) was carried out for 2 (Short-term memory) or 24 hours (Long term memory) later. During the Short-term memory retention, objects A and B were placed in the maze replacing one of them (A and B or B and A), and the times that the animal took to explore the new object (TN) and the old object (TO) were recorded. 24 hours after the acquisition trial, the mice were tested again, with a new object and an object identical to the new one in the previous trial (A and C or B and C). The time that mice explored the Novel object (TN) and Time that mice explored the Old object (TO) were measured from the video recordings from each trial session. A Discrimination index (DI) was defined as (TN-TO)/(TN+TO). Exploration of an object by a mouse was defined as pointing the nose towards the object at a distance ≤ 2 cm and/or touching it with the nose. Turning or sitting around the object was not considered exploration. In order to avoid object preference biases, objects A and B were counterbalanced so that one half of the animals in each experimental group were first exposed to object A and then to object B, whereas the other half first saw object B and then object

A. The maze, the surface, and the objects were cleaned with 70% ethanol between the animals' trials to eliminate olfactory cues.

Brain sampling

Mice were sacrificed under deep anesthesia (i.p. injection of diazepam/ketamine) and transcardially perfused with saline and 4% paraformaldehyde. Brains were harvested and embedded in paraffin to obtain coronal sections (30 µm thick) using a cryostat LEICA CM3050 S (Leica Microsystems, Wetzlar, Germany).

Neuronal primary cultures

To prepare primary cultures of striatal neurons, brains from fetuses of pregnant C57BL/6J mice were removed (gestational age: 19 days). Neurons were isolated as described in Hradsky et al[44]. Briefly, the samples were dissected and, after a careful removal of the meninges, digested for 20 min at 37ºC with 0.25% trypsin. Trypsinization was stopped by adding an equal volume of culture medium (supplemented DMEM). Cells were brought to a single cell suspension by repeated pipetting followed by passage through a 100 µm-pore mesh. Pelleted (7 min, 200×g) cells were resuspended in supplemented DMEM and seeded at a density of 3.5×10^5cells/mL in 6-well plates. The day after, medium was replaced by neurobasal medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin and 2 % (v/v) B27 medium (GIBCO). Neuronal cultures were assayed 12 days after. Using NeuN as a marker, it was detected a percentage of neurons in the culture >90%.

Preparation of Human α-synuclein Fibrils

α-synuclein fibrils were prepared by shaking purified recombinant α-synuclein as described[43,45]. Briefly, purified recombinant α-synuclein (5 mg/ml) containing 30 mM Tris–HCl (pH 7.5), 10 mM DTT, and 0.1% sodium azide were incubated for 7 days at 37ºC in a horizontal shaker at 200 rpm, then ultracentrifuged at 113,000 × g for 20 min at 25ºC. The pellets were washed with saline and ultracentrifuged as before. The resulting pellets were collected as α-synuclein fibrils and resuspended in 30 mM Tris–HCl (pH 7.5). The fibrils were fragmented using a cup horn sonicator (Sonifier® SFX, Branson) at 35% power for 180 s (total 240 s, 30 s on, 10 s off)[43]. Before use, aliquots were left at room temperature and placed in PBS 1× (pH 7.2) to a final concentration of 0.1 µg/µL. These preparations were subjected to 60 pulses of sonication (runtime 30 s: 0.5 s on, 0.5 s off in a BBR03031311digital SONIFIER sonicator). Sonicated fibril preparations were diluted in pre-warmed medium and immediately added to cells.

Immunohistochemistry

Brain slices were fixed in 4% paraformaldehyde for 15 min and then washed twice with PBS containing 20 mM glycine before permeabilization with the same buffer containing 0.2% Triton X-100 (15 min incubation). The samples were treated for

1 h with blocking solution (PBS containing 1% bovine serum albumin) and labeled with monoclonal mouse anti-Arginase I (1/100, ref. 610708, BD Bioscience), monoclonal mouse anti-iNOS (1/200, MA5-17139, Invitrogen), polyclonal goat anti-GFAP (1/100, PA5-18598, Invitrogen), polyclonal goat anti-Iba1 (1/100, ab107159, Abcam) or monoclonal mouse anti-SOX10 (1/200, ab216020, Abcam) as primary antibodies and a Cy3-conjugated anti-mouse IgG (1/200, 715-166-150, Jackson ImmunoResearch) or a Cy3-conjugated anti-goat IgG (1/200, 705-167-003, Jackson ImmunoResearch) as secondary antibodies. The samples were washed several times and mounted with 30% Mowiol (Calbiochem, San Diego, CA, USA). Nuclei were stained with Hoechst (1/100). Samples were observed under a Zeiss 880 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Viability assay

Neurons were resuspended with neurobasal medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin and 2 % (v/v) B27 (GIBCO). Trypan blue staining was performed mixing 1 part of 0.4% trypan blue and 1 part of cell suspension in a plastic tube. After ~3 min of incubation at room temperature, 10 µl of the mixture was sampled in a Neubauer chamber. The unstained (viable) and stained (nonviable) cells were counted separately, and the percentage of viability was calculated as: total number of viable cells/total number of cells x 100.

Protein trafficking

Microfluidic standard neuronal devices (with 150 µm microgroove barriers located in the area between the channels; AXISTM AXon Investigation System, EMD Millipore) were handled following the manufacturer's protocol.

For neuronal primary cultures, the pure cortical neurons from mouse embryos (E19) were isolated. Before neuronal seeding, each assembled device was coated with poly-D-lysine (0.1 mg/mL, Gibco A3890401) for 1 h. After, ten microliters of cell suspension (5-6 million neurons/mL) were introduced to both chambers of each device by passive pumping. After 30 minutes incubation at 37ºC to allow cell attachment, 200 µL of neurobasal medium (supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, and 2% (v/v) B27 supplement (Gibco)) was added. Neurons were maintained at 37 °C in humidified 5% CO_2 atmosphere. Medium was replaced every three days in each device (a 50 µL difference in media volume was maintained to prevent spontaneous diffusion).
On DIV 10, once the axons fully crossed the microgrooves (150 µm distance) into the axonal compartment of a device, Aß, Tau, pTau or α-synuclein were added into the compartment A of each device and mantained for 24 h. On DIV 11, neurons were treated with CBD (200 nM) or vehicle. Neurons were labeled with a rabbit anti-Aß antibody (1/100, ab201060), a rabbit anti-tau antibody (1/100, abcam ab32057), a rabbit anti-phospo-tau (S396) antibody (1/100, abcam ab109390) or a mouse anti-α-synuclein antibody (1/100, ab1903) and subsequently marked with a Cy3 anti-rabbit (1/200, Jackson InmunoResearch) secondary antibody (red). Following 2 h of incubation, cells were washed and

subsequently imaged using confocal microscope with 25X (yellow squares) and 40X (green squares) objectives (Zeiss LSM 880).

Polymerase chain reaction (PCR)

The hippocampus and cortex were isolated from C57BL/6J mice at age 4-month-old. After decapitation, the hippocampus and cortex were dissected and immediately transferred to PBS. mRNAs were isolated with TRIzol™ Reagent protocol and treated with RNase-Free DNase (Qiagen). The purity was verified with a NanoDrop 2000 Spectrophotometer (Thermo Scientific). Single strand cDNA was synthesized from the extracted RNA (2 µg) using a MLV-reverse transcriptase (Fisher Scientific), Random Hexamers and oligo-dT. The qPCR was conducted with the cDNA and PowerUp SYBR Green Master Mix (AppliedBiosystems). Determinations were conducted using real-time PCR technique on an Applied Biosystems QuantStudio3 device in 96-well plates.

The primer pair for the gene that codifies for CB_1R was CCAAGAAAAGATGACGGCAG (forward) and AGGATGACACATAGCACCAG (reverse). The primer pair for the gene that codifies for CB_2R was GGGTCGACTCCAACGCTATC (forward) and AGGTAGGCGGGTAACACAGA (reverse). Primers for beta-actin were used as an internal control (forward, TTGACATCCGTAAAGACCTC; backward, AGGAGCCAGAGCAGTAAT). Each experiment included a template-free control. The PCR products were analyzed by the DNA melting curve. The relative quantities of Cnr1 and Cnr2 PCR products were estimated with respect to the amount of the house keeping gene beta-actin product using the ΔCt method: %beta-actin = (2Ct of Gapdh – Ct of CB_2R) × 100.

Neurite Patterning Determination

Cortical neuronal primary cultures were treated with Aß (500 nM), Tau (1 µM), pTau (1 µM) or α-synuclein (4 µM) for 24 h on DIV 10. Next day, neurons were treated with CBD (200 nM) or vehicle. Then, cells were fixed in 4% paraformaldehyde for 15 min and then washed twice with PBS containing 20 mM glycine followed by permeabilization with the same buffer containing 0.2% Triton X-100 (15 min incubation). Samples were treated for 1 h with blocking solution (PBS containing 1% bovine serum albumin) and labeled with polyclonal rabbit anti-Nectin 3 antibody (Abcam, 1/1000). Neurons were detected with 3 anti-F-actin antibody fused to an Alexa 488 fluorophore (ThermoFisher, 1/400). Then, sections were incubated at RT for 2 h with a Cy3-conjugated anti-rabbit secondary antibody (1/200, 711-166-152, Jackson ImmunoResearch). The samples were washed several times with PBS and mounted with 30% Mowiol (Calbiochem, San Diego, CA, USA). Nuclei were stained with Hoechst (1/100). Samples were observed under a Zeiss 880 confocal microscope (Leica Microsystems, Wetzlar, Germany). Quantification of neurite formation was performed over segments of 15 µm. Each red dot represents a neurite formation.

Data Handling and Statistical Analysis

Data were analyzed blindly. Data are presented as the mean ± SEM. Statistical analysis was performed with SPSS 18.0 software. The test of Kolmogorov–Smirnov with the correction of Lilliefors was used to evaluate normal distribution and the test of Levene to evaluate the homogeneity of variance. Significance was analyzed by one-way ANOVA, followed by Bonferroni's multiple comparison post hoc test. Significance was considered when $p < 0.05$.

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3.6 Ghrelin and Cannabinoid Functional Interactions Mediated by Ghrelin/CB_1 Receptor Heteromers That Are Upregulated in the Striatum From Offspring of Mice Under a High-Fat Diet.

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L'efecte orexigènic del consum de cànnabis es troba àmpliament descrit. La regulació de la gana és un procés molt complex en el qual intervenen molts factors. La grelina però, té un paper protagonista en aquest procés. La grelina és produïda a les glàndules gàstriques i augmenta la seva alliberació al torrent sanguini en condicions de dejú o de restricció calòrica, regulant la sensació de gana i l'homeòstasi energètica i modulant el metabolisme mitjançant l'activació de circuits orexigènics. En aquest article ens vam plantejar si aquest efecte dels cannabinoides es podria explicar per una interacció entre els receptors CB_1 i GHS-R1a. En un sistema d'expressió heteròleg, es va demostrar mitjançant la tècnica de BRET que ambdós receptors interaccionen formant complexos heteromèrics. La interacció entre el CB_1R i el GHS-R1a comporta un bloqueig de la senyalització de CB_1R. L'heteròmer CB_1R-GHS-R1a presenta unes propietats particulars quant a la senyalització a través de Gq. El tractament amb cannabinoides provoca un increment de l'efecte de la grelina quant a la mobilització del calci intracel·lular. Aquesta potenciació era major a baixes concentracions de l'agonista de CB_1R, araquidonil-2'-cloroetilamida (ACEA). L'expressió de CB_1R-GHS-R1a en neurones d'estriat de ratolí es va confirmar per mitjà de la tècnica de PLA. En cultius primaris realitzats en la progènie de ratolines alimentades amb una dieta alta en greixos (HFD) es va observar una sobreexpressió de l'heteròmer. De manera interessant, la seva expressió també es va veure incrementada després d'un tractament amb grelina i ACEA a 100 nM.

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Ghrelin and Cannabinoid Functional Interactions Mediated by Ghrelin/CB_1 Receptor Heteromers That Are Upregulated in the Striatum From Offspring of Mice Under a High-Fat Diet

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There is evidence of ghrelinergic-cannabinoidergic interactions in the central nervous system (CNS) that may impact on the plasticity of reward circuits. The aim of this article was to look for molecular and/or functional interactions between cannabinoid CB_1 and ghrelin GHS-R1a receptors. In a heterologous system and using the bioluminescence resonance energy transfer technique we show that human versions of cannabinoid CB_1 and ghrelin GHS-R1a receptors may form macromolecular complexes. Such receptor heteromers have particular properties in terms of CB_1/G_i-mediated signaling and in terms of GHS-R1a-G_q-mediated signaling. On the one hand, just co-expression of CB_1R and GHS-R1a led to impairment of cannabinoid signaling. On the other hand, cannabinoids led to an increase in ghrelin-derived calcium mobilization that was stronger at low concentrations of the CB_1 receptor agonist, arachidonyl-2'-chloroethylamide (ACEA). The expression of CB_1-GHS-R1a receptor complexes in striatal neurons was confirmed by *in situ* proximity ligation imaging assays. Upregulation of CB_1-GHS-R1a- receptor complexes was found in striatal neurons from siblings of pregnant female mice on a high-fat diet. Surprisingly, the expression was upregulated after treatment of neurons with ghrelin (200 nM) or with ACEA (100 nM). These results help to better understand the complexities underlying the functional interactions of neuromodulators in the reward areas of the brain.

Keywords: CB1 cannabinoid receptor, hunger hormone, marihuana consumption, orexigenic, obesity, addiction, ghrelin receptor (GHS-R1a), cannabinoids

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INTRODUCTION

Cell surface cannabinoid receptors were identified as targets of natural compounds present in Cannabis sativa, specially of Δ^9-tetrahydrocannabinol (Δ^9-THC; (6a*R*, 10a*R*)-6,6,9-trimethyl-3-pentyl-6a,7,8,10a-tetrahydro-6H-benzo[c]chromen-1-ol; CAS registry number: #1972-08-3). So far, two cannabinoid receptors have been cloned and pharmacologically characterized, the CB_1 and the CB_2 receptors. They belong to class A rhodopsin-like G-protein coupled receptors (GPCRs) and both have Gi as the canonical heterotrimeric G protein to which they couple (Alexander et al., 2021). Subsequent to the discovery of cannabinoid receptors, the main compounds that act as endogenous agonists were identified, 2-arachidonoylglycerol (2-AG) and anandamide (N-arachidonoylethanolamine). Further components of the endocannabinoid system are the enzymes that synthesize and degrade 2-AG and anandamide (Lu and Mackie, 2016). Cannabis smoking leads to psychotropic events that are due to Δ^9-THC acting on the CB_1 receptor (CB_1R), which is reportedly the most abundant GPCR in the central nervous system, being expressed in almost any region of the brain and both in neurons and glia (Elphick and Egertová, 2001; Mackie, 2005). In addition, it is well established that cannabis use has orexigenic properties (Pagotto et al., 2006).

Ghrelin has been considered as the "hunger hormone" (Funahashi et al., 2003; Abizaid and Horvath, 2008; Schellekens et al., 2010; Cassidy and Tong, 2017). Ghrelin, a 28-amino acid peptide produced by specialized cells of the gastrointestinal tract, activates central mechanisms that control food intake (Funahashi et al., 2003; Abizaid and Horvath, 2008; Schellekens et al., 2010; Cassidy and Tong, 2017). However, in mammals, there are overlapping mechanisms, both central and peripheral, that control food intake. Ghrelin acts *via* a specific receptor, GHS-R1a, that belongs to the family of GPCRs, couples to G_q heterotrimeric G protein and is expressed in a variety of cells and tissues (Pradhan et al., 2013; Alexander et al., 2019). In previous studies, we have reported physiologically relevant interactions in which the GHS-R1a receptor is involved. A functional unit composed of GHS-R1a and the dopamine D_1 receptor mediates, at least in part, the hunger-suppressing actions of cocaine; in a macromolecular complex that also includes the non-GPCR sigma-1 receptor, a dual coupling is possible; that is, the coupling to two G proteins makes it possible for ghrelin to act through cAMP rather than through Ca^{2+} and dopamine to signal *via* increases in cytoplasmic Ca^{2+} rather than through cAMP (Casanovas et al., 2021). The ghrelin receptor is also able to interact with the CB_2 cannabinoid receptor in both heterologous cells and in cells of the central nervous system. Cannabinoids acting on the CB_2 receptor do not alter the cytosolic Ca^{2+} increases triggered by ghrelin. However, ghrelin receptor activation led to a blockade of CB_2 receptor-mediated Gi-dependent signaling (Lillo et al., 2021). The aim of this article was to investigate the potential molecular and/or functional interactions between CB_1 and GHS-R1a receptors. As the risk of obesity is higher in the progeny of obese parents, the interaction between these two receptors was also studied in neurons isolated from fetuses of mothers on a high-fat diet (Abu-Rmeileh et al., 2008).

MATERIALS AND METHODS

Reagents

ACEA, ghrelin (human), rimonabant hydrochloride, and YIL 781 hydrochloride were purchased from Tocris Bioscience (Bristol, United Kingdom). Concentrated (10 mM) stock solutions prepared in DMSO, milli-Q® H_2O (Merck/Millipore, Darmstadt, Germany), or ethanol were stored at −20°C. In each experimental session, aliquots of concentrated solutions of compounds were thawed and conveniently diluted in the appropriate experimental solution.

Diet-Induced Obesity Model

C57BL/6J female mice were used for the experiments. All animals were subjected to a 12 h/12 h light/ dark cycle in a temperature- and humidity-controlled room and were allowed free access to water and standard laboratory chow. C57BL/6J mice were randomly assigned to a high-fat diet (HFD; 60% kcal from fat; catalog no. D12492, Research Diets, New Brunswick, NJ, USA) or standard diet (STD; 10% kcal from fat; catalog no. D12450B, Research Diets) for 60 days. Primary striatal neurons were obtained from fetuses of mothers on STD or HFD diets. Pregnant animals were killed by cervical dislocation during the light phase. All animal procedures were performed in agreement with European guidelines (2010/63/EU) and approved by the University of Barcelona Ethical Committee, which reports to the regional Government (Protocol #9659; Generalitat de Catalunya, May 24, 2019).

Cell Culture and Transient Transfection

Human embryonic kidney HEK-293T (lot 612968) cells were acquired from the American Type Culture Collection (ATCC). They were amplified and frozen in liquid nitrogen in several aliquots. Cells from each aliquot were used until passage 19. HEK-293T cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, Scotland, United Kingdom) supplemented with 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, MEM Non-Essential Amino Acid Solution (1/100), and 5% (v/v) heat-inactivated Fetal Bovine Serum (FBS; all supplements were from Invitrogen, Paisley, Scotland, United Kingdom). Cells were maintained in a humid atmosphere of 5% CO_2 at 37°C.

Cells were transiently transfected with the corresponding cDNAs using the PEI (PolyEthylenImine, Sigma-Aldrich, St. Louis, MO, USA) method as previously described (Carriba et al., 2008; Hradsky et al., 2011; Navarro et al., 2012). Four hours after transfection, growth medium was replaced by a complete medium. Experiments were carried out 48 h later.

To prepare primary striatal neurons, brains from fetuses of pregnant mice were removed (gestational age: 17 days). Neurons were isolated as described in Hradsky et al. (2013) and plated at a confluence of 40,000 cells/0.32 cm^2. Briefly, the samples were dissected and, after careful removal of the meninges, digested for 20 min at 37°C with 0.25% trypsin. Trypsinization

was stopped by adding an equal volume of culture medium (Dulbecco's modified Eagle medium-F-12 nutrient mixture, Invitrogen). Cells were brought to a single cell suspension by repeated pipetting followed by passage through a 100 μm-pore mesh. Pelleted (7 min, 200× *g*) cells were resuspended in supplemented DMEM and seeded at a density of 3.5×10^5 cells/ml. The next day, the medium was replaced by neurobasal medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 2% (*v*/*v*) B27 medium (Gibco). Neuronal cultures were used for assays after 15 days of culture. Using NeuN as a marker, the percentage of neurons in the cultures was >90%.

Expression Vectors

The human cDNAs for the CB_1R, GHS-R1a, and D_1R cloned in pcDNA3.1 were amplified without their stop codons using sense and antisense primers. The primers harbored either unique BamHI and KpnI sites for CB_1R and HindIII and BamHI sites for GHS-R1a and D_1R. The fragments were subcloned to be in frame with an enhanced yellow fluorescent protein (pEYFP-N1; Clontech, Heidelberg, Germany) and the Renilla luciferase protein (Rluc; pRluc-N1; PerkinElmer, Wellesley, MA) on the C-terminal end of the receptor to produce CB_1R-YFP, D_1R-Rluc, and GHS-R1a-Rluc.

Immunofluorescence

HEK-293T cells transfected with cDNAs for CB_1R-YFP and GHS-R1a-Rluc were fixed in 4% paraformaldehyde for 15 min and then washed twice with PBS containing 20 mM glycine before permeabilization with the same buffer containing 0.2% Triton X-100 (5 min incubation). The samples were treated for 1 h with blocking solution (PBS containing 1% bovine serum albumin) and labeled with a mouse anti-Rluc (1/100; MAB4400, Millipore, Burlington, MA, USA) as primary antibody and subsequently treated with Cy3-conjugated anti-mouse IgG (1/200; 715-166-150; Jackson ImmunoResearch) as the secondary antibody (1 h each). The samples were washed several times and mounted with 30% Mowiol (Calbiochem, San Diego, USA). Nuclei were stained with Hoechst (1/100). Samples were observed under a Zeiss 880 confocal microscope (Carl Zeiss, Oberkochen, Germany).

Bioluminescence Resonance Energy Transfer (BRET) Assay

HEK-293T cells growing in 6-well plates were transiently cotransfected with a constant amount of cDNA encoding for GHS-R1a fused to Renilla luciferase (GHS-R1a-Rluc) and with increasing amounts of cDNA corresponding to CB_1 receptor fused to the yellow fluorescent protein (CB_1R-YFP). For negative control, cells were cotransfected with a constant amount of cDNA encoding for D_1R-Rluc and with increasing amounts of cDNA for CB_1R-YFP. Forty-eight hours post-transfection cells were washed twice in quick succession with HBSS (137 mM NaCl; 5 mM KCl; 0.34 mM Na_2HPO_4; 0.44 mM KH_2PO_4; 1.26 mM $CaCl_2$; 0.4 mM $MgSO_4$; 0.5 mM $MgCl_2$; and 10 mM HEPES, pH 7.4) supplemented with 0.1% glucose (w/v), detached by gently pipetting and resuspended in the same buffer. To have an estimation of the number of cells per plate, protein concentration was determined using a Bradford assay kit (Bio-Rad, Munich, Germany) with bovine serum albumin dilutions for standardization. To quantify YFP-fluorescence expression, cells were distributed (20 μg protein) in 96-well microplates (black plates with a transparent bottom; Porvair, Leatherhead, UK). Fluorescence was read using a Mithras LB 940 (Berthold, Bad Wildbad, Germany) equipped with a high-energy xenon flash lamp, using a 10-nm bandwidth excitation and emission filters at 485 and 530 nm, respectively. YFP-fluorescence expression was determined as the fluorescence of the sample minus the fluorescence of cells expressing only protein-Rluc. For the BRET measurements, the equivalent of 20 μg of cell suspension was distributed in 96-well microplates (white plates; Porvair), and 5 μM coelenterazine H was added (PJK GMBH, Kleinblittersdorf, Germany). Then, 1 min after coelenterazine H addition, the readings were collected using a Mithras LB 940 (Berthold, Bad Wildbad, Germany), which allowed the integration of the signals detected in the short-wavelength filter at 485 nm (440–500 nm) and the long-wavelength filter at 530 nm (510–590 nm). To quantify receptor-Rluc expression, luminescence readings were collected 10 min after 5 μM coelenterazine H addition. The net BRET is defined as [(long-wavelength emission)/(short-wavelength emission)]-Cf where Cf corresponds to [(long-wavelength emission)/(short-wavelength emission)] for the Rluc construct expressed alone in the same experiment. The BRET curves were fitted assuming a single phase by a non-linear regression equation using the GraphPad Prism software (San Diego, CA, USA). BRET values are given as milli-BRET units (mBU: 1000 × net BRET).

cAMP Determination

HEK-293T cells transfected with the cDNAs for CB_1R (1 μg) and/or GHS-R1a (1.5 μg) and neuronal primary cultures were plated in 6-well plates. Two hours before initiating the experiment, the cell-culture medium was replaced by the non-supplemented DMEM medium. Then, cells were detached, resuspended in the non-supplemented DMEM medium containing 50 μM zardaverine, and plated in 384-well microplates (2,500 cells/well). Cells were pretreated (15 min) with the corresponding antagonists (1 μM rimonabant for CB_1R and 1 μM YIL 781 for GHS-R1a) or vehicle and stimulated with agonists (1 nM, 10 nM, 100 nM, and 1 μM ACEA for CB_1R or 200 nM ghrelin for GHS-R1a; 15 min) before the addition of 0.5 μM FK or vehicle. Finally, the reaction was stopped by the addition of the Eu-cAMP tracer and the ULight-conjugated anti-cAMP monoclonal antibody prepared in the "cAMP detection buffer" (PerkinElmer). All steps were performed at 25°. Homogeneous time-resolved fluorescence energy transfer (HTRF) measures were performed after 60 min incubation at RT using the Lance Ultra cAMP kit (PerkinElmer, Waltham, MA, USA). Fluorescence at 665 nm was analyzed on a PHERAstar Flagship microplate reader equipped with an HTRF optical module (BMG Lab Technologies, Offenburg, Germany).

MAPK Phosphorylation Assays

To determine extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation, HEK-293T transfected cells and primary striatal neurons were plated (50,000 cells/well) in transparent Deltalab 96-well plates and kept in the incubator for 15 days. Two hours before the experiment, the medium was replaced by non-supplemented DMEM medium. Next, the cells were pre-treated at RT for 10 min with antagonists (1 μM rimonabant for CB_1R and YIL 781 for GHS-R1a) or vehicle and stimulated for an additional 7 min with selective agonists (1 nM, 10 nM, 100 nM, 1 μM ACEA for CB_1R and 200 nM ghrelin for GHS-R1a). Then, cells were washed twice with cold PBS before the addition of 30 μl/well "Ultra lysis buffer" -PerkinElmer- (15 min treatment). Afterward, 10 μl of each supernatant was placed in white ProxiPlate 384-well plates and ERK1/2 phosphorylation was determined using an AlphaScreen®SureFire® kit (PerkinElmer), following the instructions of the supplier, and using an EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). The reference value (100%) was the value achieved in the absence of any treatment (basal). Agonist effects were given in percentage with respect to the basal value.

Real-Time Determination of Calcium Ion Cytoplasmic Level Variation

HEK-293T cells were transfected with the cDNAs for CB_1R (1 μg) and/or GHS-R1a (1.5 μg) in the presence of 1 μg cDNA for the calmodulin-based calcium GCaMP6 sensor (Chen et al., 2013) using the PEI method. 48 h after transfection, cells were detached using Mg^{+2}-free Locke's buffer (pH 7.4; 154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5.6 mM glucose and 5 mM HEPES) supplemented with 10 μM glycine. 1,500 cells per well were plated in 96-well black, clear-bottom, microtiter plates. Then, cells were incubated for 10 min with the CB_1R and GHS-R1a antagonists (1 μM rimonabant or 1 μM YIL 781), and subsequently stimulated with selective agonists (1 nM, 10 nM, 100 nM, 1 μM ACEA, or 200 nM ghrelin). Upon excitation at 488 nm, real-time 515 nm fluorescence emission due to calcium-ion complexed GCaMP6 was recorded on the EnSpire® Multimode Plate Reader (every 5 s, 100 flashes per well).

Proximity Ligation Assays (PLAs)

Physical interaction between CB_1R and GHS-R1a was detected using the Duolink *in situ* PLA detection Kit (OLink; Bioscience, Uppsala, Sweden) following the instructions of the supplier. Primary neurons were grown on glass coverslips, fixed in 4% paraformaldehyde for 15 min, washed with PBS containing 20 mM glycine to quench the aldehyde groups, and permeabilized with the same buffer containing 0.05% Triton X-100 (20 min). Then, samples were successively washed with PBS. After 1 h incubation at 37°C with the blocking solution in a pre-heated humidity chamber, primary neurons were incubated overnight in the antibody diluent medium with a mixture of equal amounts of mouse anti-CB_1R (1/100; sc-293419, Santa Cruz Technologies, Dallas, TX, USA) and rabbit anti-GHS-R1a (1/100; ab95250, Abcam, Cambridge, United Kingdom) to detect CB_1R-GHS-R1a complexes. Neurons were processed using the PLA probes detecting primary antibodies (Duolink II PLA probe plus and Duolink II PLA probe minus) diluted in the antibody diluent solution (1:5). Ligation and amplification were done as indicated by the supplier. Samples were mounted using the mounting medium with Hoechst (1/100; Sigma-Aldrich) to stain nuclei. Samples were observed in a Zeiss 880 confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with an apochromatic 63× oil immersion objective (N.A. 1.4) and a 405 nm and a 561 nm laser lines. For each field of view, a stack of two channels (one per staining) and four Z stacks with a step size of 1 μm were acquired. The number of neurons containing one or more red spots vs. total cells (blue nucleus) was determined, and the unpaired t-test was used to compare the values (red dots/cell) obtained.

RESULTS

The CB_1R May Interact With the GHS-R1a

Cannabis sativa L consumption has an orexigenic effect *via* a mechanism in which a hormone of the endocrine system, ghrelin, participates. To identify whether or not there are functional interactions between the cannabinoidergic and the orexinergic systems, we first tested a potential interaction between the CB_1R and the functional form of the ghrelin receptor, GHS-R1a. Immunocytochemical assays in HEK-293T cells transfected with the cDNA of the GHS-R1a fused to Renilla luciferase (Rluc) and/or the cDNA for the CB_1R fused to the Yellow Fluorescent Protein (YFP) led to detect the receptors at the plasma membrane level with a marked colocalization when coexpressed (**Figure 1A**).

As colocalization may be found for proteins that are close (approximately 200 nm apart) but may not be directly interacting, a Bioluminescence Resonance Energy Transfer (BRET) assay was performed in HEK-293T cells cotransfected with a constant amount of the cDNA for GHS-R1a-Rluc and increasing amounts of cDNA for CB_1R-YFP. A saturation curve ($BRET_{max}$ = 44 ± 4 mBU, $BRET_{50}$ = 280 ± 70) was obtained, demonstrating a direct interaction between the two receptors in this heterologous expression system (**Figure 1B**). When the same experiment was performed in cells pretreated for 30 min with the selective CB_1R agonist, arachidonyl-2'-chloroethylamide (ACEA, 100 nM), no significant differences were observed ($BRET_{max}$ = 47 ± 4 mBU, $BRET_{50}$ = 400 ± 10). This result indicates that CB_1R-GHS-R1a interaction is not affected by the activation of the CB_1R. As a negative control, HEK-293T cells were transfected with a constant amount of dopamine D_1 receptor-Rluc cDNA and increasing amounts of CB_1R-YFP cDNA; the nonspecific linear signal indicates a lack of interaction between these two proteins (**Figure 1C**).

CB_1R-Mediated Signaling Is Blocked in the CB_1-GHS-R1a Receptor Heteromer (CB_1R-GHS-R1aHet)

After identifying a direct interaction between CB_1R and GHS-R1a, the functional consequences of the interaction were investigated. Signaling assays were performed considering that

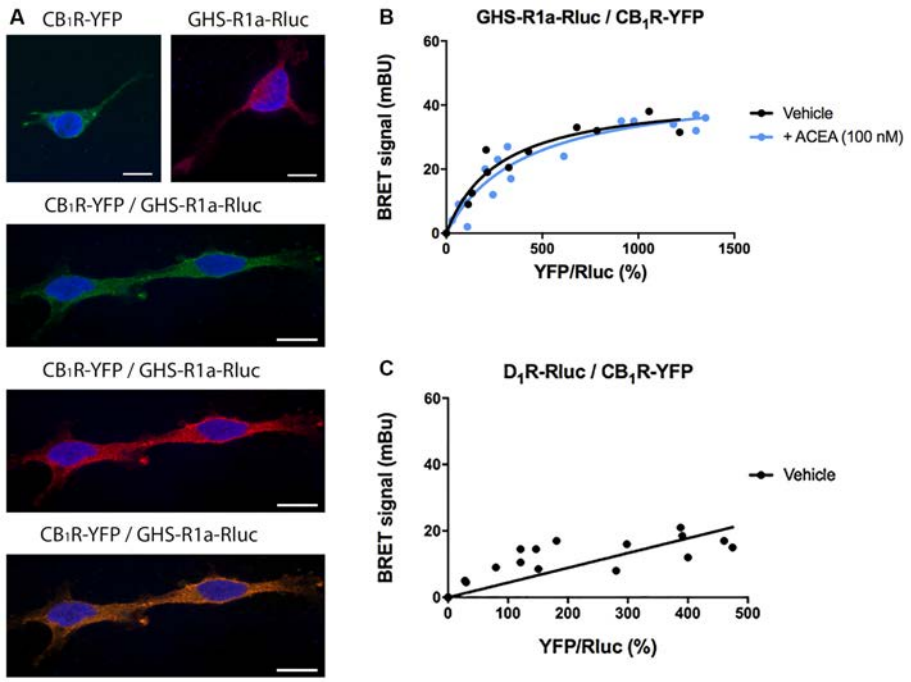

FIGURE 1 | Molecular interaction between GHS-R1a and CB_1 receptors expressed in HEK-293T cells. **(A)** Confocal microscopy images of HEK-293T cells transfected with cDNAs for GHS-R1a-Rluc (1.5 μg) and/or for CB_1R-YFP (1 μg). GHS-R1a-Rluc (red) was identified by immunocytochemistry using an anti-Rluc antibody (Merck-Millipore, 1/100). The CB_1R-YFP (green) was identified by the fluorescence due to YFP. Colocalization is shown in yellow. Cell nuclei were stained with Hoechst (blue). Scale bar: 10 μm. **(B,C)** BRET saturation experiments were performed using HEK-293T cells co-transfected with a constant amount of GHS-R1a-Rluc cDNA (1.5 μg) and increasing amounts of CB_1R-YFP cDNA (0–2 μg) and treated with ACEA (100 nM) or vehicle. As a negative control, HEK-293T cells were transfected with a constant amount of D_1R-Rluc cDNA (1.5 μg) and increasing amounts of CB_1R-YFP cDNA (0–2 μg). BRET data are expressed as the mean ± SEM of eight independent experiments performed in duplicates. mBU: milliBRET units.

the CB_1R couples to G_i and that, although the canonical protein that couples to GHS-R1a receptor is G_q, the ghrelin receptor may also couple to G_i. The activation of any of the receptors in the presence of forskolin (FK), which activates adenylate cyclase, led to a decrease in cytosolic cAMP levels in HEK-293T cells expressing CB_1R or GHS-R1a. In cells expressing the CB_1R, the selective agonist, arachidonyl-2'-chloroethylamide (ACEA), led to a dose-response decrease in (FK)-stimulated cAMP levels that were not affected by pretreatment with ghrelin. Moreover, ghrelin treatment induced no effect over cannabinoid CB_1R, demonstrating ligand selectivity. The effect of ACEA was specific as it was blocked by a selective CB_1R antagonist, rimonabant (**Figure 2A**). In cells expressing the GHS-R1a, 200 nM ghrelin induced a significant (circa 30%) decrease of FK-induced cAMP level that was not affected by ACEA but that was completely counteracted by YIL 781, a selective GHS-R1a receptor antagonist (**Figure 2B**). It was confirmed that ACEA did not induce any effect over the GHS-R1a. When the G_q coupling was assayed using the GCaMP6 sensor of cytoplasmic Ca^{2+}, no signal was obtained in cells expressing the CB_1R (**Figure 2C**), while in cells expressing the GHS-R1a receptor ghrelin lead to a transient peak of cytosolic [Ca^{2+}] that was not modified by preincubation with ACEA but that was prevented upon preincubation with the ghrelin receptor antagonist (**Figure 2D**).

In HEK-293T cells expressing CB_1 and GHS-R1a receptors, the ghrelin-induced decrease of FK-stimulated cAMP levels was significant although lower than that in cells only expressing the ghrelin receptor (37% vs. 15%; **Figure 3A**). Interestingly, the presence of the ghrelin receptor uncoupled CB_1R activation from G_i protein, at least at a functional level. In fact, ACEA even at the highest concentration (1 μM) was not able to significantly reduce FK-induced cAMP levels. Functionality was, however, found when cells were treated with ghrelin and there was no additive effect at concentration of 10 nM ACEA or higher; the lower concentration of ACEA (1 nM) showed a trend to increase the ghrelin-induced effect (**Figure 3A**). Results related to G_q coupling were noteworthy as ACEA, which did not lead to calcium ion mobilization by itself, significantly increased the effect of ghrelin. Remarkably, the potentiation was much stronger at low doses of ACEA (1 nM) than at higher concentrations (**Figure 3B**). In particular, the 40 s post-activation increase provided by the presence of 1 nM, 10 nM or 100 nM ACEA over the signal provided by 200 nM ghrelin, was (in percentage), 140 ± 30, 65 ± 30, and 71 ± 15, respectively. Finally, as GHS-R1a and CB_1 receptor agonists lead to activation of the mitogen-activated protein kinase (MAPK) pathway (Mousseaux et al., 2006; Daigle et al., 2008; Navarro et al., 2018b), we tested the properties of the heteromer in the link to the MAPK signaling pathway. Again, the effect of

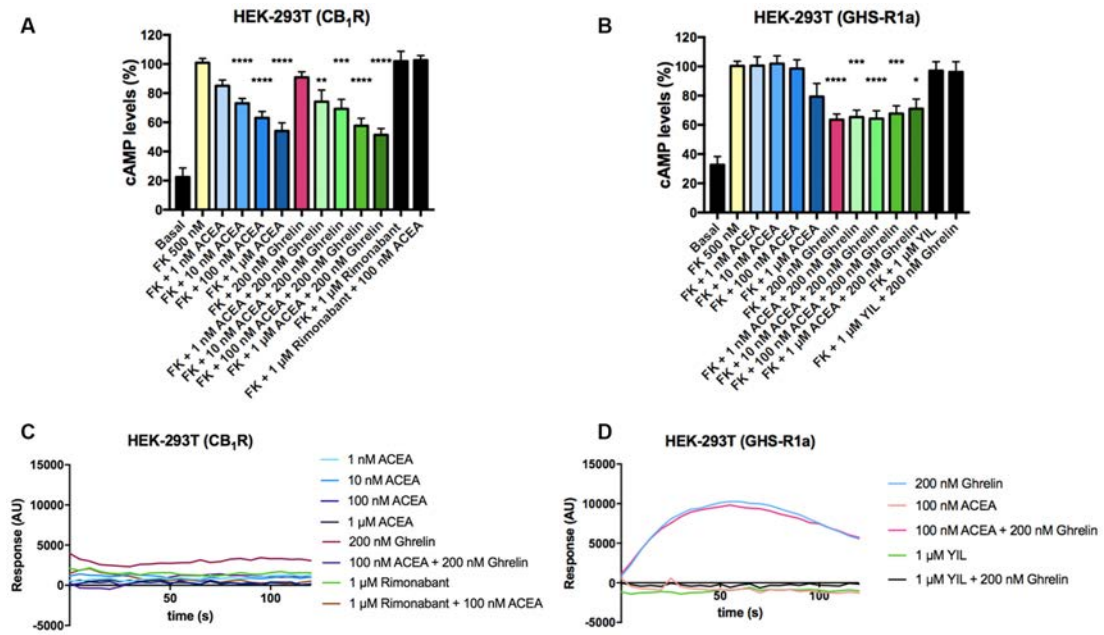

FIGURE 2 | Functional characterization of GHS-R1a and CB_1 receptors expressed in HEK-293T cells. **(A,B)** HEK-293T cells transfected with cDNA encoding for either CB_1R (1 µg; **A**) or GHS-R1a (1.5 µg; **B**) were pre-treated with selective antagonists, 1 µM rimonabant -CB_1R- or 1 µM YIL 781 -GHS-R1a-, and subsequently treated with the selective agonists, ACEA (1 nM, 10 nM, 100 nM, 1 µM) -CB_1R- or ghrelin (200 nM) -GHS-R1a-. cAMP levels after 0.5 µM forskolin (FK) stimulation were detected by the Lance Ultra cAMP kit. Results are expressed in % respect to levels obtained upon FK stimulation (100%). The values are the mean ± SEM of 10 independent experiments performed in triplicates. One-way ANOVA followed by Bonferroni's multiple comparison *post-hoc* test were used for statistical analysis. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. FK treatment. **(C,D)** HEK-293T cells expressing an engineered calcium sensor, 6GCaMP, and CB_1R **(C)** or GHS-R1a **(D)** were pre-treated with selective antagonists for 10 min followed by agonist stimulation. Real-time traces of cytoplasmic Ca^{2+} levels detected by EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA, USA) over time are shown (six independent experiments).

the cannabinoid receptor agonist was suppressed when the two receptors were co-expressed, while the effect of ghrelin was not modified by low doses of ACEA but was diminished when higher doses were used (**Figure 3C**). In a control assay, it was confirmed that ACEA does increase ERK1/2 phosphorylation, as previously reported (Navarro et al., 2018a,b). These data demonstrate that cannabinoids may regulate GHS-R1a function depending on the concentration and that GHS-R1a expression suppresses cannabinoid receptor-mediated events supposedly by the establishment of heteromeric complexes.

Cross-Talk Characterization

In the 90s different laboratories proved interactions between GPCRs to form heteromeric complexes. These complexes can be detected by energy transfer techniques in heterologous expression systems or by proximity ligation assays (PLA) in natural sources, either primary cultures or tissue sections. An often-found property of a heteromer formed by two GPCRs is that the antagonist of one of the receptors not only blocks the signaling originated at the receptor but also the signaling originated at the partner receptor. Such cross-antagonism, which is due to conformational changes transmitted from one receptor to the other, may serve as a print to detect the heteromer. Another possibility is that coactivation leads to a smaller effect than that obtained upon activating only one receptor of the complex; this phenomenon is known as negative crosstalk. Finally, in some cases, the antagonist of one receptor may restore the signaling *via* the partner receptor in the heteromer.

To study the effect of antagonists, HEK-293T cells expressing CB_1 and GHS-R1a receptors were pretreated with the selective antagonists before agonist stimulation. As observed in **Figure 4A**, the CB_1R antagonist, rimonabant, did not block G_i-mediated ghrelin-induced effect, whereas the GHS-R1a antagonist, YIL 781, which completely blocked ghrelin-induced decrease of FK-induced cAMP levels, did not restore the CB_1R-G_i coupling. Accordingly, there was no cross-antagonism in GHS-R1a/G_i-mediated signaling when the CB_1 receptor was blocked by a selective antagonist. Similar were results in **Figure 4B**, i.e., there was no cross-antagonism in GHS-R1a receptor/G_q-mediated signaling when the CB_1R receptor was blocked by a selective antagonist. In summary, neither cross-antagonism nor restoration of CB_1R-G_i coupling was observed when addressing direct G_i- or direct G_q-induced outputs using selective antagonists.

Effect of Ghrelin and/or ACEA in Primary Striatal Neurons

After demonstrating the functionality of CB_1R-GHS-R1aHet in transfected HEK-293T cells, we moved to a more physiologic environment to look for the expression of the receptor complex. We investigated the expression and function of

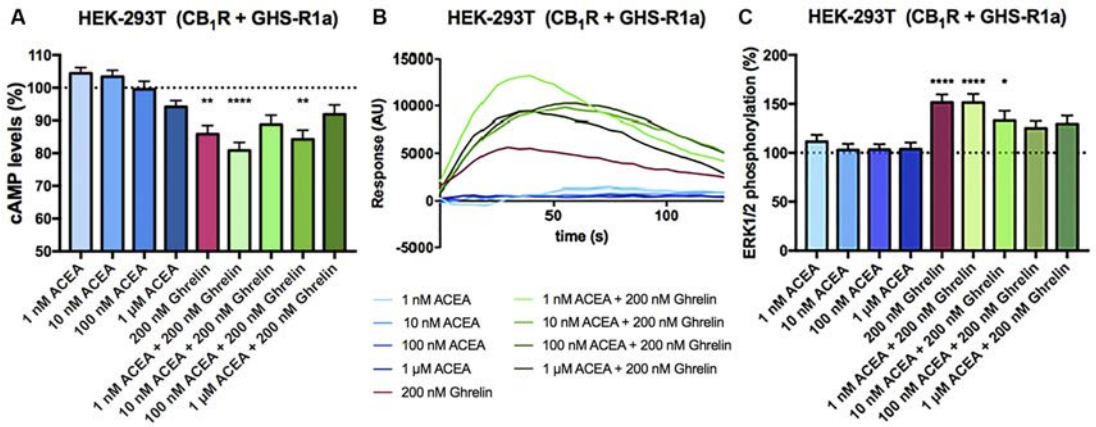

FIGURE 3 | Functional characterization of the CB_1-GHS-R1aHet expressed in HEK-293T cells. **(A–C)** HEK-293T cells were transfected with cDNAs encoding for GHS-R1a (1.5 μg) and for CB_1R (1 μg; **A,C**) or cDNAs encoding for GHS-R1a (1.5 μg), CB_1R (1 μg) and the 6GCaMP calcium sensor (1 μg; **B**) and stimulated with selective agonists, 200 nM ghrelin -for GHS-R1a- and 1 nM, 10 nM, 100 nM, and 1 μM ACEA -for CB_1R-, individually or in combination. cAMP levels were analyzed by the Lance Ultra cAMP kit and results are expressed in % respect to levels obtained upon 0.5 μM FK stimulation (**A**; 100%, dotted line). Representative traces of intracellular Ca^{2+} responses over time are shown (eight independent experiments; **B**). ERK 1/2 phosphorylation was determined by an AlphaScreen®SureFire® kit (PerkinElmer) using an EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA, USA; **C**); (basal is represented as 100%, dotted line). In cAMP accumulation and MAPK pathway signaling-related assays, the values are the mean ± SEM of eight independent experiments performed in triplicates. One-way ANOVA followed by Bonferroni's multiple comparison *post-hoc* test were used for statistical analysis. $^*p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$; vs. FK treatment in cAMP or vs. basal in ERK1/2 phosphorylation assays.

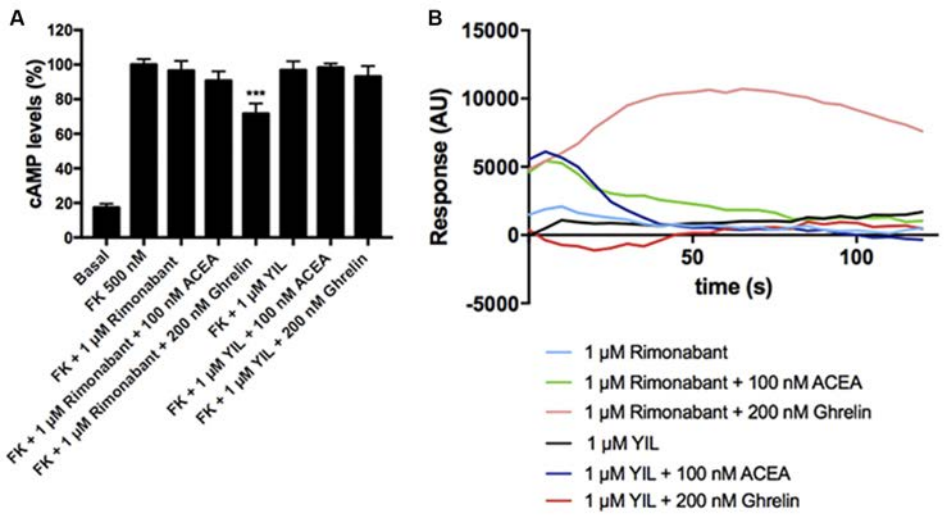

FIGURE 4 | Effect of antagonists in the functionality of the CB_1-GHS-R1aHet. HEK-293T cells were transfected with cDNAs encoding for GHS-R1a (1.5 μg) and for CB_1R (1 μg; **A**) or with cDNAs encoding for GHS-R1a (1.5 μg), CB_1R (1 μg), and the 6GCaMP calcium sensor (1 μg; **B**). Cells were pre-treated with antagonists, 1 μM YIL 781 -for GHS-R1a- or 1 μM rimonabant -for CB_1R-, and subsequently stimulated with selective agonists, 200 nM ghrelin -for GHS-R1a- or 100 nM ACEA -for CB_1R-. cAMP levels were analyzed by the Lance Ultra cAMP kit and results were expressed in % respect to levels obtained upon 0.5 μM FK stimulation (**A**; 100%). Representative real- time traces of cytoplasmic Ca^{2+} responses are shown (eight independent experiments; **B**). In cAMP accumulation assays, the values are the mean ± SEM of eight independent experiments performed in triplicates. One-way ANOVA followed by Bonferroni's multiple comparison *post-hoc* test were used for statistical analysis. $^{***}p < 0.001$; vs. FK treatment.

CB_1R-GHS-R1aHet in primary striatal neurons. First, we analyzed the effect of ACEA and ghrelin on FK-induced cAMP. As observed in **Figure 5**, ACEA was able to induce a dose-dependent effect. Ghrelin also decreased the FK-induced cAMP levels. Coactivation with ghrelin and 1 nM ACEA resulted in an additive effect, but not at higher ACEA concentrations. Moreover, the effect of ACEA was not affected by the antagonists of the GHS-R1a receptor and, reciprocally, the effect of ghrelin was not counteracted by the antagonists of the CB_1R (**Figure 5B**). On the other hand, when analyzing ERK1/2 phosphorylation in striatal primary neurons, both ACEA and ghrelin led to a significant increase

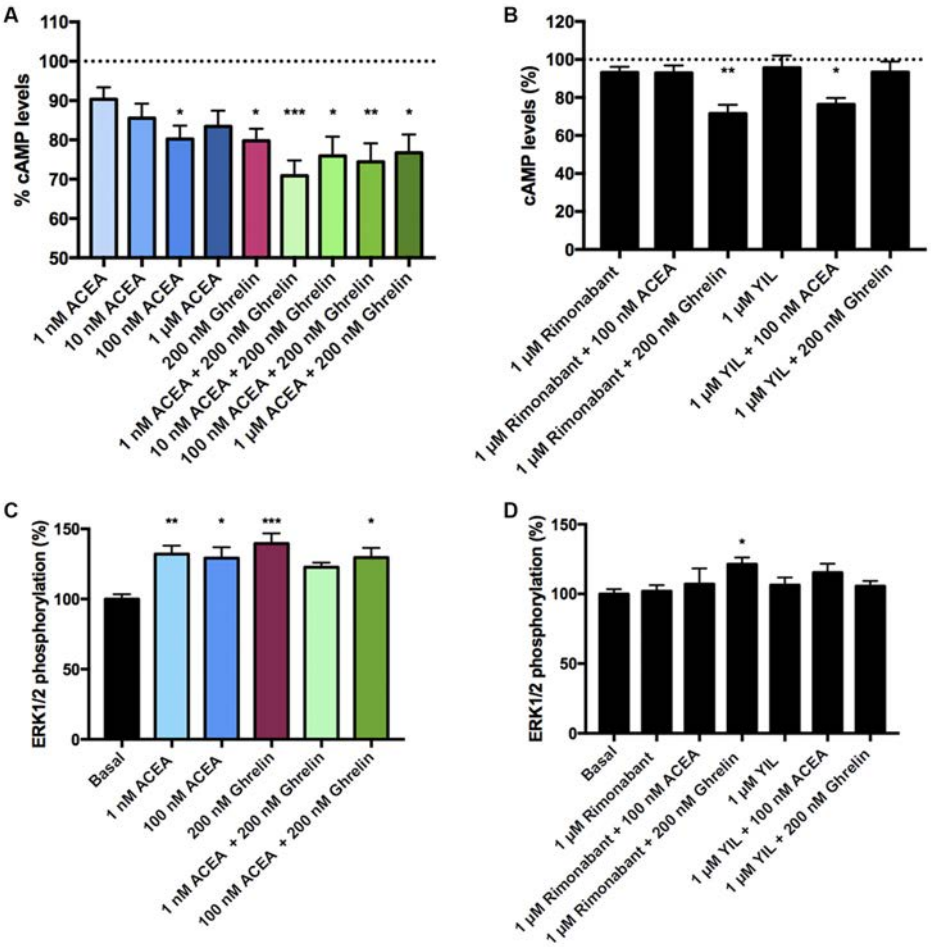

FIGURE 5 | CB_1R-GHS-R1aHet signaling in primary striatal neurons from C57BL/6J mice. Primary striatal neurons obtained from C57BL/6J brain fetuses were pre-treated with selective antagonists, 1 μM YIL 781 -for GHS-R1a- or 1 μM rimonabant -for CB_1R- or vehicle and subsequently stimulated with selective agonists, 200 nM ghrelin -for GHS-R1a- or 1 nM, 10 nM, 100 nM, and 1 μM ACEA -for CB_1R-, individually or in combination. cAMP levels **(A,B)** were collected by the Lance Ultra cAMP kit and results are expressed in % respect to levels obtained upon 0.5 μM FK stimulation (100%, dotted line). ERK1/2 phosphorylation **(C,D)** was analyzed using an AlphaScreen® SureFire® kit (PerkinElmer) and results are expressed in % respect to basal levels. Values are the mean ± SEM of six independent experiments performed in triplicates. One-way ANOVA followed by Bonferroni's multiple comparison *post-hoc* tests were used for statistical analysis. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; vs. FK treatment **(A,B)** or vs. basal **(C,D)**.

in phosphorylation levels. In addition, no cross-antagonism was detected in primary neurons pretreated with rimonabant followed by ghrelin stimulation (**Figures 5C,D**). These results suggest that the proportion of CB_1R-GHS-R1aHets in primary striatal cultures is relatively low and that CB_1 and GHS-R1a receptors may be forming complexes with other GPCRs (see "Discussion" section).

The CB_1R-GHS-R1aHet Is Overexpressed in Striatal Neurons Isolated From the Brain of the Progeny of Mothers Under a High-Fat Diet

One of the aims of this study was to correlate the expression of CB_1R-GHS-R1a receptor complexes in a situation of unbalanced energy homeostasis. For this purpose, we used primary striatal neurons isolated from fetuses of mothers fed a standard (STD) or high fat (HFD) diet (see "Materials and Methods" section). The expression of CB_1R-GHS-R1aHets was assessed by *in situ* proximity ligation assay (PLA). An important increase in the expression of CB_1-GHS-R1a complexes was observed in striatal neurons from fetuses of HFD mothers (18 red dots/cell vs. 3 red dots/cell in neurons from fetuses of STD mothers; **Figures 6A,B**). Moreover, when primary neurons from fetuses of HFD mothers were treated with 1 nM ACEA, a significant decrease in complex expression (12 red dots/cell) was observed; however, a higher concentration of the compound, 100 nM, led to a marked increase in the number of complexes (36 red dots/cell). Remarkably, treatment with 200 nM ghrelin produced a robust increase in CB_1R-GHS-R1a complex expression (>40 red dots/cell; **Figure 6C**). In summary, the expression of the CB_1R-GHS-R1aHet was higher in the progeny of HFD mothers and

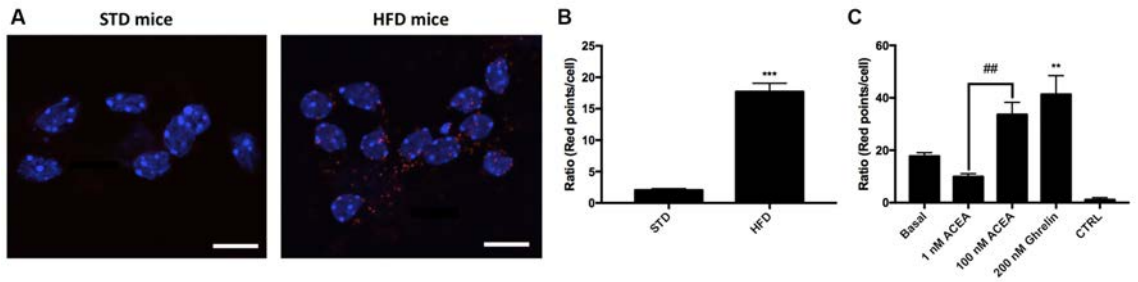

FIGURE 6 | Expression and function of CB_1-GHS-R1aHets in primary striatal neurons isolated from fetuses of pregnant C57BL/6J female mice subjected to a standard (STD) or high-fat (HFD) diet. **(A)** CB_1-GHS-R1aHets were detected by the *in situ* proximity ligation assay (PLA) in primary striatal neurons isolated from the striatum of fetuses from STD or HFD pregnant mothers. Experiments were performed in samples from six animals. **(B,C)** The number of red dots/cell was quantified using Andy's algorithm Fiji's plug-in; nuclei were stained using Hoechst (blue). In **(B)** the number of dots/cell in HFD samples are compared with that in STD samples. In **(C)** neurons obtained from fetuses from HFD mothers were stimulated with 200 nM ghrelin -for GHS-R1a- or 1 nM and 100 nM ACEA -for CB_1R- and analyzed by PLA **(C)**. The negative control was obtained omitting one of the primary antibodies. Scale bar: 10 μm. One-way ANOVA followed by Bonferroni's multiple comparison *post-hoc* test was used for statistical analysis on comparing to basal or to STD: $^{***}p < 0.001$, vs. STD **(B)** $^{**}p < 0.01$, vs. basal **(C)**. Bonferroni's multiple comparison test showed significance in comparing results from ACEA treatments: $^{\#\#}p < 0.01$ **(C)**.

may be regulated by ghrelin and, differentially, by cannabinoid concentration.

DISCUSSION

Cannabis has been known and used for years by various civilizations and is used even today. Cannabis use is perceived in two almost opposite ways. One is related to the psychotropic properties of one of its components, (6a*R*, 10a*R*)-6,6,9-trimethyl-3-pentyl-6a,7,8,10a-tetrahydro-6H-benzo[c]chromen-1-ol, commonly known as THC (or Δ^9THC; CAS registry number #1972-08-3). It is under question whether the exposure to THC is addictive although it is known that the compound affects homeostatic synaptic plasticity. The good side is the possibility that compounds in *Cannabis sativa L* may be useful to combat a variety of diseases. For instance, dronabinol (Marinol®), with an identical chemical structure to THC but of synthetic origin, has been already approved for human use. Also approved are a preparation having an equal amount of THC and cannabidiol (Sativex®) and a preparation of pure cannabidiol in vegetable oil (Epidiolex®). The main therapeutic indications of these drugs are anti-emetic and spasticity management. Interestingly, a CB_1R antagonist, rimonabant, was approved to combat obesity. The discovery that blockade of the CB_1R could lead to a drop in body weight by reducing food consumption correlated with the well-known orexigenic properties of Cannabis consumption. Unfortunately, the compound, which is brain permeable, was withdrawn due to serious adverse events related to alterations in central functions. Although it was hypothesized that CB_1R antagonists unable to cross the blood-brain barrier could overcome the side effects, there is evidence that the anti-orexigenic actions of CB_1R antagonists are due to, at least in part, the blockade of receptors in the central nervous system where the CB_1 is the most abundant GPCR (Carai et al., 2006; Christensen et al., 2007; Sam et al., 2011; Tudge et al., 2015). In this scenario, we aimed at defining possible interactions between cannabinoid receptors and the receptor for the so-called "hunger" hormone, ghrelin. In a recent article, we have shown the interaction of the cannabinoid CB_2 receptor (CB_2R) with the GHS-R1a receptor in a heterologous expression system and in physiological cell models (Lillo et al., 2021). Therefore, the first aim of the present study was to address the possible interaction between the ghrelin receptor and the CB_1R and to characterize the functional consequences of such interaction. Both, the previous and the present studies demonstrate that the GHS-R1a receptor may interact with either CB_1 or CB_2 receptors and that the resulting heteromers may occur in physiological environments.

At the functional level, allosteric interactions within CB_1R-GHS-R1aHets and of CB_2R-GHS-R1aHets lead to the blockade of cannabinoid receptor/G_i-mediated signaling. The blockade occurs just by simple co-expression, i.e., it does not require the activation of the GHS-R1a receptor. Taking these results together, cells expressing cannabinoid and ghrelin receptors heteromers on the cell surface would not be responsive to cannabinoids, or even to endocannabinoids, unless there is a pool of cell surface CB_1Rs that are not interacting with the ghrelin receptor.

Several phytocannabinoids are able to cross the blood-brain barrier (Sagredo et al., 2007; Lafuente et al., 2011; Espejo-Porras et al., 2013; García et al., 2016; Libro et al., 2016; Palomo-Garo et al., 2016; Zeissler et al., 2016; Valdeolivas et al., 2017; Haider et al., 2018; Franco et al., 2020) and reach the brain reward circuits in which ghrelin acts. Ghrelin receptors in reward circuits mediate the control of food intake (Guan et al., 1997; Funahashi et al., 2003; Geelissen et al., 2003; Argente-Arizón et al., 2015; Cassidy and Tong, 2017). Our data indicate a blockade of G_i/CB_1R coupling but a potentiation induced by cannabinoids of the GHS-R1a/G_q-mediated events, namely a potentiation in calcium-mediated signaling. In our opinion, the higher potentiation at low doses of the CB_1R agonist (1 nM) is physiologically relevant. It is tempting to speculate that the atypical effect depending on the dose, i.e., higher potentiation at lower doses underlies previous results in humans and mice showing that low doses of THC are associated with hyperphagia,

whereas high doses suppress it (Simon and Cota, 2017). As previously highlighted, endocannabinoid tone may be important in controlling the inputs received by the reward circuits and that impact on food intake, especially as it relates to the hedonic part of eating (Coccurello and Maccarrone, 2018). Whereas cannabinoids acting on the CB_1 receptor affected calcium mobilization mediated by GHS-R1a-G_q coupling, this is not the case for the CB_2R-GHS-R1aHet (Lillo et al., 2021). As further discussed below, caution must be taken when trying to make general conclusions as the allosteric-cross interactions will occur in neurons expressing CB_1R and GHS-R1a receptors and also CB_1R-GHS-R1aHets; i.e., not all neurons express the two receptors, and a given neuron may express the CB_1R-GHS-R1aHet plus other CB_1R-containing heteromers (see, in http://www.gpcr-hetnet.com/, GPCRs that interact with the CB_1R; accessed on October 22, 2021).

As the risk to be obese is higher in families with a history of overweighed individuals, we reasoned that the expression of the heteromer could be altered in the offspring of high-fat-diet mouse mothers as they have more risk to be obese. Compared with samples from fetuses of mothers subjected to STD, there was a marked increase of CB_1R-GHS-R1aHets expression in striatal neurons from siblings of pregnant female mice under a high-fat diet. Such an increase in heteromer expression might be implicated in the obesity predisposition of the progeny of obese parents (Abu-Rmeileh et al., 2008). Upregulation of the CB_1R-GHS-R1aHet in siblings of mothers fed with HFD suggests that already at birth, these mice have a compromised CB_1R function. In addition, we observed that the number of heteromers markedly increased by activation of either the CB_1 with 100 nM ACEA or the GHS-R1a receptors with ghrelin. The upregulation induced by ACEA treatment in neurons did not occur in transfected HEK-293T cells, where ACEA pretreatment does not alter the BRET saturation curve. Also noteworthy is the fact that treatments are short, i.e., changes in expression are not due to regulation of gene expression but to conformational rearrangements within the receptor-receptor and receptor-G protein interactions. Another piece of information is that, in our hands, the CB_1R/G_i-mediated effect of 100 nM ACEA observed in striatal primary neurons was completely blocked in transfected HEK-293T cells expressing the CB_1R-GHS-R1aHet. This result indicates that not all striatal neurons express the heteromer and/or that the CB_1R in a given neuron may be interacting with receptors other than the GHS-R1aHet, i.e., with receptors that are not allosterically blocking CB_1R-mediated signaling. In the so called "*hedonic eating*" by Coccurello and Maccarrone (2018), dopamine plays a key role in the reward circuits. Accordingly, dopamine receptors may be considered in the overall picture. In this sense, the CB_1R may interact with dopamine receptors; it has been reported that the cannabinoid receptor may, at least, interact with the dopamine D_2 receptor (Jarrahian et al., 2004; Navarro et al., 2008; Marcellino et al., 2008; Przybyla and Watts, 2010; Khan and Lee, 2014; Köfalvi et al., 2020), reviewed in García et al. (2016). Cannabinoid CB_1/dopamine D_2 receptor-receptor interaction is bidirectional and may result in functional antagonism, i.e., a CB_1R agonist blocking the D_2R-mediated modulation of locomotor activity (Marcellino et al., 2008) or in a shift from G_i to G_s coupling and signaling (Bagher et al., 2017).

In conclusion, the benefits of cannabinoids acting on populations of striatal neurons expressing CB_1R-GHS-R1aHet would be lost by the blockade exerted by the ghrelin receptor, thus prevailing the effect of such cannabinoids on other systems such as the dopaminergic. On the other hand, the CB_1R-GHSR1aHet coactivated by ghrelin and cannabinoids provides a more robust calcium response. Such bursts in the concentration of cytosolic calcium must be relevant since calcium regulates almost any event of neuronal physiology. Our results suggest the potential for GHS-R1a receptor antagonists, which could offer a double benefit: (i) reduce food intake and (ii) revert the detrimental effects of HFD on the functionality of the CB_1R in striatal neurons.

It is quite likely that the CB_1R-GHSR1aHet does occur in given subpopulations of neurons. Therefore, the next stage would be an accurate description, at the anatomical and cellular levels, of the regions and specific neurons where the two receptors are co-expressed.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Barcelona Ethical Committee, which reports to the regional Government (Protocol #9659; Generalitat de Catalunya, May 24, 2019).

AUTHOR CONTRIBUTIONS

GN and RF: conceptualization, supervision, and writing—original draft. RF: data curation. AL, JL, and GN: formal analysis. AL, JL, IR, FD, CM, and GN: investigation, writing—review and editing. AL, JL, and IR: methodology. AL: project administration. CM, GN, and RF: resources. GN: validation. All authors have read and agreed to the published version of the manuscript. All authors contributed to the article and approved the submitted version.

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3.7 Identification of the Ghrelin and Cannabinoid CB_2 Receptor Heteromer Functionality and Marked Upregulation in Striatal Neurons from Offspring of Mice under a High-Fat Diet.

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Un dels efectes més coneguts del cànnabis és el de la inducció de la gana. La regulació de la ingesta és un procés on intervenen molts factors, però la grelina, també anomenada hormona de la gana, hi juga un paper crucial. L'objectiu d'aquest article va ser determinar si aquesta capacitat orexigènica dels cannabinoides es podria explicar per una interacció entre els receptors CB_2 i GHS-R1a. Emprant l'assaig de BRET es va identificar la formació de l'heteròmer CB_2R-GHS-R1a produint com a conseqüència el bloqueig de CB_2R per part del receptor GHS-R1a de la via mediada per Gi. De manera interessant, aquest bloqueig es veia suprimit en presència d'antagonistes del receptor de grelina, com per exemple el YIL 781. L'assaig de PLA va confirmar l'expressió de l'heteròmer CB_2R-GHS-R1a en cultius primaris de neurones d'estriat. En comprovar si els nivells d'expressió de l'heteròmer es veurien alterats en la progènie d'una ratolina alimentada amb dieta alta en greixos (HFD) vam observar una marcada sobreexpressió de CB_2R-GHS-R1a en aquest model animal en comparació amb el grup control.

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Identification of the Ghrelin and Cannabinoid CB_2 Receptor Heteromer Functionality and Marked Upregulation in Striatal Neurons from Offspring of Mice under a High-Fat Diet

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Abstract: Cannabinoids have been reported as orexigenic, i.e., as promoting food intake that, among others, is controlled by the so-called "hunger" hormone, ghrelin. The aim of this paper was to look for functional and/or molecular interactions between ghrelin GHSR1a and cannabinoid CB_2 receptors at the central nervous system (CNS) level. In a heterologous system we identified CB_2-GHSR1a receptor complexes with a particular heteromer print consisting of impairment of CB_2 receptor/G_i-mediated signaling. The blockade was due to allosteric interactions within the heteromeric complex as it was reverted by antagonists of the GHSR1a receptor. Cannabinoids acting on the CB_2 receptor did not affect cytosolic increases of calcium ions induced by ghrelin acting on the GHSR1a receptor. In situ proximity ligation imaging assays confirmed the expression of CB_2-GHSR1a receptor complexes in both heterologous cells and primary striatal neurons. We tested heteromer expression in neurons from offspring of high-fat-diet mouse mothers as they have more risk to be obese. Interestingly, there was a marked upregulation of those complexes in striatal neurons from siblings of pregnant female mice under a high-fat diet.

Keywords: orexigenic; anorexia; marihuana; phytocannabinoids; G protein-coupled receptors; pharmacology; receptor heteromers; signaling; high-fat diet; obesity

1. Introduction

The psychotropic effects of marihuana smoking led to the discovery of cannabinoid receptors and of endocannabinoids. Since the discovery of tetrahydrocannabinol (THC) and cannabidiol (CBD) in *Cannabis sativa* L., decades of research have led to the identification of multiple phytocannabinoids that show biological activity. The characterization of cannabinoid receptors, the use of animal models of disease, and the experience of marihuana users have led to the discovery of the health-promoting benefits of natural cannabinoids. In addition to the already approved cannabinoid-containing drugs (Dronabinol/Marinol® -LGM Pharma Boca Raton, USA)- and Sativex® and Epidiolex® -Jazz Pharmaceuticals, Dublin, Ireland-), non-psychotropic compounds such as cannabidiol and cannabigerol

extracted from *Cannabis sativa* L. have been proposed to aid in a variety of diseases (see [1,2] for review). Although cannabinoids can act via a variety of targets (GPR55, GPCR18, peroxisome proliferator-activated receptor gamma, serotonin receptors, etc.), their canonical targets are the cannabinoid CB_1 and CB_2 receptors. Both belong to the superfamily of G-protein-coupled-receptors (GPCRs) and both couple to the G_i heterotrimeric protein, i.e., receptor activation leads to G_i-mediated deactivation of adenylyl cyclase and reduction of intracellular cAMP levels. The CB_1 receptor (CB_1R), expressed mainly in neurons, is the most abundant GPCR in the nervous system; while the CB_2R is expressed at lower levels both in glia and neurons located in specific regions of the brain [3–7]. The CB_2R is considered a target for neuroprotection, especially in diseases coursing with neuroinflammation. For instance, cannabinoids acting on the CB_2R in striatal neural cells reduce the neuronal loss in synucleinopathies such as Parkinson's disease [8–13]. The underlying mechanism is mainly related to the reduction of inflammation, but the CB_2R expressed in some striatal neurons may also have a relevant role [3,7,14–16]. A selective antagonist of the CB_1R, rimonabant, approved to combat obesity was, unfortunately, withdrawn due to side effects [17–20]. The anti-obesity potential of targeting the CB_2R has not yet been addressed.

Although the molecular mechanism has not been fully elucidated, the orexigenic properties of marihuana consumption are well known [21]. Ghrelin is often considered the "hunger" hormone because its appearance in blood leads to an increase under food intake [22,23]. Ghrelin, a 28-amino acid peptide, mainly produced by specialized cells of the gastrointestinal act, may reach the central nervous system where it activates central mechanisms that control food intake [24–27]. Its main target there is the ghrelin GHSR1a receptor, also a member of the GPCR superfamily [28]. The aim of this paper was to investigate the molecular and/or functional interactions between CB_2 and GHS1a receptors that could explain the orexigenic effects of marihuana consumption and whether ghrelin affects the neuroprotective effects induced by cannabinoids acting on the CB_2R. As offspring from obese mothers have more risk to be obese, the potential expression of complexes formed by these two receptors was also studied in neurons isolated from fetuses of pregnant female mice in a high-fat diet.

2. Results

2.1. The CB_2R May Interact with the GHSR1a

Consumption of *Cannabis sativa* L. increases appetite via a mechanism in which ghrelin, a hormone of the endocrine system, participates. To investigate whether there may be a functional interaction between the cannabinoidergic and the orexinergic systems, we first investigated a potential interaction between the cannabinoid receptor CB_2 and the functional form of the ghrelin receptor, GHSR1a. Immunocytochemical assays in HEK-293T cells transfected with the cDNA for the CB_2R fused to the Green Fluorescent Protein (GFP^2) and the cDNA of the GHSR1a fused to Renilla luciferase (RLuc) led to detect the receptors at the plasma membrane level with a marked colocalization (Figure 1A). As colocalization may be found for proteins that are close (approximately 200 nm apart) but not directly interacting, a Bioluminescence Resonance Energy Transfer (BRET) assay was performed in HEK-293T cells cotransfected with a constant amount of the cDNA for GHSR1a-RLuc and increasing amounts of cDNA for CB_2R-GFP^2. A saturation curve ($BRET_{max}$: 550 ± 50 mBU, $BRET_{50}$: 18 ± 4) was obtained, demonstrating a direct interaction between the two receptors in the heterologous expression system (Figure 1B). As negative control, HEK-293T cells were transfected with a constant amount of $GABA_BR$-RLuc cDNA and increasing amounts of GHSR1a-GFP^2 cDNA; the nonspecific linear signal indicated a lack of interaction between these two proteins.

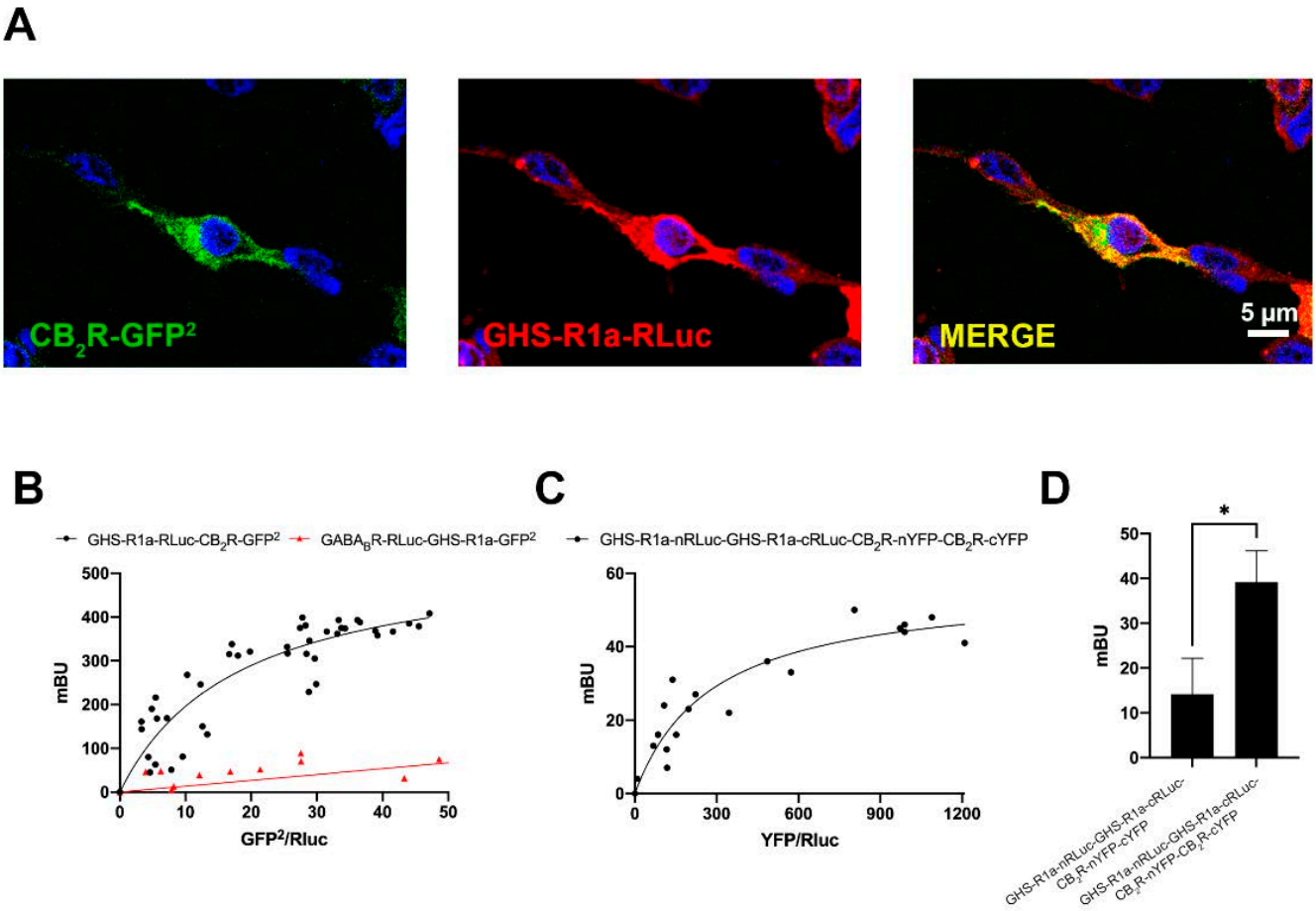

Figure 1. Molecular interaction between GHS1a and CB_2 receptors expressed in transfected HEK-293T cells. (**A**) Confocal microscopy images of HEK-293T cells co-transfected with cDNAs for GHSR1a-RLuc (0.75 µg) and for CB_2R-GFP^2 (0.5 µg). GHSR1a-RLuc (red) was identified by immunocytochemistry using an anti-RLuc antibody (Merck-Millipore, 1/100). The CB_2R-GFP^2 (green) was identified by the fluorescence due to GFP^2. colocalization is shown in yellow (image in the right). Cell nuclei were stained with Hoechst (blue). Scale bar: 5 µm. (**B**) BRET saturation experiments were performed using HEK-293T cells co-transfected with (i) a constant amount of GHSR1a-RLuc cDNA (1.5 µg) and increasing amounts of CB_2R-GFP^2 cDNA (0–2 µg) or (ii) a constant amount of $GABA_B$-RLuc cDNA (0.75 µg) and increasing amounts of GHSR1a-GFP^2 cDNA (0–2 µg) as negative control. BRET data are expressed as the mean ± SEM of 8 different experiments performed in duplicates. (**C**) Bimolecular luminescence and fluorescence complementation (BiLFC) assays were performed in HEK-293T cells co-transfected with a constant amount of GHSR1a-cRLuc and GHSR1a-nRLuc cDNAs (1.5 µg each) and increasing amounts of CB_2R-cYFP and CB_2R-nYFP cDNAs (0–3 µg each). (**D**) HEK-293T cells were co-transfected with 1.5 µg of the GHSR1a-cRLuc and GHSR1a-nRLuc cDNAs and 3 µg of the CB_2R-nYFP and CB_2R-cYFP cDNAs or with the non-fused cYFP as negative control. BRET data are expressed as the mean ± SEM of 7 different experiments performed in duplicates. * $p < 0.05$. mBU: milliBret units.

It is well known that GPCRs may form homodimers of heterodimers and higher order structures. One of the first examples has been provided for the adenosine A_1 and A_2A receptors that can form tetrameric structures complexed with two heterotrimeric G proteins, one G_i and one G_s [29,30]. To investigate the possible formation of tetramers formed by CB_2 and GHS1a receptors, BRET with bimolecular complementation experiments was performed in HEK-293T cells transfected with a constant amount of Rluc8 N/C hemiproteins fused to GHSR1a (GHSR1a-cRLuc and GHSR1a-nRLuc) and increasing amounts of YFP N/C hemiproteins fused to CB_2R (CB_2R-cYFP and CB_2R-nYFP). As observed in Figure 1C, both CB_2R and GHSR1a were able to reconstitute RLuc and YFP proteins, emitting, respectively, bioluminescence and fluorescence. This result proves homodimerization. However, a saturation BRET curve was possible in cells expressing a

constant amount of donor complemented hemiproteins and increasing levels of acceptor complemented hemiproteins ($BRET_{max}$: 56 ± 5 mBU, $BRET_{50}$: 280 ± 70 mBU). Taken together, these results constitute evidence of heterotetramer formation with two protomers of each receptor, i.e., a heterotetramer constituted by two homodimers. As a negative control, we obtained a negligible signal when substituting one of the fused proteins by the non-fused version (one example is provided in Figure 1D that shows data in assays where CB_2R-cYFP cDNA was substituted by that of cYFP).

2.2. CB_2R-Mediated Signaling Is Blocked in the CB_2-GHSR1a Receptor Heteromer (CB_2-GHSR1aHet)

Once a direct interaction between CB_2R and GHSR1a was identified, we investigated the functional consequences of interprotomer cross-talk within the heteromer. Signaling assays were performed taking into account that both CB_2 and GHS1a receptors may couple to G_i. The activation of the receptors leads to reduced adenylate cyclase activity and a decrease in cytosolic cAMP levels. Accordingly, intracellular cAMP levels following receptor activation were measured in HEK-293T cells expressing CB_2R, GHSR1a or both. In cells expressing the GHSR1a, ghrelin induced a 25% decrease of forskolin-induced cAMP levels that was completely counteracted by YIL 781 (1 μM), the selective GHSR1a antagonist. Moreover, the selective CB_2R agonist, JWH-133 induced no effect, demonstrating the ligand selectivity and lack of nonspecific effects (Figure 2A). On the other hand, in cells expressing the CB_2R, 200 nM JWH-133 induced a 30% decrease with respect to forskolin-induced cAMP levels, an effect that was completely blocked by pretreatment with the selective antagonist (AM 630, 1 μM). The control that was performed with 200 nM ghrelin induced no effect (Figure 2B). In HEK-293T cells expressing both receptors, the effect of ghrelin was similar to that found in cells only expressing the ghrelin receptor (Figure 3A); remarkably, the selective CB_2R agonist, JWH-133, did not induced any effect suggesting that CB_2R, in the heteromeric context, is not functionally coupled to G_i. Simultaneous treatment with both agonists resulted in a 37% decrease of the intracellular cAMP levels, i.e., similar to levels obtained upon ghrelin treatment. However, when the same cells were pretreated with ghrelin selective antagonist (YIL 781, 1μM) JWH-133 was able to allow the CB_2R-G_i coupling (Figure 3A). Additionally, the finding that 1 μM AM 630 in co-transfected HEK-293T cells potentiated GHSR1a-mediated signaling was also noticeable. These results indicate that (i) the basal structure restrains activation and/or decreases the functional signal, and (ii) the CB_2R blockade disappears in the presence of the selective antagonists of the partner receptor in the macromolecular complex.

We next analyzed the possibility of calcium mobilization upon activation of receptors. It is well known that GHSR1a receptor may couple to G_q, thus being linked to phospholipase C activation and calcium release from endoplasmic reticulum stores towards the cytosol. In GHSR1a-expressing HEK-293T cells, 200 nM ghrelin led to calcium mobilization (Figure 2B), that was counteracted by the pretreatment of the GHSR1a antagonist but not by the CB_2R antagonist (and did not occur in cells only expressing the CB_2R). In GHSR1a- or in CB_2-receptor-expressing cells, JWH-133 treatment did not lead to any effect (Figure 2D).

When calcium mobilization was assayed in cotransfected cells, ghrelin treatment resulted in a characteristic curve of calcium mobilization that was not significantly modified upon simultaneous treatment with JWH-133 (Figure 3B). Pretreatment with the CB_2R antagonist, AM 630, partially blocked the ghrelin effect. This phenomenon, known as cross-antagonism is, often, a print of the heteromer that is instrumental to detect it in natural sources. In these cells the CB_2R was not coupled to G_q, i.e., JWH-133 did not lead to any signal related to changes in cytosolic levels of the calcium ion.

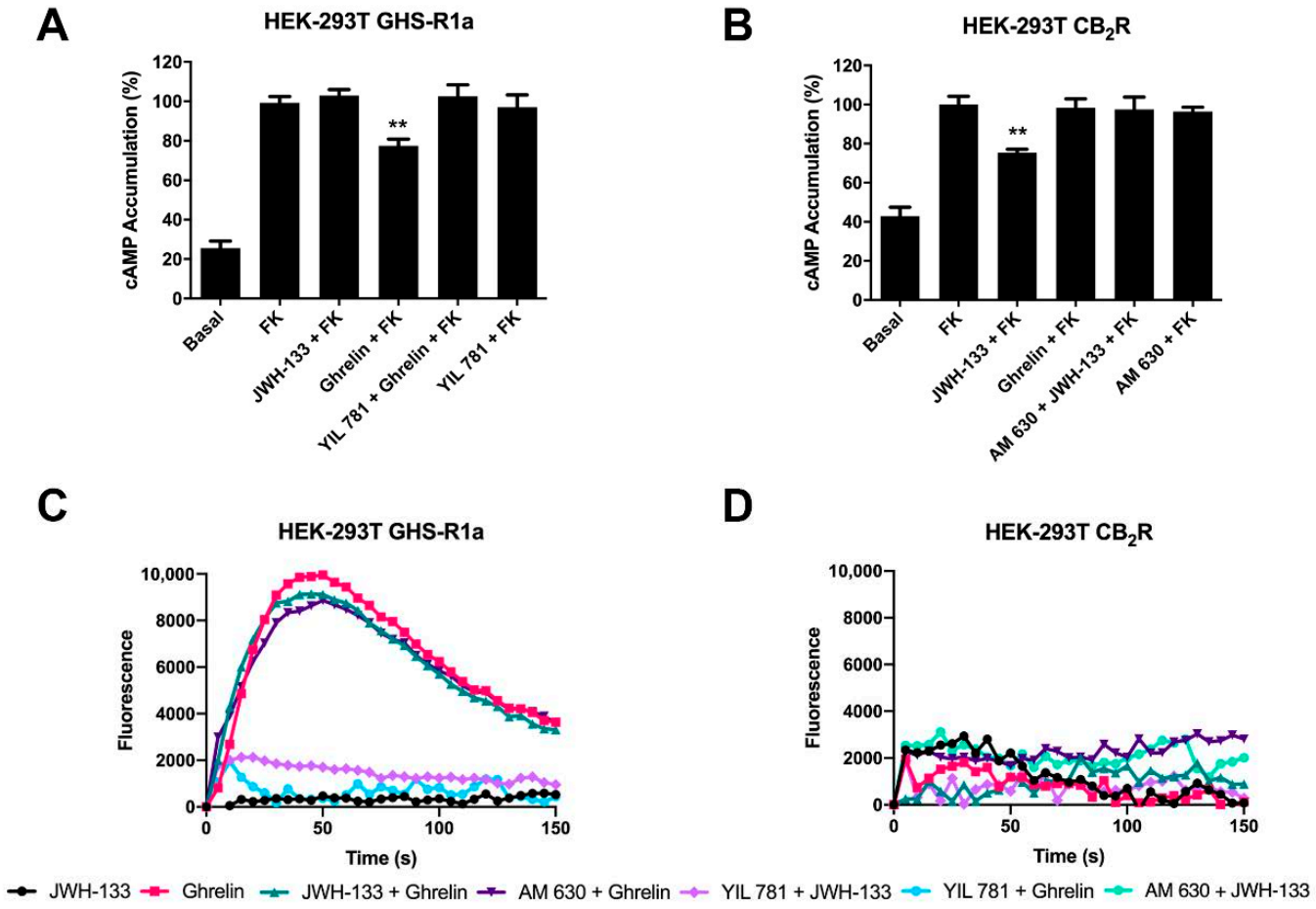

Figure 2. Functional characterization of GHS1a and CB_2 receptors expressed in HEK-293T cells. (**A**,**B**) HEK-293T cells transfected with plasmids encoding for either GHSR1a (1.5 μg) (**A**) or CB_2R (1 μg) (**B**) were pre-treated with selective antagonists, 1 μM YIL 781-GHSR1a- or 1 μM AM 630-CB_2R, and subsequently treated with the selective agonists, 200 nM ghrelin -GHSR1a- or 200 nM JWH-133 -CB_2R-. cAMP levels after 0.5 μM forskolin stimulation were detected by the Lance Ultra cAMP kit and the results were expressed in % respect to levels obtained upon forskolin stimulation. The values are the mean ± SEM of 10 different experiments performed in triplicates. One-way ANOVA followed by Dunnett's multiple comparison post-hoc test were used for statistical analysis. ** $p < 0.01$, versus forskolin treatment. (**C**,**D**) HEK-293T cells expressing an engineered calcium sensor, GCaMP6 and GHSR1a (**C**) or CB_2R (**D**) were pre-treated with selective antagonists for 10 min followed by agonist stimulation. Representative traces of intracellular Ca^{2+} responses over time are shown (6 independent experiments).

Finally, as CB_2R and GHSR1a activation leads to activation of the mitogen-activated protein kinase (MAPK) pathway [31,32], we tested the properties of the heteromer in the link to the MAPK signaling cascade. Hence, we measured ERK1/2 phosphorylation in HEK-293T cells expressing the two receptors. Treatment with 200 nM ghrelin resulted in a significant signal whereas 200 nM JWH-133 did not induce any effect (Figure 3C). Interestingly, pretreatment with the ghrelin receptor antagonist, YIL 781, allowed the activation of the MAPK pathway via the CB_2R. This action that results from antagonizing the GHSR1a was similar to that found in cAMP assays, i.e., blockade of the ghrelin receptor releases the brake posed on CB_2R-mediated signaling.

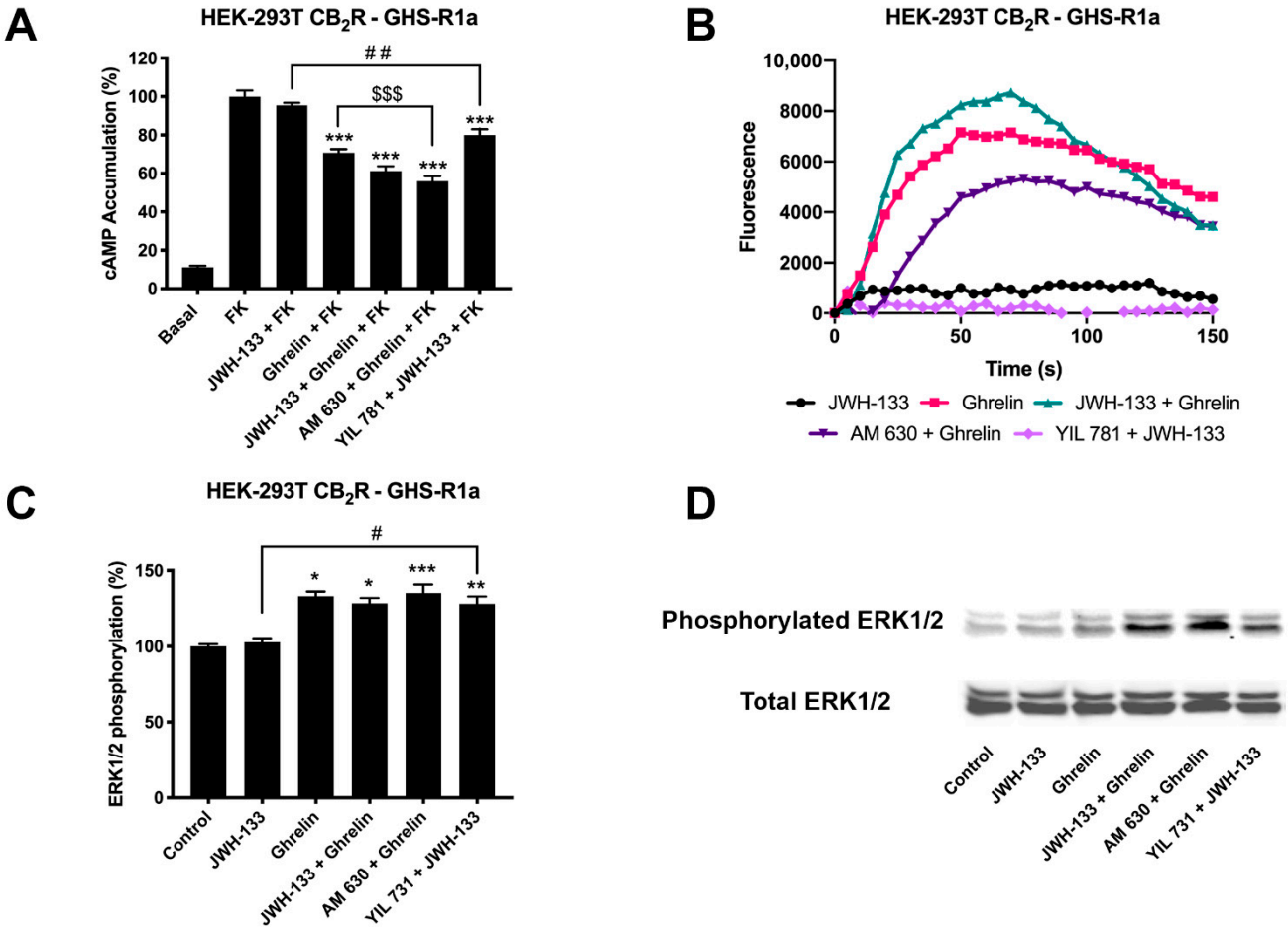

Figure 3. CB_2-GHSR1aHet-mediated signaling in transfected HEK-293T cells. Panels (**A**–**C**) HEK-293T cells were transfected with cDNAs encoding for GHSR1a (1.5 μg) and CB_2R (1 μg) (**A,C**) or with GHSR1a (1.5 μg), CB_2R (1 μg) and the GCaMP6 calcium sensor (**B**) and pre-treated with antagonists, 1 μM YIL 781 -for GHSR1a- and 1 μM AM 630 -for CB_2R, and subsequently stimulated with selective agonists, 200 nM ghrelin -for GHSR1a- and 200 nM JWH-133 -for CB_2R, individually or in combination. cAMP levels were analyzed by the Lance Ultra cAMP kit and results were expressed in % respect to levels obtained upon 0.5 μM forskolin stimulation (**A**). Representative traces of intracellular Ca^{2+} responses over time are shown (9 independent experiments) (**B**). ERK 1/2 phosphorylation was determined by immunoblot using the Odyssey infrared scanner (LI-COR Biosciences) (**C,D**). In cAMP accumulation and MAPK pathway signaling-related assays, the values are the mean ± SEM of 8 different experiments performed in triplicates. One-way ANOVA followed by Dunnett's multiple comparison post-hoc test were used for statistical analysis..* $p < 0.05$, ** $p < 0.01$, versus basal in pERK1/2 assays; *** $p < 0.001$; versus forskolin treatment in cAMP or versus basal in pERK1/2 assays, # $p < 0.05$ versus JWH-133 treatment in pERK assays, ## $p < 0.01$ versus JWH-133+FK treatment in cAMP assays; $$$ $p < 0.001$; versus ghrelin+FK treatment.

2.3. CB_2R Activation Is Blocked in Striatal Neurons via Formation of Heteromers of CB_2R and GHSR1a (CB_2R-GHSR1aHets)

Upon the demonstration that CB_2R-GHSR1aHets may be formed in a heterologous system and upon the detection of particular heteromer prints, we undertook the search for detecting the prints in a more physiological context; for such purpose we used primary striatal neurons isolated from fetuses of mothers under a standard (STD) diet (see Section 4, Materials and Methods). Primary striatal neurons from fetuses of pregnant female mothers were isolated and cultured for 15 days prior to undertake signaling assays.

First, in regard to the forskolin-induced levels, the cAMP assays showed that stimulation of CB_2R with JWH-133 did not induce any significant effect (Figure 4A). These findings may be due to lack of CB_2R or to the presence of CB_2R-GHSR1aHets. However, the results using antagonists did show that the CB_2R is expressed and that is likely forming complexes

with the ghrelin receptor. In fact, YIL 781 allowed the G_i coupling of the CB_2R as JWH-133 was then able to decrease the forskolin-induced cytosolic cAMP levels (Figure 4A). These results are fully consistent with those obtained in the heterologous expression system (HEK-293T cells). In addition, analysis of the link to the MAPK pathway showed the effect of ghrelin whereas the effect of JWH-133 was only possible in cells pretreated with the selective ghrelin receptor antagonist (Figure 4B).

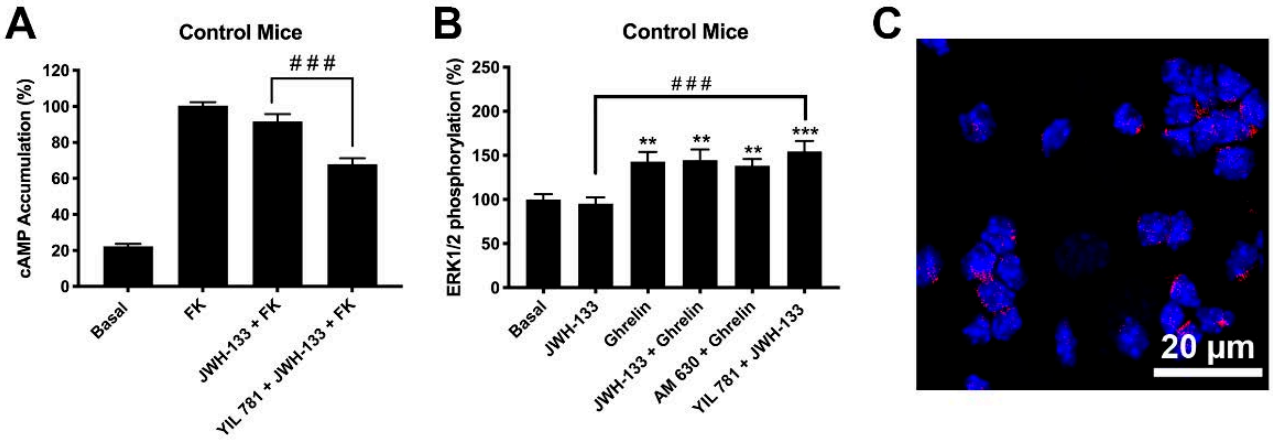

Figure 4. Expression and function of CB_2R-GHSR1aHets in primary neurons from C57BL/6J mice. (**A**,**B**) Primary striatal neurons isolated from C57BL/6J mice were pre-treated with antagonists, 1 µM YIL 781 -for GHSR1a- or 1 µM AM 630 -for CB_2R-, and subsequently stimulated with selective agonists, 200 nM ghrelin -for GHSR1a- or 200 nM JWH-133 -for CB_2R-, individually or in combination. cAMP levels were determined using the Lance Ultra cAMP kit and results were expressed in % respect to levels obtained upon 0.5 µM forskolin stimulation (**A**), while ERK1/2 phosphorylation was analyzed using the AlphaScreen®SureFire® kit (PerkinElmer; Wellesley, MA, USA) (**B**). Values are the mean ± SEM of 6 different experiments performed in triplicates. One-way ANOVA followed by Dunnett's multiple comparison post-hoc tests were used for statistical analysis. ** $p<0.01$, *** $p < 0.001$ versus basal, ### $p < 0.001$; versus JWH-133+FK treatment in cAMP or versus JWH-133 treatment in p-ERK1/2 assays. (**C**) CB_2R-GHSR1aHets were detected in primary striatal neurons by the in situ proximity ligation assay (PLA) using specific antibodies. Cell nuclei was stained with Hoechst (blue). Samples from 5 different animals were processed and analyzed and quantitation is shown in Figure 5. Scale bar: 20 µm.

Finally, we used an imaging technique, the Proximity Ligation Assay (PLA), to demonstrate the occurrence of CB_2R-GHSR1aHets in striatal neurons. PLA has been instrumental for detecting, in natural scenarios (cells or tissues), complexes formed by two proteins. Clusters of macromolecules formed by two different proteins appear as red dots using a confocal microscope (see Section 4, Materials and Methods for details); such red dots, which surrounded Hoechst stained nuclei, demonstrated the existence in striatal neurons of complexes of CB_2R and GHSR1a (Figure 4C).

2.4. Expression of CB_2R and GHSR1a Complexes Is Increased in Neurons of Progeny from Mothers on a High-Fat Diet

The ghrelin GHS1a receptor has an important role in controlling food intake, with some authors referring to ghrelin as the peripheral "hunger hormone" [22]. Moreover, it is known both an association between child obesity and maternal body mass index [33] and that diet-induced obesity leads to neuroinflammation and synapsis structure modification [34]. Herein, we investigated the expression of the CB_2R-GHSR1aHet in striatal neurons of the progeny from female mice under a high-fat (HFD) diet. Primary striatal neurons from fetuses of pregnant HFD mothers were isolated and cultured for 15 days prior to undertaking signaling assays, and the results were compared with those obtained from neurons isolated from fetuses of mothers under a standard diet (see results presented in the previous section).

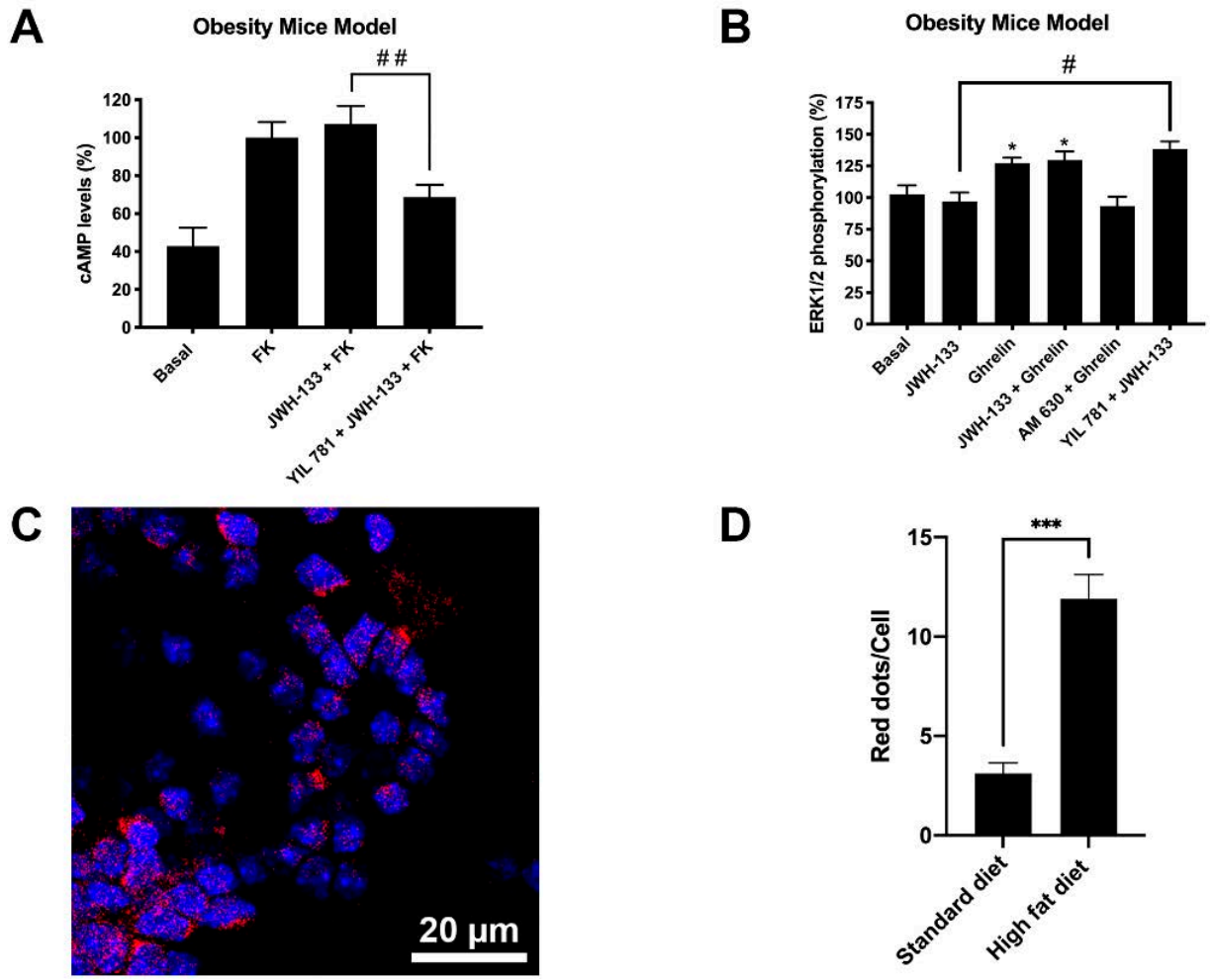

Figure 5. Expression and function of CB_2R-GHSR1aHets in primary neurons isolated from fetuses of pregnant C57BL/6J female mice subjected to a high-fat diet. (**A**,**B**) Primary striatal neurons isolated from fetuses of pregnant females mice subjected to a high-fat diet for 60 days were pre-treated with antagonists, 1 µM YIL 781 -for GHSR1a- or 1 µM AM 630 -for CB_2R-, followed by stimulation with selective agonists, 200 nM ghrelin -for GHSR1a- or 200 nM JWH-133 -for CB_2R-, individually or in combination. cAMP levels were determined using the Lance Ultra cAMP kit and results were expressed in % versus 0.5 µM forskolin treatment (**A**) while ERK1/2 phosphorylation was analyzed using the AlphaScreen® SureFire® kit (PerkinElmer) (**B**). Values are the mean ± SEM of 5 different experiments performed in triplicates. One-way ANOVA followed by Dunnett's multiple comparison post-hoc test were used for statistical analysis. # $p < 0.05$, ## $p < 0.005$ versus JWH-133+FK treatment in cAMP or versus JWH-133 treatment in p-ERK1/2 assays. * $p < 0.05$, basal. (**C**). CB_2R-GHSR1aHets were detected by the in situ proximity ligation assay (PLA) in primary striatal neurons; the negative control undertaken by omitting one of the primary antibodies is shown in Supplementary Figure S1. Experiments were performed in samples from 5 animals. The number of red dots/cell was quantified using the Andy's algorithm Fiji's plug-in and represented over cell stained nuclei with Hoechst (blue) (**D**). The number of dots-clusters/cell were compared to those in neurons from control mice (mice fed with standard diet). Unpaired t-test was used for statistics analysis. *** $p < 0.001$, versus control. Scale bar: 20 µm.

By analyzing the cAMP levels in primary striatal neurons from mothers in a HFD (Figure 5A) the results were qualitatively similar to those observed in the control group (Figure 4A) although more marked, i.e., the decreases obtained with respect to forskolin-induced cAMP levels were higher. One print of the CB_2R-GHSR1aHet was noticed as JWH-133 was only efficacious in the presence of the selective GHS1a receptor antagonist (YIL 781). The results related to the phosphorylation of pERK1/2 (Figure 5B) were virtually identical to those found in the samples of the control group (Figure 4B), being necessary YIL 781 to observe an effect of JWH-133.

Finally, when analyzing by PLA the CB_2R-GHSR1aHet expression, a marked increase in samples from the HFD group was noticed. An average of 12 red dots per Hoechst stained nuclei were observed in neurons derived from the HFD group, whereas neurons derived from the standard diet group only presented three red dots per stained nuclei (Figures 4C and 5C,D the negative control is provided in Supplementary Figure S1). Remarkably, the striatal neurons of the siblings of HFD mothers show a much higher number of CB_2R-GHSR1aHets than neurons of the siblings of mothers in standard diet, suggesting an enhanced suppression of CB_2R function in HFD mother's siblings.

3. Discussion

There is interest in the potential of targeting cannabinoid receptors for combating a variety of diseases. Despite targeting the CB_1R was the main objective in cannabinoid-related drug discovery, the psychotropic action of some cannabinoids acting on its receptors and the side effects of a CB_1R antagonist approved to combat obesity, rimonabant, has shifted the focus toward the CB_2R. The limited expression of the CB_2R in some CNS regions and its upregulation in activated glial cells have led to propose this receptor as target to limit neuroinflammation, to limit neurotoxicity induced by oxidative stress, to afford neuroprotection and/or the increase neurogenesis/gliogenesis [11,35–41]. Medications targeting cannabinoid receptors have been approved for a very limited therapeutic interventions (Sativex®, Marinol®, Epidiolex®; mainly to combat spasticity and emesis). The potential is higher and there are clinical trials already running or in preparation to test the efficacy of cannabidiol, an allosteric modulator of the CB_2R [42], and of other cannabinoids for treating from the hypoxia of the neonate to improving the course of amyotrophic lateral or multiple sclerosis [15,43–51]. The number of registered clinical trials indicated in https://clinicaltrials.gov/ for testing cannabidiol in a variety of pathological conditions is 321 (date: 1 July 2021). As commented in the introduction, the CB_2R is, also, an attractive target to afford neuroprotection in Parkinson's disease [3,7–14].

The ghrelin receptor expressed in the hypothalamus and in reward circuits of the brain is key to mediating the control of food intake [24,25,52–54]. Several of the known phytocannabinoids are able to enter the brain where they exert multiple actions [1,12,55–62]. This study was undertaken to identify possible interactions between the orexinergic and the cannabinoidergic systems. The discovery of complexes formed by GHS1a and CB_2 receptors and their identification in striatal neurons show that the ghrelin receptor modulates the effect of cannabinoids in the brain. From the molecular point of view our results suggest a tetrameric structure in complex with, at least, one G_i and one G_q protein. These results are qualitatively similar to those reported in the first reliable structural model of two interacting GPCRs, namely A_1 and A_{2A} adenosine receptors that arrange into a tetramer formed by two homodimers and are coupled to two different G proteins (one G_s and one G_i) [29,30]. In this example, the activation of one receptor blocks the activation of the partner receptor in the heteromer. However, the allosteric interactions within the CB_2R-GHSR1aHet are such that CB_2R-mediated signaling is blocked even in the absence of ghrelin, i.e., irrespective of the presence of the hormone, the CB_2R cannot be activated within the CB_2R-GHSR1aHet. Cannabinoid receptor activation is only possible in the presence of a selective GHS1a receptor antagonist, YIL 781. Although this atypical behavior is not found in many of the already identified GPCR heteromers (http://www.gpcr-hetnet.com/, accessed on 24 June 2021; [63]), it has been reported that the mere presence of the A_{2B} receptor and the formation of A_{2A}–A_{2B} receptor heteromers decreases both agonist affinity and function of the A_{2A} receptor. At present, the only reasonable hypothesis to explain the physiological role of the of CB_2R-GHSR1aHet and of $A_{2A}R$-A_{2B}RHet is that they are formed to suppress the functionality of one of the two receptors in the heteromer.

Heteromer formation in GPCRs appear as a means for achieving functional diversification [64–66], i.e., heteromers are functional units that behave differently than individually-expressed receptors. There have been few ghrelin receptor-containing heteromers reported in the literature. To our knowledge, GHSR1a may interact with the class A dopamine

D_1 and D_2 receptors or with class B secretin receptors [67,68]. It should be noted that cocaine interacting with sigma1-receptors modulate the GHSR1a-D_1 receptor interaction in hypothalamic cells to suppress appetite [69]. Results in the present paper show that CB_2R functionality is blunted by formation of the CB_2R-GHSR1aHet. This discovery is complemented by another result of the present study, namely that the expression of the CB_2R-GHSR1aHet in primary striatal neurons is altered in the progeny of obese mothers. The increased CB_2R-GHSR1aHets expression in samples from fetuses of mothers subjected to a HFD (when compared with samples from fetuses of mothers subjected to a STD) may explain some of the findings related to obesity and unbalanced diets. On the one hand, genetic inactivation of the gene coding for the receptor leads to improved insulin function but leads to age-related obesity [70]. On the other hand, neuroinflammation often occurs in obesity [71] and, interestingly, balancing the diet improves both maternal deficits and neuroinflammation in offspring [72]. These findings are relevant as cumulative research has found a higher proportion of obesity cases among children with obese parents [73]. Upregulation of the CB_2R-GHSR1aHet in offspring of mothers with HFD would indicate that already at birth these animals have CB_2R function compromised, i.e., the benefits of cannabinoids acting on striatal CB_2Rs would be lost by the blockade exerted by the ghrelin receptor. In addition, our results suggest that GHSR1a antagonists could have a double benefit: (i) reducing food intake and (ii) revert the detrimental effects of HFD on the functionality of striatal CB_2Rs.

4. Materials and Methods

4.1. Reagents

JWH-133, AM 630, Ghrelin and YIL 781 were purchased from Tocris Bioscience (Bristol, UK).

4.2. High Fat Diet Model Generation

C57BL/6J female mice were used for the experiments. All animals were housed on a 12 h/12 h light/ dark cycle in a temperature- and humidity-controlled room and were allowed free access to water and standard laboratory chow. C57BL/6J mice were randomly assigned to a high fat diet (HFD) (60% kcal from fat; catalog no. D12492, Research Diets, New Brunswick, NJ, USA) or standard diet (STD) (10% kcal from fat; catalog no. D12450B, Research Diets) for 60 days. Primary striatal neurons were obtained from fetuses of mother on STD or HFD. Pregnant animals were killed by cervical dislocation during the light phase. All animal procedures were performed in agreement with European guidelines (2010/63/EU) and approved by the University of Barcelona Ethical Committee, which reports to the regional Government (Protocol #9659; Generalitat de Catalunya, 24 May 2019).

4.3. Cell Culture and Transient Transfection

Human embryonic Kidney HEK-293T (lot 612968) cells were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA). They were amplified and frozen in liquid nitrogen in several aliquots. Cells from each aliquot were used until passage 19. HEK-293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Waltham, MA, USA) supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, MEM Non-Essential Amino Acid Solution (1/100) and 5% (*v*/*v*) heat-inactivated fetal bovine serum (FBS) (all supplements were from Invitrogen, Paisley, Scotland, UK) and maintained in a humid atmosphere of 5% CO_2 at 37 °C.

Cells were transiently transfected with the corresponding cDNAs using the PEI (Poly EthylenImine, Sigma-Aldrich, St. Louis, MO, USA) method as previously described [74,75]. 4 h after transfection, growth medium was replaced by complete medium. Experiments were carried out 48 h later.

To prepare primary striatal neurons, brains from fetuses of pregnant C57/BL6 mice were removed (gestational age: 17 days). Neurons were isolated as described in Hrad-

sky et al. [1] and plated at a confluence of 40,000 cells/0.32 cm^2. Briefly, the samples were dissected and, after careful removal of the meninges, digested for 20 min at 37 °C with 0.25% trypsin. Trypsinization was stopped by adding an equal volume of culture medium (Dulbecco's modified Eagle medium-F-12 nutrient mixture, Invitrogen). Cells were brought to a single cell suspension by repeated pipetting followed by passage through a 100 µm-pore mesh. Pelleted (7 min, 200× *g*) cells were resuspended in supplemented DMEM and seeded at a density of 3.5 × 10^5 cells/mL in 6-well plates. The day after, medium was replaced by neurobasal medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin and 2% (*v*/*v*) B27 medium (GIBCO). Neuronal cultures were used for assays after 15 days of culture. Using NeuN as a marker, the percentage of neurons in the cultures was >90%.

4.4. Expression Vectors

The human cDNAs for the CB_2, GHS1a and $GABA_B$ receptors cloned in pcDNA3.1 were amplified without their stop codons using sense and antisense primers. The primers harbored either unique BamHI and HindIII sites for GHS1a and $GABA_B$ receptors and BamHI and KpnI sites for the CB_2R. The fragments were subcloned to be in frame with an enhanced green fluorescent protein (GFP^2-N2, Clontech, Heidelberg, Germany), the Renilla luciferase protein (RLuc) (pRluc-N1; PerkinElmer, Wellesley, MA, USA) or the hemiproteins nRLuc8, cRLuc8, nVenus or cVenus (pcDNA3.1-nRLuc8, pcDNA3.1-cRLuc8, pcDNA3.1-nVenus and pcDNA3.1-cVenus) on the C-terminal end of the receptor to produce CB_2R-GFP^2, GHSR1a-GFP^2, GHSR1a-RLuc, $GABA_B$R-RLuc, GHSR1a-nRLuc, GHSR1a-cRLuc, CB_2R-nYFP and CB_2R-cYFP fusion proteins.

4.5. Immunofluorescence

HEK-293T cells transfected with cDNAs for CB_2R-GFP^2 and GHSR1a-RLuc were fixed in 4% paraformaldehyde for 15 min and then washed twice with PBS containing 20 mM glycine before permeabilization with the same buffer containing 0.2% Triton X-100 (5 min incubation). The samples were treated for 1 h with blocking solution (PBS containing 1% bovine serum albumin) and labeled with a mouse anti-RLuc (1/100; MAB4400, Millipore, Burlington, MA, USA) as primary antibody and a Cy3-conjugated anti-mouse IgG (1/200; 715-166-150; Jackson ImmunoResearch) as secondary antibody. The samples were washed several times and mounted with 30% Mowiol (Calbiochem, San Diego, CA, USA). Nuclei were stained with Hoechst (1/100). Samples were observed under a Zeiss 880 confocal microscope (Leica Microsystems, Wetzlar, Germany).

4.6. Bioluminescence Resonance Energy Transfer (BRET) Assay

HEK-293T cells growing in 6-well plates were transiently co-transfected with a constant amount of cDNA encoding for GHSR1a-Rluc and with increasing amounts of cDNA for CB_2R-GFP^2. For negative control, cells were co-transfected with a constant amount of cDNA encoding for $GABA_B$R-Rluc and with increasing amounts of cDNA for GHSR1a-GFP^2. 48 h post-transfection, cells were washed twice in quick succession with HBSS (137 mM NaCl; 5 mM KCl; 0.34 mM Na_2HPO_4; 0.44 mM KH_2PO_4; 1.26 mM $CaCl_2$; 0.4 mM $MgSO_4$; 0.5 mM $MgCl_2$ and 10 mM HEPES, pH 7.4) supplemented with 0.1% glucose (*w*/*v*), detached by gently pipetting and resuspended in the same buffer. To have an estimation of the number of cells per plate, protein concentration was determined using a Bradford assay kit (Bio-Rad, Munich, Germany) using bovine serum albumin dilutions for standardization. To quantify GFP^2-fluorescence expression, cells were distributed (20 µg protein) in 96-well microplates (black plates with a transparent bottom; Porvair, Leatherhead, UK). Fluorescence was read using a fluorimeter FluoStar Optima (BMG Labtechnologies, Offenburg, Germany) equipped with a high-energy xenon flash lamp, reading at 510 nm. GFP^2-fluorescence expression was determined as the fluorescence of the sample minus the fluorescence of cells only expressing protein-RLuc. For the $BRET^2$ measurements, the equivalent of 20 µg protein of cell suspension was distributed in 96-well

microplates (white plates; Porvair), and 5 μM Deep-Blue C was added (PJK GMBH, Kleinblittersdorf, Germany). 30 s after, readings were collected using a Mithras LB 940 (Berthold, Bad Wildbad, Germany), which allowed the integration of the signals detected in the short-wavelength filter at 410 nm (400–420 nm) and the long-wavelength filter at 510 nm (500–520 nm). To quantify receptor-RLuc expression, luminescence readings were collected 10 min after 5 μM coelenterazine H addition. The net BRET is defined as [(long-wavelength emission)/(short-wavelength emission)]-Cf where Cf corresponds to [(long-wavelength emission)/(short-wavelength emission)] for the RLuc construct expressed alone in the same experiment. The BRET curves were fitted assuming a single phase by a non-linear regression equation using the GraphPad Prism software (San Diego, CA, USA). BRET values are given as milli BRET units (mBU: 1000 × net BRET).

4.7. BRET with Bimolecular Luminescence and Fluorescence Complementation (BiLFC)

For BRET with bimolecular luminescence and fluorescence complementation (BiLFC) assays, HEK-293T cells were transiently transfected with a constant amount of cDNAs for GHSR1a-cRLuc and for GHSR1a-nRLuc cDNAs and increasing amounts of cDNAs for CB_2R-cYFP and for CB_2R-nYFP cDNAs. For negative controls, the cDNA for one of the fusion proteins was substituted by the corresponding empty vector (pcDNA3.1-cYFP) maintaining the other three plasmids. Protein determination was performed as described in the previous section. 48 h post-transfection, the equivalent of 20 μg protein of cell suspension was distributed in 96-well microplates. To quantify protein-YFP expression, fluorescence was read in a Mithras LB 940 equipped with a high-energy xenon flash lamp, using a 30 nm bandwidth excitation filter at 485 nm and an emission filter at 530 nm (510–550 nm). For BRET measurements, readings were collected 1 min after the addition of 5 μM coelenterazine H (Molecular Probes, Eugene, OR, USA) using a Mithras LB 940, which allows the integration of the signals detected in the short-wavelength filter at 485 nm and the long-wavelength filter at 530 nm. To quantify protein-RLuc expression, luminescence readings were obtained 10 min after 5 μM coelenterazine H addition using a Mithras LB 940.

4.8. cAMP Determination

HEK-293T cells transfected with the cDNAs for CB_2R (0.5 μg) and/or GHSR1a (1 μg) and neuronal primary cultures were plated in 6 well plates. Two hours before initiating the experiment, neuronal culture or HEK-293T cell-culture media were exchanged to non-supplemented DMEM medium. Then, cells were detached, re-suspended in non-supplemented medium containing 50 μM zardaverine, and plated in 384-well microplates (2500 cells/well). Cells were pretreated (15 min) with the corresponding antagonists (1 μM AM 630 for CB_2R or 1 μM YIL 781 for GHSR1a) or vehicle and stimulated with agonists (200 nM JWH-133 for CB_2R or 200 nM ghrelin for GHSR1a) (15 min) before the addition of 0.5 μM forskolin or vehicle. Finally, reaction was stopped by addition of the Eu-cAMP tracer and the ULight-cAMP monoclonal antibody prepared in the "cAMP detection buffer" (PerkinElmer). All steps were performed at 25º. Homogeneous time-resolved fluorescence energy transfer (HTRF) measures were performed after 60 min incubation using the Lance Ultra cAMP kit (PerkinElmer, Waltham, MA, USA). Fluorescence at 665 nm was analyzed on a PHERAstar Flagship microplate reader equipped with an HTRF optical module (BMG Lab technologies, Offenburg, Germany).

4.9. MAPK Phosphorylation Assays

To determine MAP kinase 1/2 (ERK1/2) phosphorylation, striatal neurons were plated in transparent Deltalab 96-well plates and kept in the incubator for 15 days. 2 to 4 h before the experiment, the medium was replaced by serum starved medium. Next, the cells were pre-treated at 25 °C for 10 min with antagonists (1 μM AM 630 for CB_2R or 1 μM YIL 781 for GHSR1a) or vehicle and stimulated for an additional 7 min with selective agonists (200 nM JWH-133 for CB_2R or 200 nM ghrelin for GHSR1a). Then, neurons were washed twice

with cold PBS before the addition of 30 μL/well "Ultra lysis buffer" -PerkinElmer- (20 min treatment). Afterwards, 10 μL of each supernatant were placed in white ProxiPlate 384-well plates and ERK1/2 phosphorylation was determined using an AlphaScreen® SureFire® kit (PerkinElmer), following the instructions of the supplier, and using an EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). The reference value (100%) was the value achieved in the absence of any treatment (basal). The ligands effect was given in percentage with respect to the basal value.

On the other hand, HEK-293T cells were cultured into 25 cm^2 flasks and transfected with the cDNAs for CB_2R (0.5 μg) and/or GHSR1a (1 μg). Two hours before initiating the experiment, cell-culture medium was exchanged to serum-starved DMEM medium. The cells were, subsequently, pre-treated at 25 °C for 10 min with antagonists (1 μM AM 630 for CB_2R or 1 μM YIL 781 for GHSR1a) or vehicle and stimulated for an additional 7 min with selective agonists (200 nM JWH-133 for CB_2R or 200 nM ghrelin for GHSR1a). Stimulation was ended by a rapid rinse with ice-cold PBS, and the cell lysis was performed by the addition of 250 μL of ice-cold lysis buffer. Cellular debris were removed by centrifugation at 13,000× *g* for 5 min at 4 °C, and protein was quantified by the bicinchoninic acid method using bovine serum albumin dilutions as standard. To determine the level of ERK1/2 phosphorylation, equivalent amounts of protein (10 μg) were separated by electrophoresis on a denaturing 10% SDS-polyacrylamide gel and transferred onto PVDF-FL membranes. Membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) for 60 min and probed with a mixture of a mouse anti-phospho-ERK1/2 antibody (1:2500, Sigma-Aldrich) and rabbit anti-ERK1/2 antibody (1:40,000, Sigma-Aldrich) for 2 h. The 42 and 44 kDa bands corresponding to ERK 1 and ERK 2 were visualized by the addition of a mixture of IRDye 800 (anti-mouse) antibody (1:10,000, Sigma-Aldrich) and IRDye 680 (anti-rabbit) antibody (1:10,000, Sigma-Aldrich) for 1 h and scanned by the Odyssey infrared scanner (LI-COR Biosciences). Band densities were quantified using the scanner software and exported to Microsoft Excel. The level of phosphorylated ERK 1/2 was normalized for differences in loading using the total ERK1/2 protein band intensities.

4.10. Intracellular Calcium Mobilization

HEK-293T cells were co-transfected with cDNAs for CB_2R (0.5 μg) and/or GHSR1a (1 μg) in the presence of 1 μg cDNA for the calmodulin-based calcium GCaMP6 sensor. Forty-eight hours after transfection, cells were detached using Mg^{2+}-free Locke's buffer pH 7.4 (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5.6 mM glucose and 5 mM HEPES) supplemented with 10 μM glycine. 1500 cells per well were plated in 96-well black, clear bottom, microtiter plates. Then, cells were incubated for 10 min with the CB_2R and GHSR1a antagonists (1 μM AM 630 or 1 μM YIL 781), and subsequently stimulated with selective agonists (200 nM JWH-133 or 200 nM ghrelin). Upon excitation at 488 nm, real-time 515 nm fluorescence emission due to calcium-ion complexed GCaMP6 was recorded on the EnSpire® Multimode Plate Reader (every 5 s, 100 flashes per well).

4.11. Proximity Ligation Assays (PLAs)

Physical interaction between CB_2R and GHSR1a were detected using the Duolink in situ PLA detection Kit (OLink; Bioscience, Uppsala, Sweden) following the instructions of the supplier. Primary neurons were grown on glass coverslips, fixed in 4% paraformaldehyde for 15 min, washed with PBS containing 20 mM glycine to quench the aldehyde groups and permeabilized with the same buffer containing 0.05% Triton X-100 (20 min). Then, samples were extensively washed with PBS. After 1 h incubation at 37 °C with the blocking solution in a pre-heated humidity chamber, primary cultures were incubated overnight in the antibody diluent medium with a mixture of equal amounts of mouse anti-CB_2R (1/100; sc-293188, Santa Cruz Technologies, Dallas, TX, USA) and rabbit anti-GHSR1a (1/100; ab95250, Abcam, Cambridge, United Kingdom) to detect CB_2R–GHSR1a complexes. Neurons were processed using the PLA probes detecting primary antibodies (Duolink II PLA probe plus and Duolink II PLA probe minus) diluted in the antibody

diluent solution (1:5). Ligation and amplification were done as indicated by the supplier. Samples were mounted using the mounting medium with Hoechst (1/100; Sigma-Aldrich) to stain nuclei. Samples were observed in a Leica SP2 confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with an apochromatic 63× oil immersion objective (N.A. 1.4) and 405 and a 561 nm laser lines. For each field of view, a stack of two channels (one per staining) and four Z stacks with a step size of 1 µm were acquired. The number of neurons containing one or more red spots versus total cells (blue nucleus) was determined, and the unpaired Student's t-test was used to compare the values (red dots/cell) obtained in the two groups.

Supplementary Materials: Supplementary materials can be found at https://www.mdpi.com/article/10.3390/ijms22168928/s1.

Author Contributions: Conceptualization, G.N. and R.F.; Data Curation, R.F.; Formal Analysis, J.L., A.L. and G.N.; Investigation, J.L., A.L., D.A.Z., C.M. and G.N.; Methodology, J.L., A.L. and R.R.-S.; Project Administration, R.R.-S.; Resources, C.M., N.C., G.N. and R.F.; Software, R.R.-S.; Supervision, G.N. and R.F.; Validation, G.N.; Writing—Original Draft, G.N. and R.F.; Writing—Review and Editing, J.L., A.L., C.M., R.R.-S., N.C. and G.N. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Ethics Committee (Protocol #9659; Generalitat de Catalunya).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available upon reasonable request to corresponding authors. A Western blot compilation file accompanies the submission and may be found via IJMS.

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3.8 Expression of GPR55 and either cannabinoid CB_1 or CB_2 heteroreceptor complexes in the caudate, putamen, and accumbens nuclei of control, parkinsonian, and dyskinetic non-human primates.

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Els endocannabinoides són neuromoduladors que actuen sobre els receptors CB_1 i CB_2, representant un gran potencial terapèutic per malalties neurodegeneratives. Aquests compostos també regulen l'activitat de GPR55, un receptor que interacciona amb CB_1R i CB_2R. La nostra hipòtesi consisteix en que aquests heteròmers poden ser considerats com a diana per tractar la malaltia de Parkinson (PD). En aquest article es van determinar els nivells d'expressió de l'heteròmer CB_1R-GPR55 i CB_2R-GPR55 en estriat de macacos Parkinsonians amb i sense discinèsia induïda per Levodopa. D'aquesta manera vam realitzar l'assaig de PLA en teixit d'animals tractats amb MPTP, detectant la presència de complexos heteromèrics tant en animals control com discinètics. Comparant amb els nivells d'expressió basal de l'heteròmer CB_1R-GPR55 vam observar un increment de l'expressió en ganglis basals dels animals Parkinsonians. Aquest increment de CB_1R-GPR55 va veure's revertit en animals discinètics tractats amb Levodopa. En relació a l'heteròmer CB_2R-GPR55, vam obtenir resultats molt similars: Nivells d'expressió equivalents en animals control i discinètics, amb un increment dels nivells d'expressió en els animals tractats amb MPTP. En conjunt, aquest increment de l'expressió dels heteròmers entre receptors cannabinoides i GPR55, apunta a emprar aquests complexos de GPCR com a potencials dianes per tractar el Parkinson amb una teràpia no dopaminèrgica.

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ORIGINAL ARTICLE

Expression of GPR55 and either cannabinoid CB_1 or CB_2 heteroreceptor complexes in the caudate, putamen, and accumbens nuclei of control, parkinsonian, and dyskinetic non-human primates

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Abstract

Endocannabinoids are neuromodulators acting on specific cannabinoid CB_1 and CB_2 G-protein-coupled receptors (GPCRs), representing potential therapeutic targets for neurodegenerative diseases. Cannabinoids also regulate the activity of GPR55, a recently "deorphanized" GPCR that directly interacts with CB_1 and with CB_2 receptors. Our hypothesis is that these heteromers may be taken as potential targets for Parkinson's disease (PD). This work aims at assessing the expression of heteromers made of GPR55 and CB_1/CB_2 receptors in the striatum of control and parkinsonian macaques (with and without levodopa-induced dyskinesia). For this purpose, double blind in situ proximity ligation assays, enabling the detection of GPCR heteromers in tissue samples, were performed in striatal sections of control, MPTP-treated and MPTP-treated animals rendered dyskinetic by chronic treatment with levodopa. Image analysis and statistical assessment were performed using dedicated software. We have previously demonstrated the formation of heteromers between GPR55 and CB_1 receptor (CB_1-GPR55_Hets), which is highly expressed in the central nervous system (CNS), but also with the CB_2 receptor (CB_2-GPR55_Hets). Compared to the baseline expression of CB_1-GPR55_Hets in control animals, our results showed increased expression levels in basal ganglia input nuclei of MPTP-treated animals. These observed increases in CB_1-GPR55_Hets returned back to baseline levels upon chronic treatment with levodopa in dyskinetic animals. Obtained data regarding CB_2-GPR55_Hets were quite similar, with somehow equivalent amounts in control and dyskinetic animals, and with increased expression levels in MPTP animals. Taken together, the detected increased expression of GPR55-endocannabinoid heteromers appoints these GPCR complexes as potential non-dopaminergic targets for PD therapy.

Keywords G-protein coupled receptor (GPCR) heteromer · Levodopa · Parkinson's disease · Proximity ligation assay (PLA) · Striatum

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Abbreviations

CB_1-GPR55_Hets	Complexes formed by CB_1 and GPR55 receptors
CB_1R	CB_1 receptors
CB_2-GPR55_Hets	Complexes formed by CB_2 and GPR55 receptors
CB_2R	CB_2 receptors
CLAHE	Contrast Limited Adaptive Histogram Equalization
CNS	Central nervous system
GPCRs	G-protein-coupled receptors
LPI	Lysophosphatidylinositol
PD	Parkinson's disease
PLA	In situ proximity ligation assay
SN	Substantia nigra

Introduction

Endocannabinoids and their specific G-protein-coupled receptors (GPCRs), CB_1 and CB_2, are known to regulate neurotransmission and synaptic plasticity in the basal ganglia [see (Giuffrida and Seillier 2012) for review]. Consequently, synthetic or natural (e.g. Sativex® or Epidiolex®) cannabinoids have been proposed as novel drug compounds for Parkinson's disease (PD) treatment. Neuroprotective and/or anti-symptomatic mechanisms of cannabinoids may result from activation/blockade of CB_1 receptors (CB_1R, mainly expressed in neurons), of CB_2 receptors (CB_2R, expressed in microglia and in some neuronal populations (Onaivi 2006; Onaivi et al. 2006; Brusco et al. 2008; Lanciego et al. 2011)), or be receptor-independent [see (Fernández-Ruiz et al. 2011) for review].

Despite lack of consensus, GPR55 was deorphanized to be preliminarily considered as a receptor for lysophosphatidylinositol (LPI) (Oka et al. 2007). GPR55 is widely expressed in the central nervous system (CNS). Thus, mRNA transcripts were found by Northern blot in human caudate and putamen nuclei, and by in situ hybridization in hippocampus, thalamic nuclei and midbrain regions of rodent brains (Sawzdargo et al. 1999; Wu et al. 2010). Moreover, it is also expressed in microglia (Pietr et al. 2009), similarly to CB_2R (Núñez et al. 2004). GPR55 is still poorly characterized due to a variety of factors. Firstly identified as a putative cannabinoid receptor because of similar amino acid sequence in the binding region (Baker et al. 2006), extensive characterization at GlaxoSmithKline and AstraZeneca pharmaceutical companies led to propose that the receptor was responsible for the blood pressure lowering properties of cannabinoids. However, whereas GPR55 seems to be activated by endogenous or exogenous (plant and synthetic) cannabinoids, research studies using GPR55 knockout mice showed that the receptor does not mediate vasodilator effects (Johns et al. 2009). Interestingly, phenotypic characterization of these animals guided to the discovery of receptor involvement in motor control (Wu et al. 2013). In fact, the knockout mouse showed impaired motor coordination providing the basis of a relevant role of the receptor in basal ganglia neural circuits.

$G_{i/o}$ are the cognate heterotrimeric proteins of cannabinoid receptors (Alexander et al. 2017) (https://www.guidetopharmacology.org/). In the case of GPR55, these proteins are of the G_q/G_{11} and G_{12}/G_{13} families (Alexander et al. 2017) (https://www.guidetopharmacology.org/) leading to a quite complex pharmacology. In this sense, not only cytosolic calcium increases resulting from receptor activation are slower than for other G_q-coupled receptors but it has been reported that the receptor activation may engage multiple signaling pathways (Henstridge et al. 2010). Among the mechanisms underlying such pleiotropic actions, receptor heteromerization is included. Dimer/oligomerization is now considered a general phenomenon for practically all cell surface receptors. Noteworthy, we have previously demonstrated that CB_1R and CB_2R may heteromerize with GPR55 and that heteromerization affects receptor functionality (Balenga et al. 2014; Martínez-Pinilla et al. 2014). Indeed, as a general rule, GPCR heteromers are currently considered as novel molecular entities, with signaling and ligand characteristics different than those for each GPCR when considered individually (Ferré et al. 2009; Franco et al. 2016, 2018).

The aim of this study was to assess, from a cell biology and anatomical perspective, whether the striatal expression of GPR55-containing heteromers is altered in Parkinsonism and in levodopa-induced dyskinesia. For this purpose, in situ proximity ligation (PLA), a technique instrumental for detecting receptor-receptor interactions and their precise anatomical localization, was used in samples from the MPTP monkey PD model. The results obtained showed that whereas both heteroreceptor complexes increase in caudate, putamen and accumbens nuclei of parkinsonian animals, chronic levodopa treatment tuned down these increases back to normal expression levels.

Material and methods

This manuscript adheres to the guidelines detailed in (Curtis et al. 2018). Studies were designed to generate groups of equal sizes, using randomization and blinded analysis. Immunological based assays were conducted according to the guidelines detailed in (Alexander et al. 2018).

Generation of parkinsonian animals and levodopa treatment

A total of 9 naïve young adults male *Macaca fascicularis* primates (body weight 3.5–4.7 kg) were used in this study.

Animal handling was conducted at all times in accordance with the European Council Directive 2010/63/UE as well as in keeping with current Spanish legislation (RD53/2013). The experimental design was reviewed and approved by the Ethical Committee for Animal Testing of the University of Navarra (Protocol Ref: 009/12). All animals were captive-bred and supplied by R. C. Hartelust (Leiden, The Netherlands).

To induce a bilateral parkinsonian syndrome, 6 monkeys were systemically administered 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, Sigma Aldrich, St. Louis, USA) (Rico et al. 2010). Animals received a weekly injection of MPTP (0.2 mg/kg i/v; accumulated doses ranging from 5 to 7 mg/kg) until reaching a non-reversible, stable parkinsonian syndrome. The severity of the MPTP-induced Parkinsonism was evaluated using a clinical rating scale (Kurlan et al. 1991). This scale rates parkinsonian motor symptoms such as resting tremor (0–3), action or intention tremor (0–3), facial expression (0–3), posture (0–2), balance coordination (0–3), gait (0–3), bradykinesia (0–4), defense reaction (0–2), and gross motor skills of the upper (0–3) or lower limb (0–3); using this scale the highest severity corresponds to the maximal score, 29. Once primates reached a score of at least 21 points, the treatment was discontinued for a wash-out period of 2 months before performing any further assay. At the end of the stabilization period, the PD scores were in the 21–24 range. Dyskinesia was induced in 3 animals scoring 21, 22 and 24 points in the Kurlan's scale through oral chronic administration of levodopa/benserazide (200:50 Roche) at a dose of 25 mg/kg daily. Levodopa effects were monitored by motor scoring (Kurlan et al. 1991) and by assessing the "off/on" states and the duration of the "on" response (Lanciego et al. 2008). Dyskinesias were evaluated using the scale included in the "Core Assessment Program for Intracerebral Transplantation for Parkinson's disease" (Langston et al. 1992), subsequently modified and validated for the assessment of dyskinesias in patients (Goetz et al. 1994). Accordingly, they were rated as severe when dyskinesias were continuous, generalized and perturbing motor behavior; moderate if were presented during most of the "on" period without interfering with voluntary movements; and mild, when happened only under a stress challenge. By the time of sacrifice, all 3 monkeys treated with levodopa showed severe dyskinesia. These primates entered in the "on" state 30 min post-levodopa oral delivery and the duration of the "on" period was maintained for 2.5–3 h. A mild dyskinetic syndrome was displayed by the end of the first month of treatment, whereas severe dyskinetic symptoms appeared later and remained stable until sacrifice. Levodopa-treated animals were euthanized in the "on" state (peak-of-dose dyskinesia).

Tissue processing

Animals were anesthetized with an overdose of 10% chloral hydrate and perfused transcardially. The perfusate was made of a saline Ringer solution followed by 3000 mL of fixative solution containing 4% paraformaldehyde and 0.1% glutaraldehyde in 0.125 M phosphate buffer (PB) at neutral pH. Perfusion was continued with 1000 mL of a cryoprotective solution made of 10% glycerin and 1% of dimethyl sulfoxide (DMSO) in 0.125 M PB, pH 7.4. Once perfusion was completed, the brain was removed and stored in a cryoprotective solution containing 20% glycerin and 2% DMSO for 48–72 h. Finally, 10 series of frozen coronal adjacent sections (40 µm-thick) were obtained in a sliding microtome. These series were used for (1) in situ PLA for CB_1-GPR55_Hets counterstained with Topro-3, (2) in situ PLA for CB_2-GPR55_Hets counterstained with Topro-3, (3) immunohistochemical detection of tyrosine hydroxylase, and (4) control stains assessing the specificity of the PLA method for the detection of CB_1-GPR55_Hets. The remaining 6 series of sections were stored at − 80 ºC as backup materials for further processing, if needed. Areas of interest for PLA stains (pre-and post-commissural caudate, putamen and accumbens nuclei), were selected according to the stereotaxic atlas of (Lanciego and Vázquez 2012).

In situ proximity ligation assays (PLA)

PLA allows the ex vivo detection of molecular interactions between two endogenous proteins. Assays were performed in samples from 3 monkeys per group (control, parkinsonian and dyskinetic). Proximity probes consisted of affinity-purified antibodies modified by covalent attachment of 5′ end of various nucleotides to each primary antibody. PLA probes were prepared by conjugating a rabbit anti-CB_1 antibody (Ref: PA1-745, Thermo Scientific, Rockford, USA) with a PLUS oligonucleotide (Duolink In Situ Probemaker PLUS ref: DUO92009; Sigma) and a rabbit anti-GPR55 antibody (Ref: 10224; Cayman Chemicals; Ann Arbor, MI, USA), raised against the human 207–219 sequence, with a MINUS oligonucleotide (Duolink In Situ Probemaker MINUS Ref: DUO92010; Sigma) according to manufacturer's guidelines. For CB_2-GPR55_Hets detection, a rabbit anti-CB_2 antibody (Ref: 1001012; Cayman Chemicals) was conjugated with a PLUS oligonucleotide, whereas the rabbit anti-GPR55 antibody was conjugated as described above. Tissue sections containing the caudate and putamen nuclei were used for the detection of either CB_1-GPR55_Hets or CB_2-GPR55_Hets with the PLA technique. Briefly, free-floating sections were incubated for 1 h at 37 ºC with the blocking solution, followed by overnight incubation at 4 ºC with the PLA probe-linked antibodies described above (at a final concentration

of 75 µg/mL). After washing with buffer A (wash buffer A, ref: DUO82047; Sigma), samples were immersed for 1 h in a 1:400 solution of Topro-3 (ref: T3605; Molecular Probes-Invitrogen). GPCR heteromers were detected using the Duolink II in situ PLA detection kit (Duolink In Situ Detection Reagents Red ref: DUO92008; Sigma). Sections were washed with buffer A and incubated with the ligation solution for 1 h at 37 ºC in a humidity chamber. After washing with buffer A again, sections were incubated with the amplification solution for 100 min at 37 ºC and then washed with buffer B (Wash buffer B, Ref: DUO82048; Sigma). Sections were finally mounted using an aqueous mounting medium. Appropriate negative control assays were carried out to ensure that there was a lack of non-specific labeling and amplification.

Statistical analysis on the receptor heteromer densities were conducted using dedicated software (Duolink Image Tool, Ref: DUO90806; Sigma-Olink). This software has been developed for quantification of PLA signals and cell nuclei in images generated from fluorescent confocal microscopy. Regarding selected regions of interest (ROIs), and for each field of view, a stack of two channels (one per staining) and 9–15 Z stacks with a step size of 1 µm were acquired. A quantification of cells containing one or more red spots *versus* total cells (blue nucleus), and the ratio *r* (number of red spots/cell) in cells containing spots were determined considering a total of 300–400 cells from six different fields within basal ganglia from three different animals per group using the Andy's algorithm and the procedure detailed elsewhere (Law et al. 2017). Nuclei and red spots were counted on the maximum projections of each image stack. After getting the projection, each channel was processed individually. The nuclei were segmented by filtering with a median filter, subtracting the background, enhancing the contrast with the Contrast Limited Adaptive Histogram Equalization (CLAHE) plug-in and finally applying a threshold to obtain the binary image and the ROI around each nucleus. Red spots images were also filtered and thresholded to obtain the binary images. Red spots were counted in each of the ROI obtained in the nuclei images.

It should be noted that the experiments were achieved in a blind basis; the experimenter was not aware of the label and the conditions (control, parkinsonian or dyskinetic) when images were taken. Moreover, the experimenter who made the analysis did not know the exact nature of the analyzed samples.

Data analysis

Data collected in samples from 3 animals per group (control, parkinsonian and dyskinetic) were the mean ± SEM. One- or two-way ANOVA followed by Bonferroni's post hoc multiple comparison tests were used to compare the values (% of positive cells or *r* spots/cell) obtained for each pair of receptors in different disease states or in different striatal regions. The normality of populations and homogeneity of variances were tested prior to ANOVA. Differences were considered significant when $p \leq 0.05$. Statistical analysis were carried out with GraphPad Prism software version 5 (San Diego, CA, USA). Outlier tests were not used; all data points were used for analysis.

Results

Expression of CB_1R-GPR55 heteromers in control, parkinsonian and dyskinetic macaques

The PLA technique enables the detection of receptor-receptor interactions in a native system. Red clusters detected in PLA assays proved the occurrence of complexes formed by CB_1 and GPR55 receptors (CB_1-GPR55_Hets) in all samples analyzed. PLA data was collected from different fields in samples of pre- and post-commissural basal ganglia input nuclei, which included caudate, putamen and accumbens. The areas that were considered in the study are delineated in Fig. 1 and the color code was used in subsequent figures.

A representative image of results of CB_1-GPR55_Het expression in all analyzed areas is given in Fig. 2, where the increase in the amount of red signal in samples from parkinsonian animals is already noticed. Analysis using the Andy's algorithm (see "Materials and methods") confirmed that the number of red dots per cell was significantly higher in the parkinsonian conditions. Remarkably, the level of expression in dyskinetic animals was similar to that found in control animals (Fig. 3a). These quantitative findings (higher density of CB_1-GPR55_Het clusters) in parkinsonian conditions were similar in post- and pre-commissural areas (Fig. 3b). Also relevant was that all regions showed significant increase of CB_1-GPR55_Het expression in samples from the PD animal model (Fig. 3c).

Expression of CB_2R-GPR55 heteromers in control, parkinsonian and dyskinetic macaques

Analogous analysis performed in the same regions but addressing CB_2-GPR55_Het expression led to similar findings. Under the blind-like conditions of the experiments we discovered that in every animal in a given group the expression was similar in all analyzed regions, and that there was an increase in samples from parkinsonian animals that returned to "normal" after levodopa-induced dyskinesia (Figs. 4 and 5). The results were similar in pre- and post-commissural areas, and all regions showed significant increase of CB_2-GPR55_Het expression in samples from the PD animal model (Fig. 5b, c). A difference was the higher

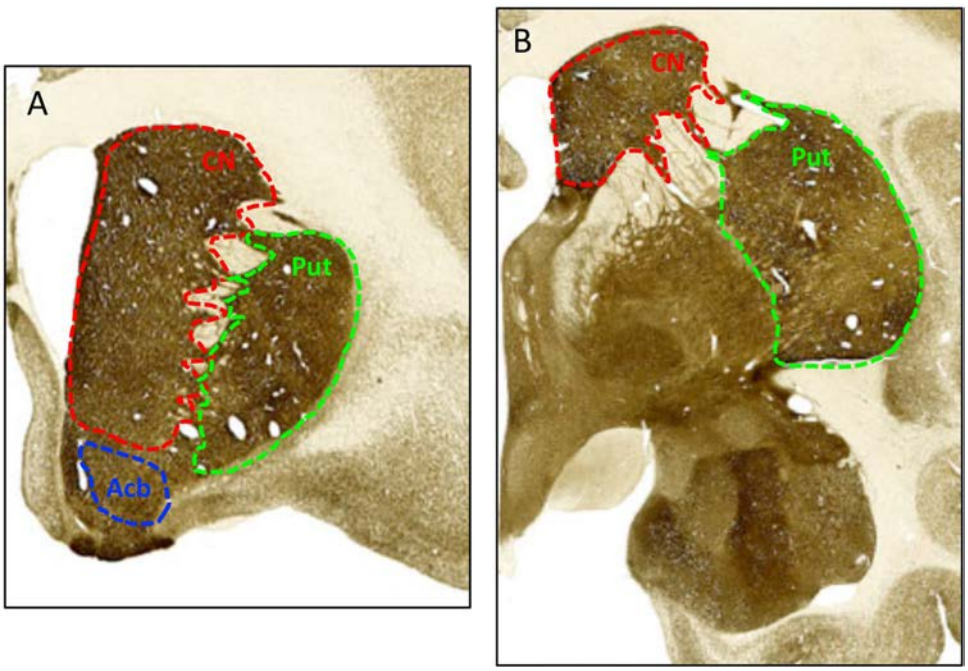

Fig. 1 Delineation of pre- (**a**) and postcommissural (**b**) areas of interest in the *Macaca fascicularis* brain. Caudate (CN, red), putamen (Put, green) and accumbens (Acb, blue) are indicated in a color code that will be used in Figs. 3c and 5c

number of red clusters per cell if compared with the results concerning the CB_1-GPR55_Het.

Discussion

The present study was aimed at knowing whether Parkinsonism correlates with altered expression of CB_1-GPR55_Hets and/or CB_2-GPR55_Hets. The results here reported are quite clear in showing that the expression of both heteromers was significantly higher in MPTP-treated monkeys in all analyzed nuclei (caudate, putamen and accumbens). Increases of striatal expression in PD models have been similarly demonstrated for other heteromers (Rodríguez-Ruiz et al. 2017). Also interesting is the disruption of heteromers here shown when parkinsonian animals are rendered dyskinetic after chronic levodopa treatment; a similar alteration has been demonstrated for adenosine A_{2A}/dopamine D_2/cannabinoid CB_1 heteroreceptor complexes (Armentero et al. 2011; Pinna et al. 2014; Bonaventura et al. 2014). Disorganization of dopamine receptor heteromers has been associated to differential effects exerted by cocaine action (Perreault et al. 2016). The trend is not unspecific as there are cases of increased heteromer expression in dyskinesia. One relevant example, due to the possibility of alleviate dyskinesias by dopamine D_3 receptor blockade, is the increase of striatal expression of the D_3 receptor itself and of dopamine D_1–D_3 receptor heteromers in dyskinetic macaques (Marcellino et al. 2008; Fuxe et al. 2015; Farré et al. 2015).

In a recent report, we have addressed the expression of CB_1-GPR55_Hets in the basal ganglia input nuclei both in interneurons and in projection neurons in samples from naïve *Macaca fascicularis*. By using a tracer, delivered to the internal or to the external subdivisions of the globus pallidus, and immunohistochemical techniques we showed that heteromers are expressed in both striatofugal projection neuron types. CB_1-GPR55_Hets were not found in dendrites but in the cell somata. Triple immunofluorescent stains identified heteromers in parvalbumin, calretinin and nitric oxide positive interneurons. In contrast, cholinergic interneurons lacked heteromer expression (Martínez-Pinilla et al. 2020).

For drug development, the CB_1R was first considered as better alternative than CB_2R due to its higher expression in neurons. However, potential psychotropic effects of drugs acting on the CB_1R have led to focus more on the CB_2R. Our results would indicate that, to counteract the increase in receptor expression associated to PD, the most appropriate intervention would be the use of CB_1R and/or GPR55 antagonists. While GPR55 has been poorly addressed in drug discovery, the serious side effects of rimonabant, a CB_1R antagonist, led to regulatory bodies to withdrawn this anti-obesity drug (Christensen et al. 2007). Alternatively, the benefits of targeting CB_2R are mainly based on the upregulation of the receptor in activated glial cells that is concomitant with dopaminergic neurodegeneration (Price et al. 2009; Palomo-Garo et al. 2016; Navarro et al. 2016). Our results open perspectives for the non-dopaminergic management of PD patients.

As indicated in the introduction, the presence of CB_2R in neurons is scarce. Although the receptor is significantly expressed in some pallidal, cerebellar and cortical neurons, striatal ones have negligible levels. Consistent with

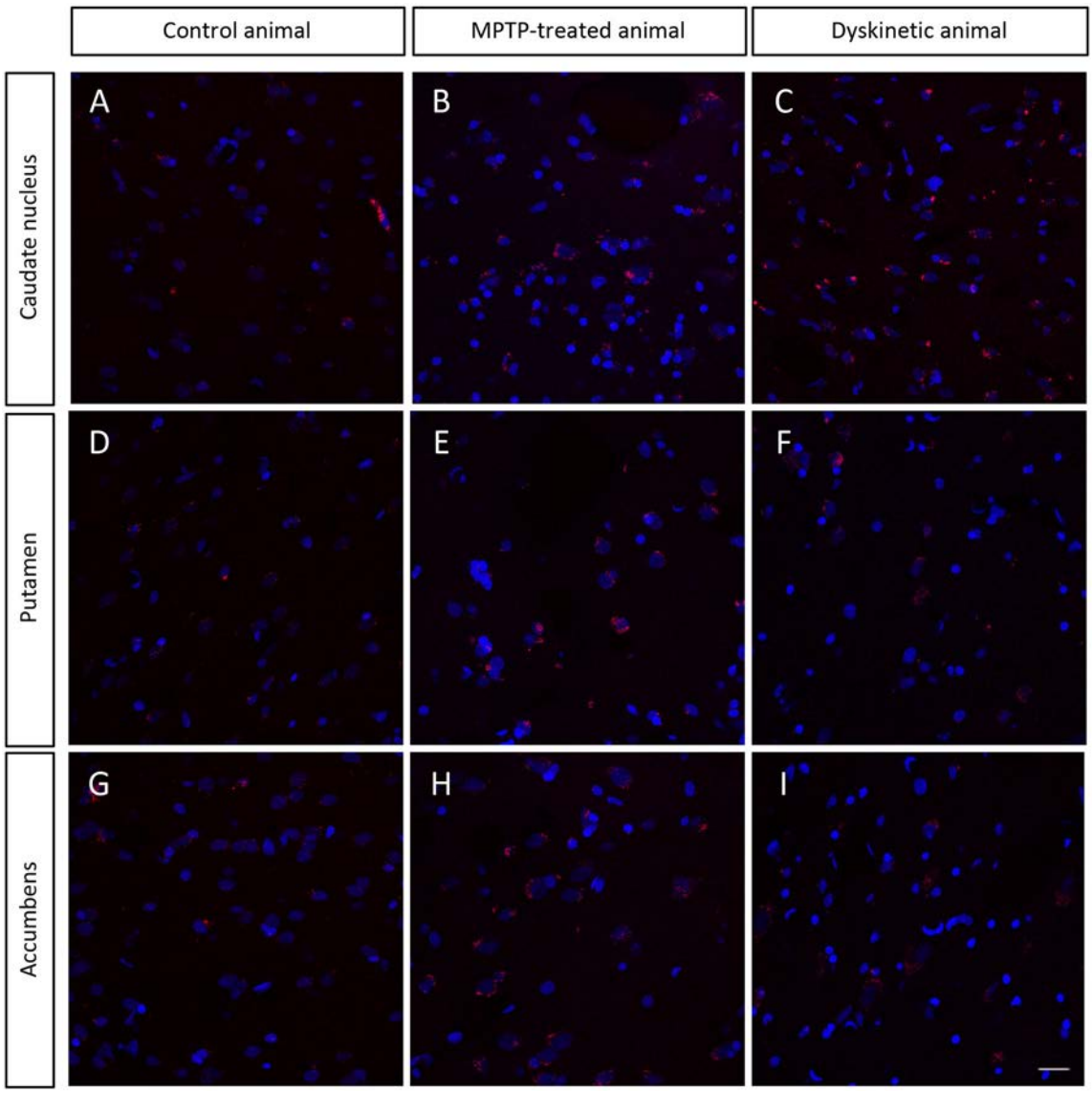

Fig. 2 CB_1-GPR55_Hets detected by the in situ proximity ligation assay (PLA) in striatal regions of the monkey brain. Representative images showing PLA label for CB_1-GPR55_Hets in the regions of interest: caudate, putamen and accumbens (see Fig. 1 legend). Experiments were performed in samples from the 3 animal groups namely control, parkinsonian (MPTP-treated) and dyskinetic. Quantification of red dots/cell is shown in the bar graphs displayed in Fig. 3

neuroinflammation occurring in PD and with upregulation of the receptor in microglia, it is likely that the increase in CB_2-GPR55_Hets in parkinsonian animals is due to activated (reactive) microglia. Cannabinoids are important regulators of neuroinflammation associated with neurodegenerative diseases (Palazuelos et al. 2009; Stella 2010; Kong et al. 2014; Janssen et al. 2016, 2018; Tao et al. 2016; Attili et al. 2019). Approaches to skew the phenotype of microglial cells from the M1 proinflammatory to the M2 neuroprotective (Franco and Fernández-Suárez 2015) are being actively sought. In this sense, there is the possibility for cannabinoids to regulate the M1/M2 balance (Galve-Roperh et al. 2008; Stella 2009; Aso and Ferrer 2014; Mecha et al. 2015, 2016; Tao et al. 2016). Interestingly, there are already data showing that GPR55 may be a non-dopaminergic therapeutic target in PD (Wu et al. 2013; Celorrio et al. 2017).

The above-commented study of identification of neurons expressing CB_1-GPR55_Hets in the striatum of naïve animals also concluded that the heteromers are not only expressed on cell surface but in intracellular structures (Martínez-Pinilla et al. 2020). In this regard, it has been reported that the CB_1R may be present in mitochondria and may mediate regulation of mitochondrial function by cannabinoids (Bénard et al. 2012; Hebert-Chatelain et al. 2014; Melser et al. 2017; Gutiérrez-Rodríguez et al. 2018). There are huge technical challenges to detect heteromers, either CB_1-GPR55_Hets

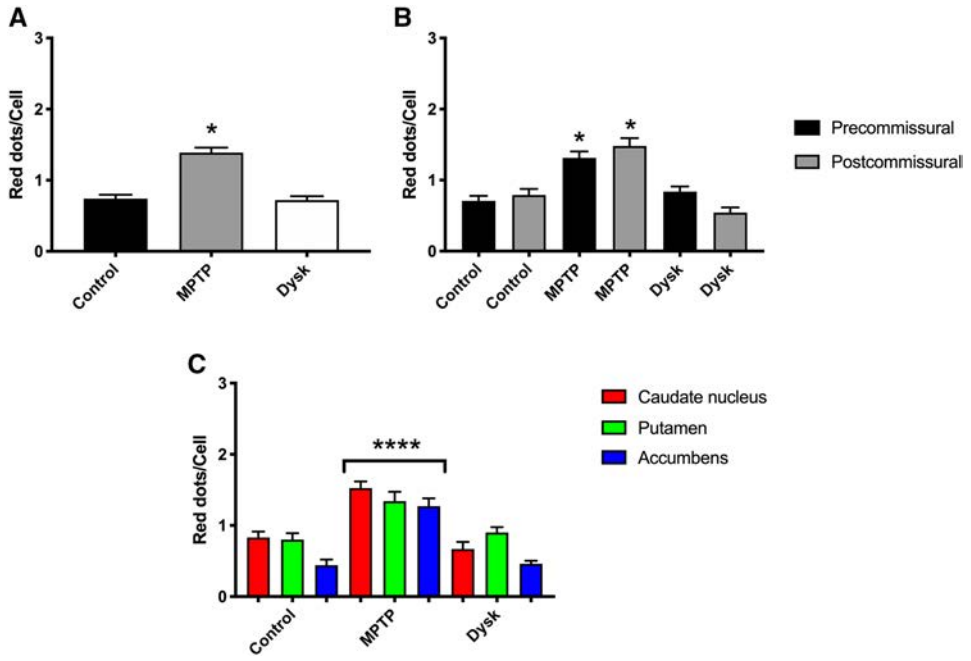

Fig. 3 Quantitation of CB_1-GPR55_Hets performed according to the Andy's algorithm (see "Materials and methods") in all striatal areas (caudate, putamen and accumbens nuclei) and in samples from control, MPTP-treated and dyskinetic animals. **a** Quantitation adding up the PLA label in all regions and both in pre- and post-commissural locations. **b** Quantitation in pre- *versus* quantitation in post-commissural areas. **c** Quantitation in each area (caudate, putamen and accumbens nuclei) following the color code indicated in Fig. 1. Data are the mean ± SEM (150 data points from 3 sections, 6 fields and $n = 3$ per group). Significant differences were analyzed by a one- or two-way ANOVA followed by post-hoc Bonferroni's test. $^*p < 0.05$ compared with control; in **c**: $^{****}p < 0.0001$: every region in MPTP-treated animals *versus* both control and dyskinetic

or CB_2-GPR55_Hets, but it is worth knowing whether cannabinoids may affect neural cell energy production via these GPR55-containing receptor complexes and whether expression alterations during PD correlate with changes in the expression of heteromers at the mitochondrial level. Indeed, altered mitochondrial functionality is raising as a culprit in sporadic PD cases (Macdonald et al. 2018; Ben-Shachar 2019). Therefore, targeting intracellular receptors by plasma-membrane-permeant cannabinoids could be a good possibility to revert mitochondrial malfunctioning and prevent neuronal death.

The physiological role of the interactions, i.e. the properties of the heteromers in terms of signaling, must be taken into account in order to design the most effective approach. In this sense, CB_2R agonists or allosteric modulators such as cannabidiol, a very safe compound, may be useful (Fernández-Ruiz et al. 2011; Pertwee 2012; Noel 2017; Martínez-Pinilla et al. 2017; Navarro et al. 2018; Fraguas-Sánchez and Torres-Suárez 2018). In contrast, more experimental effort is needed to know the most convenient way to approach GPR55 targeting, apart from doubts on which is mechanistically the best option, i.e. to measure the efficacy of agonists, antagonists or allosteric modulators in PD models. The safety of GPR55 ligands must be also tested.

With regard to GPR55, it is important to note that the affinity, the potency and the selectivity of different natural and synthetic cannabinoids are inconsistent across studies. For instance, whereas Kapur et al. (2009) and Yin et al. (2009) reported agonist activity of rimonabant using a β-arrestin reporter assay, Lauckner et al. (2008) showed that the drug behaved as GPR55 antagonist in intracellular Ca^{2+} mobilization assays. GPR55 shows the most unique features as it seems to act primarily through the $G_{\alpha 12}$-family of proteins and RhoA (Ryberg et al. 2007; Henstridge et al. 2010; Obara et al. 2011), but it may also couple to G_q (Lauckner et al. 2008; Waldeck-Weiermair et al. 2008). In a careful study by Henstridge et al. (2010), it appears that GPR55 is linked to a range of downstream signaling events and that the activity of GPR55 ligands is influenced by the functional assay employed, with notable differences in potency and efficacy (Henstridge et al. 2010). Among these factors, the most investigated are ERK1/2 (Oka et al. 2007; Ryberg et al. 2007; Lauckner et al. 2008; Waldeck-Weiermair et al. 2008; Pietr et al. 2009; Kapur et al. 2009; Andradas et al. 2011; Piñeiro et al. 2011), and p38 MAPK (Oka et al. 2010). Importantly, it has been demonstrated that LPI-induced ERK1/2 phosphorylation is controlled, at least in part, by GPR55 coupling to $G_{\alpha 12/13}$ which indicates a crosstalk between MAPK and Rho GTPases signaling (Andradas et al. 2011; Anavi-Goffer et al. 2012). Using a variety of homogenous assays, Anavi-Goffer et al. (2012)

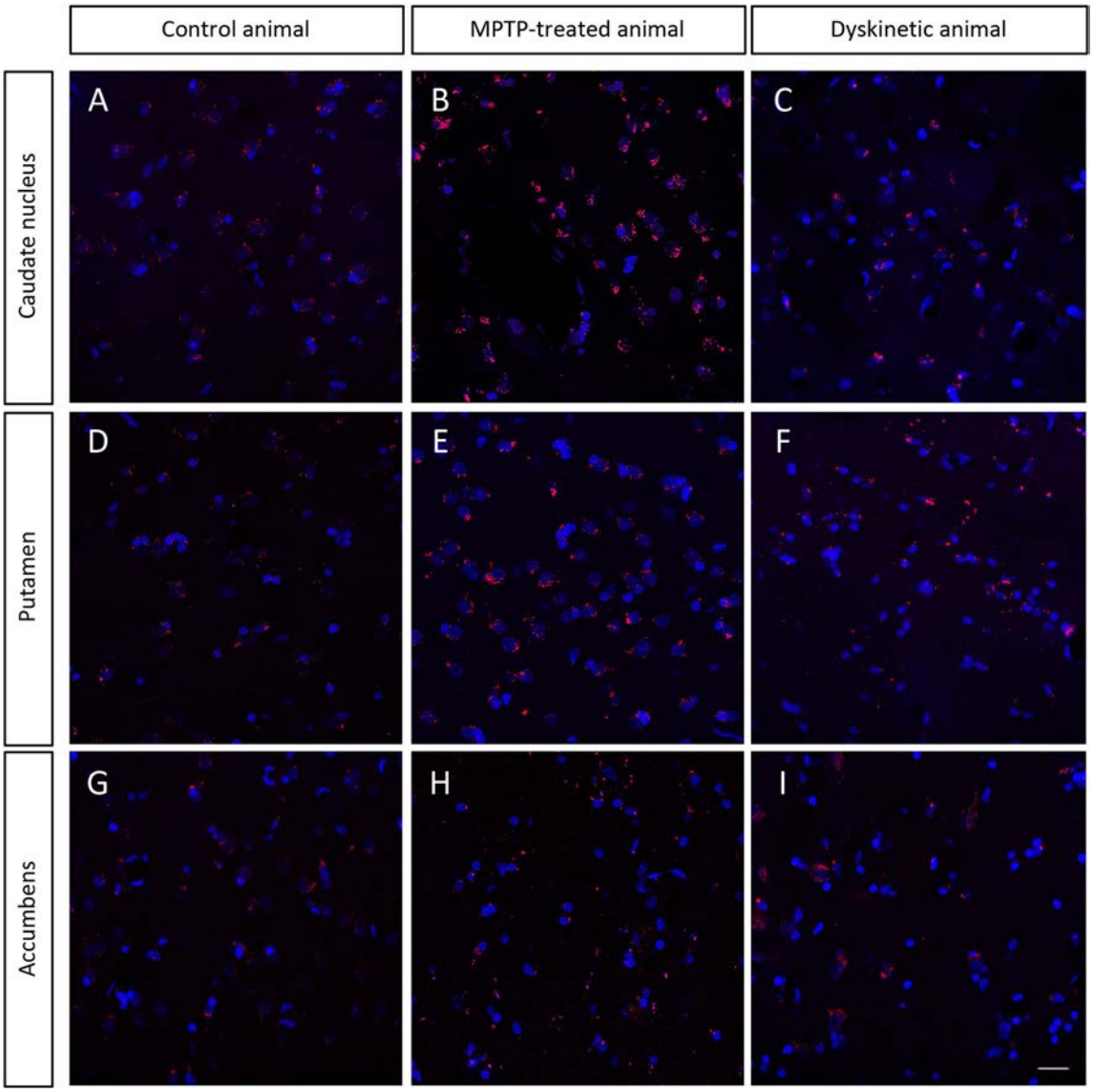

Fig. 4 CB_2-GPR55_Hets detected by the in situ proximity ligation assay (PLA) in striatal regions of the monkey brain. Representative images showing PLA label for CB_2-GPR55_Hets in the regions of interest: caudate, putamen and accumbens nuclei (see Fig. 1 legend). Experiments were performed in samples from the 3 animal groups namely control, parkinsonian (MPTP-treated) and dyskinetic. Quantification of red dots/cell is shown in the bar graphs displayed in Fig. 5

reported that certain cannabinoids can both activate GPR55 and attenuate LPI-mediated ERK1/2 phosphorylation concluding that "*cannabinoid ligands have complex interactions with the LPI/GPR55 signalling system*" (Anavi-Goffer et al. 2012). Some of the results may be attributed to CB_2-GPR55 receptor heteromerization. In fact, GPR55-mediated regulation of cannabinoid effects on CB_2R was first suggested in human neutrophils (Balenga et al. 2011; Irving 2011). Concerning CB_2-GPR55_Hets, we have previously shown that signaling by agonists of either receptor in cell models "*was governed (i) by the presence or absence of the partner receptors (with the consequent formation of heteromers) and (ii) by the activation state of the partner receptor*" (Balenga et al. 2014).

The atypical pharmacology often reported (including the above-described phenotypic profiles for antagonists) for CB_1R and CB_2R but even more extreme for GPR55 makes these receptors excellent candidates to show, as a proof-of-concept, that heteromerization influences pharmacology and the coupling to signaling pathways. In this context, additional forms of functional selectivity still need to be examined. For example, ligand-directed selectivity in G-protein-independent signaling pathways, which has been demonstrated with β-arrestin biased ligands for the β_2-adrenergic

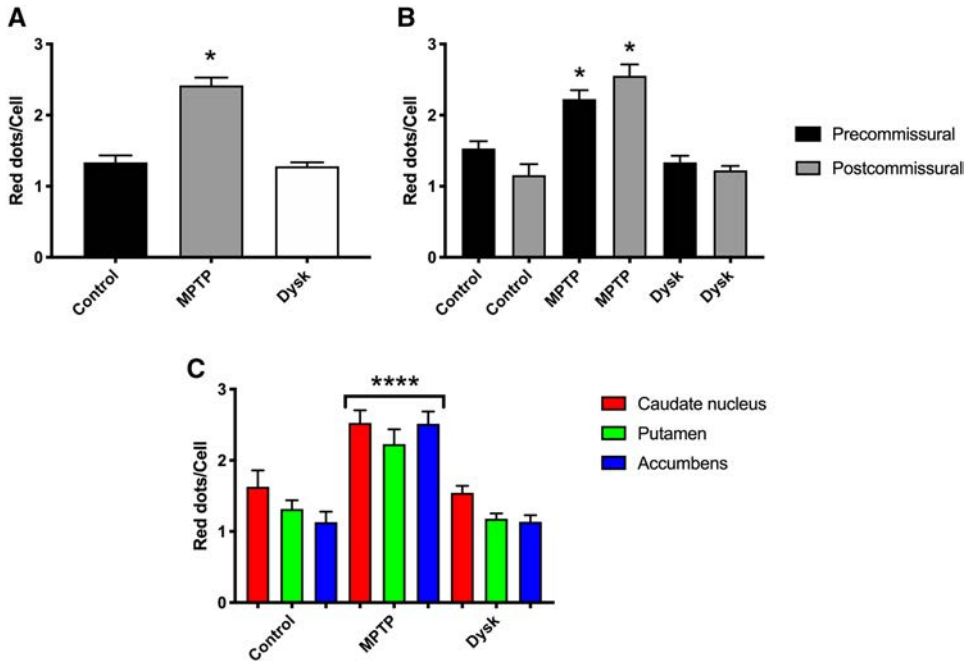

Fig. 5 Quantitation of CB_2-GPR55_Hets performed according to the Andy's algorithm (see "Materials and methods") in all striatal areas (caudate, putamen and accumbens nuclei) and in samples from control, MPTP-treated and dyskinetic animals. **a** Quantitation adding up the PLA label in all regions and both in pre- and post-commissural locations. **b** Quantitation in pre- *versus* quantitation in post-commissural areas. **c** Quantitation in each area (caudate, putamen and accumbens nuclei) following the color code indicated in Fig. 1. Data are the mean ± SEM (150 data points from 3 sections, 6 fields and $n = 3$ per group). Significant differences were analyzed by a one- or two-way ANOVA followed by post-hoc Bonferroni's test. *$p < 0.05$ compared with control; in **c**: ****$p < 0.0001$: every region in MPTP-treated animals *versus* both control and dyskinetic

receptors (Drake et al. 2008), has not been examined in full detail for CB_1R, CB_2R or GPR55.

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Author contributions JLL, GN and RF participated in the design of the project and analyzed the results; it is considered that their contribution was similar. EMP and AJR performed the majority of the experiments and took care of animals and measured ad hoc scores; it is considered that their contribution was similar. ER participated in a significant number of experiments. RRS and JL incorporated the software to analyze the PLA results, and actively participated in the PLA data analysis and in providing data for final Figures. JLL did many of the imaging assays in the confocal microscope, took images and participated in data analysis. EMP, JLL and RF wrote the first draft of the manuscript that was further edited by all co-authors, who agreed with submission.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval All experiments have been performed with approved protocols and under the regional supervision, i.e. strictly following all national and EU regulations.

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DISCUSSIÓ

4. Discussió

El Sistema Cannabinoide, que inclou els dos receptors de cannabinoides CB_1 i CB_2, els seus lligands endògens i els enzims de síntesi i degradació d'aquests, és un sistema molt complex que controla un gran nombre de processos biològics[448]. Aquest sistema té un paper important tant a nivell del Sistema Nerviós Central com Perifèric, tenint una implicació directa en la plasticitat neuronal, la neuroprotecció, la regulació del dolor, l'aprenentatge i la memòria, la resposta a l'estrès, la reproducció i la fertilitat, la resposta immune i la progressió del càncer entre d'altres[449].

Els receptors de cannabinoides CB_1 i CB_2 són ambdós, receptors acoblats a proteïna G (GPCR) que es troben implicats en un gran nombre de processos fisiològics, fet que els ha fet adquirir una elevada atenció com a prometedores dianes terapèutiques per un gran nombre de malalties[1]. Des dels anys noranta, es coneix que els GPCR poden interaccionar formant estructures de major ordre com homòmers o heteròmers. La interacció entre dos GPCR indueix a la formació de complexos amb propietats diferents de les que expressaven els receptors individualment. L'heteromerització té implicacions en l'expressió, la unió a lligand, la diversitat farmacològica, la senyalització i la internalització dels receptors que en formen part[450].

Entendre la biologia i la regulació dels heteròmers de GPCR, no només ens fa entendre el rol modulador d'aquesta interacció proteïna-proteïna en l'expressió i senyalització dels GPCR, sinó que obre una gran porta a la intervenció terapèutica. Els heteròmers de GPCR representen una diana amb molt potencial per fàrmacs que puguin actuar més enllà dels GPCR individualment. Tot i les evidències de què l'heteromerització dels GPCR produeix canvis en un gran nombre de les seves propietats, gairebé no hi ha estudis sobre fàrmacs que actuïn a nivell d'heteròmer[451].

Actualment, s'utilitzen un gran nombre de tècniques per investigar les interaccions proteïna-proteïna, com per exemple la tècnica de transferència d'energia per ressonància bioluminescent (BRET), l'assaig de lligació per proximitat (PLA) o també la complementació bimolecular per fluorescència (BiFC) en presència de pèptids transmembrana TAT[452]. Tot i que els receptors CB_1 i CB_2 no han estat tan estudiats com altres GPCR, el creixent nombre de publicacions que posen de manifest el seu rol en malalties neurodegeneratives i el seu potencial com a diana terapèutica, està conduint a que cada vegada s'estudiïn més, fent que l'interactoma dels receptors cannabinoides cada vegada sigui més gran. A dia d'avui, un gran nombre de receptors, tant ionotròpics com metabotròpics, han estat identificats com a proteïnes capaces de formar complexos heteromèrics amb els receptors de cannabinoides[453]. Entre aquests receptors es troba el receptor inotròpic NMDA. Els receptors inotròpics de glutamat són receptors essencials pel funcionament del SNC, i precisament el receptor NMDA té un paper clau en molts aspectes de les accions mediades per glutamat en el Sistema Nerviós, tant a nivell de neurotransmissió com en desenvolupament i neurogènesi[265]. Paral·lelament, el receptor NMDA també està relacionat amb l'excitotoxicitat induïda per nivells elevats de glutamat

extracel·lular, que acabarà produint una acumulació tòxica d'ions al citoplasma de les neurones[277]. En aquesta línia, es va hipotetitzar que el NMDAR podria ser una diana per tractar certes malalties neurodegeneratives, ja que la reducció de la sobreactivació del NMDAR, suprimiria l'excitotoxicitat. D'aquesta manera, el Memantine, un modulador al·lostèric negatiu del receptor NMDA va ser aprovat fa uns anys per tractar la malaltia de l'Alzheimer[454].

Tot i que lògicament l'interès de l'estudi d'aquests receptors s'ha centrat en les neurones, el NMDAR també s'expressa en cèl·lules glials, on té un paper crucial en el manteniment de l'homeòstasi cerebral.

L'article "**N-Methyl-D-aspartate (NMDA) and cannabinoid CB_2 receptors form funcional complexes in cells of the central nervous system: insights into the therapeutic potential of neuronal and microglial NMDA receptors"** es basa en la hipòtesi que la funcionalitat de NMDAR pot ser regulada pel receptor de cannabinoides CB_2 per mitjà de la formació de complexos heteromèrics entre ambdós receptors. L'estudi es va fer tant en neurones com en micròglia. El primer objectiu de l'article va ser comprovar si hi havia una interacció directa entre els receptors CB_2 i NMDA. Aquesta hipòtesi va resultar ser certa, demostrant que ambdós receptors interaccionen formant complexos heteromèrics que alteren l'efecte d'agonistes de NMDAR i CB_2R.

Aquests resultats posen de manifest la rellevància dels GPCR en el manteniment d'un correcte equilibri neuroprotector i confirma que els GPCR poden interaccionar també amb els receptors ionotròpics. Tot i que el CB_2R és menys abundant en neurones que el receptor CB_1, es pot trobar expressat en diverses regions del SNC. En aquest article s'ha detectat l'expressió de l'heteròmer CB_2R-NMDAR en cultius primaris de neurona d'hipocamp. En micròglia, aquest heteròmer també es va detectar, amb nivells més elevats en micròglia activada. Aquest augment d'expressió podria ser explicat per l'increment de l'expressió del receptor CB_2 en episodis de neuroinflamació, que en condicions normals es troba present, però en menor mesura.

La característica principal de l'heteròmer CB_2R-NMDAR, és el fenomen de *crosstalk* negatiu, on el tractament de cèl·lules HEK-293T transfectades amb els dos agonistes simultàniament, comporta l'absència de resposta tant en la via mediada per la proteïna Gi com en la via de les MAPK. L'efecte més rellevant d'aquest *crosstalk* negatiu, és la reducció de l'efecte del receptor ionotròpic NMDA, produït per agonistes del receptor CB_2. El bloqueig d'aquest receptor sembla ser degut a canvis conformacionals i canvis en les interaccions al·lostèriques produïts a conseqüència d'aquesta interacció proteïna-proteïna que impedeixen una correcta senyalització. En ratolins sans, els resultats obtinguts amb relació a la senyalització del receptor CB_2 van ser semblants als obtinguts en cèl·lules HEK transfectades. No obstant això, el *crosstalk* negatiu en coactivar ambdós receptors no es va observar en cèl·lules del ratolí transgènic $APP_{Sw/Ind}$ model d'Alzheimer. Aquest resultat demostra que en etapes primerenques del desenvolupament del SNC, les neurones d'hipocamp de ratolins WT i $APP_{Sw/Ind}$ ja presenten diferències entre si. Això és important, ja que el fenotip de la malaltia d'Alzheimer triga mesos a ser detectable. D'altra banda,

el fet que no hi hagi *crosstalk* negatiu en ratolins $APP_{Sw/Ind}$ podria indicar l'absència de l'heteròmer CB_2R-NMDAR en neurones model d'Alzheimer.

La presència de complexos heteromèrics va ser demostrada mitjançant la tècnica d'assaig de lligació per proximitat (PLA). A més, en comparar els nivells d'expressió de l'heteròmer CB_2R-NMDAR en ratolins WT i en ratolins $APP_{Sw/Ind}$, es va observar que l'heteròmer incrementa la seva expressió en el model d'Alzheimer, tant en neurones com en micròglia. Per tant, la hipòtesi més plausible per explicar l'absència de *crosstalk* negatiu en ratolins $APP_{Sw/Ind,}$ podria ser la coexistència de diferents poblacions de receptors, sent l'heteròmer CB_2R-NMDAR una d'elles. Aquesta hipòtesi explicaria per què el NMDAR no afecta els nivells d'AMPc en presència de JWH-133: o els receptors de CB_2 acoblats a proteïna Gi no interaccionen amb el NMDAR en els ratolins transgènics, o l'heteròmer CB_2R-NMDAR adopta una conformació que impedeix que la proteïna Gi s'acobli adequadament.

Aquest fenomen de *crosstalk* negatiu en GPCR que interaccionen amb el receptor NMDA ja s'havia observat altres vegades, un clar exemple és el receptor A_{2A}[455]. El receptor A_{2A} és una diana potencial per prevenir la neurodegeneració. Cada vegada hi ha més estudis que demostren que el tractament amb antagonistes d'aquest receptor afavoreixen la neuroprotecció. Tant és així que l'istradefylline, un antagonista selectiu del receptor A_{2A}, ha estat aprovat per tractar la malaltia del Parkinson[456]. L'heteròmer $A_{2A}R$-NMDAR també és capaç de modular l'activació del receptor NMDA, però mitjançant el fenomen de crosantagonisme, on l'antagonista del receptor A_{2A} és capaç de bloquejar la senyalització del receptor NMDA. Aquest fenomen, sumat a un increment en l'expressió de l'heteròmer en micròglia activada de ratolins $APP_{Sw/Ind,}$ fa que, igual que el CB_2R-NMDAR, el complex $A_{2A}R$-NMDAR sigui una diana molt atractiva per tractar la malaltia de l'Alzheimer.

L'excitotoxicitat causada per l'acumulació excessiva d'ions Ca^{2+} en el citoplasma, a causa de la sobreestimulació dels receptors NMDA, és una característica clau en la patologia de l'Alzheimer. El Ca^{2+} i l'AMPc són els dos segons missatgers més rellevants en neurones, i la seva concentració intracel·lular canvia segons l'activació dels receptors en la superfície cel·lular[264]. En aquest sentit, un elevat nombre d'investigacions s'han centrat en fenòmens de *crosstalk* entre la senyalització per AMPc i Ca^{2+}, demostrant interaccions directes entre sensors de calci i receptors metabotròpics com els GPCR. La interacció entre GPCR i sensors de calci en condicions de nivells elevats de Ca^{2+} resulta en una acusada regulació de la senyalització mediada pels receptors expressats en la superfície[457]. En l'article **"*N*-Methyl-D-Aspartate Receptor Link to the MAP Kinase Pathway in Cortical and Hippocampal Neurons and Microglia Is Dependent on Calcium Sensors and Is Blocked by α-Synuclein, Tau, and Phospho-Tau in Non-transgenic and Transgenic $APP_{Sw,Ind}$ Mice"** es descriu la interacció, en un sistema heteròleg d'expressió, dels sensors de calci calmodulina (CaM), calneuron-1, NCS1 i caldendrina, amb la subunitat GluN1 del receptor NMDA. Contràriament, la caldendrina no va ser capaç de formar complexos amb NMDAR. El receptor NMDA té un impacte en la via de senyalització de les MAPK, en aquest article es suggereix que aquest efecte podria ser degut a la seva interacció amb diferents sensors de calci.

De manera interessant, la relació entre l'activació del NMDAR i la senyalització per la via de les MAPK sembla ser deguda al sensor CaM en cultius primaris de micròglia de còrtex i hipocamp, mentre que en neurones tant de còrtex com d'hipocamp pot ser deguda pels sensors CaM i NCS1. Tot i que a dia d'avui no hi ha molts estudis sobre la relació entre els sensors de calci i els efectes fisiològics derivats dels receptors ionotròpics, ja s'havia descrit que en la depressió a llarg termini mediada per receptors NMDA intervé la CaM, mentre que en la mediada per GPCR intervé el NCS1[458].

En cultius primaris de neurones, l'efecte induït pel NMDA en la via de les MAPK es va veure bloquejat per les proteïnes α-sinucleïna, Tau i pTau, que juguen un paper crucial en la fisiopatologia de malalties com l'Alzheimer i el Parkinson. Aquest efecte va ser similar tant en neurones com en micròglia. Tenint en compte que aquest bloqueig va ser similar per les tres proteïnes, tant en tractaments crònics com aguts, i sabent que els sensors de calci modulen la unió de NMDA a la via de les MAPK, el bloqueig ha de ser degut a una interacció directa amb NMDAR o per interferència amb intermediaris de la senyalització.

En estudis previs on es van utilitzar ratolins $APP_{Sw,Ind}$, es va observar que els cultius primaris de micròglia de nounats, tenien un fenotip activat[459]. Com que els individus joves del model $APP_{Sw,Ind}$ no presenten deficiències cognitives, això podria indicar que la micròglia activada juga un paper neuroprotector en aquest model animal. D'aquesta manera, ens vam proposar estudiar diferents alteracions en el funcionament del receptor NMDA en aquestes cèl·lules. Per un costat, el NMDA és capaç d'activar la via de les MAPK, però amb diferències entre el model d'Alzheimer i el grup control. De fet, l'efecte induït per NMDA sobre els nivells de proteïna ERK1/2 fosforilada eren independents de calneuron-1 però modulats per CaM i NCS1. En animals control, l'efecte es va veure inhibit quan se silenciava la CaM en cultius de micròglia, mentre que en neurones aquest efecte es detectava silenciant tant la CaM com el NCS1.

Avaluant els nivells d'expressió dels complexos formats per NMDAR i els sensors de calci es va demostrar un increment de cèl·lules glials que expressaven NMDAR i CaM i també NMDAR i NCS1 en el model $APP_{Sw,Ind}$. En neurones, aquest increment es va veure únicament en els complexos NMDAR/NCS1. Aquests resultats obtinguts tant en neurones com en micròglia de ratolins transgènics van demostrar una alteració de l'activació de les MAPK induïda per NMDA. Això, sumat a l'efecte de les proteïnes α-sinucleïna, Tau, i pTau ens condueix a dues possibilitats: la hiperactivació dels receptors NMDA és independent dels sensors de calci o les cèl·lules neuronals són capaces de contrarestar l'efecte de la α-sinucleïna, la Tau i la pTau, incrementant la senyalització del NMDA.

El potencial de la relació entre el receptor NMDA i els sensors de calci requereix molta més investigació. El fet que en un model d'Alzheimer els complexos NMDAR–NCS1 es trobin sobreexpresats, obre les portes a futures investigacions sobre la seva rellevància en la fisiopatologia de l'Alzheimer.

Aquests resultats situen el NMDAR en el centre de la patofisiologia de la malaltia d'Alzheimer. Malauradament, els fàrmacs que afecten la seva funció, no són efectius a mitjà/llarg termini. Per tant, trobar GPCR que puguin modular l'activació del NMDAR obre les portes a trobar nous tractaments, sigui apuntant a neurones, micròglia o ambdós simultàniament. En el cas de l'heteròmer A_{2A}R-NMDAR, gràcies al fenomen de crosantagonisme, inactivar NMDAR mitjançant els antagonistes del receptor d'adenosina és una idea molt atractiva, però els compostos cannabinoides també poden produir beneficis equivalents reduint significativament l'efecte dels agonistes de NMDAR[455].

El centre ortostèric acostuma a estar molt conservat en totes les famílies de GPCR. Així, és difícil trobar alta especificitat en un receptor en concret. Per aquest motiu, s'ha investigat en la síntesi de lligands bitòpics que s'uneixen al centre ortostèric i simultàniament, a una regió menys conservada, i per tat més especifica del receptor que es troba ubicada fora del centre ortostèric[460]. En el cas del receptor CB_2, es va dissenyar un fàrmac bitòpic que s'unia al centre ortostèric i a una regió del canal que formen els dominis TM1 i TM7[461]. D'aquesta manera, un fàrmac bitòpic amb alta especificitat per CB_2R podria unir-se també al CB_2R que interacciona amb NMDAR. Aquest lligand bitòpic podria suposar una molt bona estratègia per modular l'efecte de NMDAR en diverses patologies com la de l'Alzheimer, ja que l'heteròmer CB_2R-NMDAR es troba sobreexpresat en neurones i micròglia de ratolins $APP_{Sw/Ind}$. Un gran nombre d'estudis indiquen que per modular receptors com NMDA a partir de compostos cannabinoides, es poden emprar la majoria de cannabinoides naturals ja que poden fer aquest efecte de forma segura, amb excepció del Δ^9-THC i altres compostos que produeixin efectes psicoactius per mitjà de CB_1R[462].

En els darrers anys, està havent-hi un interès creixent en l'ús de cannabinoides per tractar diferents malalties[463]. Un exemple d'aquests compostos és el CBD, el segon fitocannabinoide més abundant de la planta del cànnabis. El CBD ha estat considerat des de fa temps com una molècula amb caràcter neuroprotector i degut a l'absència d'efectes psicoactius associats al seu consum, ha esdevingut un principi actiu molt atractiu per diverses malalties. Una d'aquestes malalties on el CBD ha resultat ser útil és l'ictus isquèmic. L'any 2005 es va demostrar que el tractament amb CBD en models animals d'ictus causat per oclusió de l'artèria cerebral mitja era capaç de reduir l'àrea infartada[464]. Aquest efecte era parcialment bloquejat per WAY100135, antagonista del receptor $5HT_{1A}$.

La hipòxia en nounats pot tenir conseqüències negatives en el desenvolupament del Sistema Nerviós Central. De cara a la recuperació després d'un episodi d'ictus, és important que es recuperi l'oxigenació el més aviat possible per tal de millorar el pronòstic de l'accident. D'altra banda, també és necessari limitar el dany anatòmic i cel·lular en l'òrgan més susceptible a la falta d'oxigen, el cervell. En un model animal d'isquèmia en porcs nounats, el CBD va resultar ser efectiu en reduir el dany cerebral[465].

El CBD és un fitocannabinoide que s'uneix als receptors de cannabinoides. Aquest fitocannabinoide a elevades concentracions pot entrar al centre ortostèric tant del receptor CB_1 com CB_2, actuant com a agonista parcial, mentre que a concentracions nanomolars actua com a modulador al·lostèric[119]. A més, el CBD

a concentracions micromolars també és capaç d'activar el receptor de serotonina $5HT_{1A}$[352]. Tant el CB_2R com el $5HT_{1A}R$ són mediadors de l'efecte neuroprotector del CBD en model animal d'hipòxia-isquèmia[466].

El CB_2R i el $5HT_{1A}R$ són un altre exemple de dos GPCR que formen complexos heteromèrics. La interacció dels dos receptors ja s'havia descrit amb anterioritat, demostrant una sobreexpressió de l'heteròmer CB_2R–$5HT_{1A}R$ en porcs nounats model d'isquèmia. En l'article "**Regulation of Expression of Cannabinoid CB_2 and Serotonin $5HT_{1A}$ Receptor Complexes by Cannabinoids in Animal Models of Hypoxia and in Oxygen/Glucose-Deprived Neurons"** es va demostrar la sobreexpressió de l'heteròmer CB_2R-$5HT_{1A}R$ en animals lesionats i amb un procés d'hipòxia. Un dels resultats més rellevants d'aquest article és, possiblement, la reducció de l'expressió de l'heteròmer en animals pretractats amb CBD en el model animal d'isquèmia amb hipòxia. La sobreexpressió de l'heteròmer CB_2R-$5HT_{1A}R$ també es va detectar en cultius primaris de neurona en deprivació d'oxigen i glucosa (GOD), on de nou el tractament amb CBD va disminuir els nivells d'expressió. En aquest estudi, el fitocannabinoide CBG es va emprar en paral·lel amb el CBD, ja que en tenir diferents afinitats pel receptor CB_2, podia conduir a diferències rellevants pel que fa a l'activació de les vies de senyalització[467]. Diferents publicacions especulen que els diferents efectes beneficiosos induïts pels cannabinoides en termes terapèutics poden dependre del mode d'unió al receptor, de com es col·loca un compost en el centre ortostèric o al·lostèric i la seva afinitat[134].

Els nostres resultats comparant l'efecte del CBD i el CBG són consistents amb les seves diferències respecte al model d'unió al receptor, consistents amb les modulacions al·lostèriques produïdes per la unió dels receptors CB_2 i $5HT_{1A}$. Així, l'efecte del CBG en la regulació de l'expressió de l'heteròmer CB_2R-$5HT_{1A}R$ en cultius primaris de neurones va ser més feble que el produït pel CBD. El fet que ambdós cannabinoides reduïssin l'expressió de l'heteròmer CB_2R-$5HT_{1A}R$, i que fossin capaços de bloquejar l'efecte de la serotonina, obre les portes a la hipòtesi que en un ambient d'hipòxia/isquèmia la serotonina és perjudicial. Cada vegada hi ha més consens sobre els beneficis del CBD en la patologia d'hipòxia, on segons sembla, la supressió de la senyalització a través de $5HT_{1A}R$ podria ser realment beneficiosa. En aquest sentit, vam trobar interessant estudiar el potencial d'antagonistes del receptor $5HT_{1A}$ en cultius primaris de neurona GOD i en models animals d'ictus. Aquest estudi tenia certa complexitat, pel fet que la majoria d'antagonistes de $5HT_{1A}R$, com per exemple l'alprenolol, també interaccionen amb receptors adrenèrgics[468]. Actualment, no hi ha estudis sobre l'efecte directe d'antagonistes selectius de $5HT_{1A}R$ com el spiroxatrine o el WAY100135 en models d'ictus, però, si que hi ha estudis que indiquen que l'ús de WAY100135 és beneficiós en un model intestinal d'hipòxia[469].

Una altra hipòtesi que fa temps que està sobre la taula és que el CBD indueix els seus efectes beneficiosos actuant principalment sobre la glia, emfatitzant la possibilitat que el fitocannabinoide sigui capaç de polaritzar la microglia cap al fenotip M2, conegut per ser neuroprotector i antiinflamatori davant del fenotip M1 que és proinflamatori. Diferents estudis suggereixen que la microglia té un paper crucial en la protecció de les neurones en episodis de dany, tant agut com crònic, a nivell del Sistema Nerviós Central.

En la hipòxia neonatal, la micròglia es troba activada i precisament, en casos de supervivència i dany limitat, la micròglia és activada en pro del seu fenotip neuroprotector M2[470]. La possibilitat de polaritzar la micròglia cap a una funció neuroprotectora comporta unes implicacions terapèutiques molt importants per poder combatre diverses malalties que causen mort neuronal. Per poder explorar aquesta línia d'investigació cal definir, en diferents patologies, els marcadors que estan relacionats amb el fenotip neuroprotector M2 i proinflamatori M1 de la micròglia[126]. En l'article "**Cannabidiol skews microglia towards a neuroprotective phenotype in a model of neonatal hypoxia-ischemia**" hem estudiat els diferents marcadors en un model d'hipoxia amb isquèmia en presència i absència de CBD. Hem detectat que el CBD és capaç d'activar la micròglia i revertir el dany. Per realitzar els experiments vam emprar rates Winstar les quals se'ls va realitzar una electrocoagulació de l'artèria caròtida i van ser mantingudes en condicions d'hipòxia durant dues hores. Després, les rates van ser injectades intraperitonealment amb CBD o vehicle. El model animal d'hipòxia en nounats usat en els nostres experiments demostra una marcada activació de la micròglia amb una sobreexpressió d'iNOS, un marcador proinflamatori propi del fenotip M1. Aquesta elevada expressió d'iNOS va ser persistent i se seguia detectant 30 dies després de la lesió. Quan el model d'ictus era tractat amb CBD, els nivells d'expressió d'iNOS es van veure disminuïts i aquest efecte era detectat 1, 7 i 30 dies després del tractament. Interessantment, l'Arginasa I, considerat un marcador del fenotip M2, es trobava també sobreexpressat just després de la lesió, posant de manifest l'equilibri en la polarització entre els dos fenotips M1 i M2. Quan els animals van ser tractats amb CBD, es va veure el seu marcat potencial neuroprotector, produint un increment de l'expressió de l'Arginasa I; ja detectable el primer dia després del tractament i mantingut en el temps fins 30 dies després de la lesió.

En conjunt, aquests resultats posen en dubte la hipòtesi d'una micròglia purament proinflamatòria o purament neuroprotectora[471]. També qüestionen la visió d'un continu de fenotips que va des del més proinflamatori fins al més antiinflamatori, passant per molts estadis intermedis[472]. Els nostres resultats són més consistents amb una polarització depenent del temps que regula l'expressió de marcadors en la micròglia activada.

Els astròcits també tenen un rol molt important en processos d'hipòxia[473]. L'elevada immunoreactivitat de GFAP després de la lesió reflecteix un increment de l'expressió d'aquest marcador d'activació d'astròcits. Aquesta activació va resultar ser moderada després d'un pretractament amb CBD. El darrer marcador que es va analitzar és SOX-10. SOX-10 és un factor de transcripció que en oligodendròcits promou l'expressió de mielina i potencia la seva supervivència. Els resultats amb relació a SOX-10 poden semblar una mica contraindicatoris, ja que sembla que un increment en l'expressió de SOX-10 després d'un episodi d'ictus podria ser beneficiós en preparar els oligodendròcits per reparar les conseqüències del dany. En aquest sentit, el fet que un compost amb activitat neuroprotectora com el CBD faci disminuir els nivells d'expressió de SOX-10 és difícil d'explicar; a no ser que els resultats també estiguin detectant l'expressió d'astròcits. Per una banda, s'ha descrit que SOX-10 s'expressa en oligodendròcits, però també s'ha descrit la capacitat in vitro de SOX-10 per

convertir astròcits en oligodendròcits[474]. Per tant, podria ser que l'increment de GFAP i SOX-10 en el model d'ictus sigui degut a la formació d'astròcits i la seva conversió a oligodendròcits.

Més enllà de la patologia de l'ictus, el CBD també ha estat investigat com a potencial tractament per la patologia de l'Alzheimer gràcies a les seves propietats beneficioses en malalties neuroinflamatòries. L'Alzheimer és el tipus de demència més comú arreu del món, amb un total del 60% dels casos. Es caracteritza per la producció i acumulació de plaques β-amiloides i cabdells neurofibril·lars, produint neuroinflamació i estrès oxidatiu, amb la conseqüent mort neuronal i dèficits tant comportamentals com cognitius. Entre ells, l'apatia i la pèrdua de memòria i de la funció motora[475].

S'ha proposat que el CBD presenta propietats neuroprotectores i antioxidants en quant a la toxicitat induïda pel pèptid β-amiloide en cèl·lules de rata PC12[476]. El CBD també modula la funció de la micròglia, concretament s'ha descrit que evita l'activació induïda per l'Aβ[293]. En aquesta línia, en l'article "**Cannabidiol decreases pTau and Aβ axonal transport and improves spatial memory in 5xFAD mice model of Alzheimer's disease**" vam analitzar els diferents fenotips de la micròglia en un model de ratolí d'Alzheimer 5xFAD que expressa les mutacions M146L i L286V en el gen PSEN, i les mutacions *Swedish* (K670N/M671L), *Florida* (I716V) i *London* (V717I) en el gen APP.

Mitjançant la tècnica d'immunohistoquímica vam demostrar que el tractament amb CBD presenta un efecte neuroprotector en el model 5xFAD tant en neurones de còrtex com d'hipocamp, polaritzant la micròglia cap al seu fenotip M1, disminuint els nivells d'astròglia activada i incrementant l'expressió de precursors d'oligodendròcits en escorça.

El CBD és capaç d'unir-se tant a CB_1R com a CB_2R, però diferents estudis indiquen que el receptor CB_2 podria jugar un paper més important que CB_1R en la polarització de la micròglia cap a un fenotip neuroprotector. El JWH-133, un agonista selectiu del CB_2R, redueix l'activació de la micròglia i fa disminuir la concentració de mediadors proinflamatoris en infecció per pneumococ, tan *in vivo* com in vitro[477]. A més, en un model animal $APP_{Sw,Ind}$, l'activació del receptor CB_2 afavoreix el fenotip M2[478]. Conseqüentment, l'efecte del CBD sobre la polarització de la micròglia sembla ser induït per mitjà de l'activació del CB_2R.

El rol de les plaques amiloides i els cabdells neurofibril·lars en la patologia de l'Alzheimer és encara desconegut. Tot i així, diferents estudis proposen una estreta relació entre la formació de plaques i la fosforilació de la proteïna Tau. S'ha demostrat que els nivells d'Aβ-pE(3), una espècie truncada del pèptid β-amiloide amb elevada capacitat per formar agregats, i els de la proteïna pTau Ser202/Thr205, correlacionen tant en teixits humans com murins, suggerint que l'Aβ-pE(3) pot afavorir la fosforilació de Tau[479].

Els experts creuen que els agregats d'Aβ i de pTau contribueixen a bloquejar la comunicació neurona-neurona induint mort neuronal, causant pèrdua de memòria i produint canvis de personalitat i altres símptomes típics de la malaltia de l'Alzheimer. L'administració de Sativex, un extracte del cànnabis utilitzat per

combatre el dolor neuropàtic i altres símptomes de l'esclerosi múltiple, ha demostrat produir una reducció important dels nivells de proteïna Tau fosforilada, de l'expressió de GSK3 i dels nivells d'Aβ en ratolins transgènics i en humans[293,302]. A més, el CBD és capaç d'inhibir la hiperfosforilació de la proteïna Tau induïda pel pèptid β-amiloide i la producció d'òxid nítric[295,294].

Una de les possibilitats per aturar la progressió de l'Alzheimer consistiria a reduir el transport axonal anterògrad d'Aβ i de pTau, fent disminuir la difusió d'aquestes proteïnes cap a diferents regions neuronals i reduint la neuroinflamació i la neurodegeneració. Després d'analitzar el transport axonal d'Aβ, de Tau i de pTau des de neurones corticals fins a neurones d'hipocamp emprant xips microfluids de dues cambres, vam observar que l'Aβ, la Tau i la pTau difonien d'una neurona a una altra neurona. Interessantment, el tractament amb CBD per 24 hores, reduïa significativament el transport d'Aβ, de Tau i de pTau, obtenint el major efecte en cas de l'Aβ.

Els resultats obtinguts en aquesta Tesi Doctoral demostren que el CBD recupera la supervivència neuronal revertint la toxicitat induïda per l'Aβ i el NMDA en cultius primaris de neurona d'escorça i d'hipocamp. Aquests resultats concorden amb els publicats pel grup del Dr. Hampson, on el CBD exhibia un fort efecte antioxidant contra la toxicitat induïda per glutamat en cultius primaris de neurona[480]. En aquest context, també s'ha demostrat mitjançant microscòpia electrònica de transmissió, que el tractament amb CBD potencia l'autofàgia en neurones d'un model animal APP/PS1[481].

Diferents estudis demostren que el receptor CB2 es troba sobreexpressat en processos proinflamatoris en teixits de la perifèria en malalties com la colitis ulcerosa o la malaltia de Crohn[482] i també a nivell de Sistema Nerviós Central en malalts d'Alzheimer tant en escorça com hipocamp[483,478]. En aquesta línia hem detectat una sobreexpressió no només de CB_2R, sinó també de CB_1R en menor nivell, en ratolins 5xFAD. De manera interessant, l'expressió d'ambdós receptors es va veure reduïda fins a nivells semblants als del model control en ratolins tractats amb CBD.

Un dels altres efectes descrits del CBD consisteix en la seva capacitat per reduir l'expressió de gens associats amb l'Alzheimer, com per exemple els que codifiquen per les β- i γ-secretases o per proteïnes responsables de la fosforilació de Tau[294]. A més, en l'hipocamp de ratolins tractats amb el pèptid β-amiloide, el CBD suprimia l'expressió de pèptids glials proinflamatoris[484]. Paral·lelament, s'ha demostrat la implicació del CBD en la neurogènesi[475]. Per aprofundir en els efectes del CBD, vam analitzar la formació de neurites en cultius primaris de neurones tractats amb Aβ, Tau i pTau, en presencia i absència de CBD. En aquest sentit, vam observar que el tractament amb CBD era capaç de revertir la pèrdua de neurites provocada pel tractament amb Aβ, Tau o pTau.

Hi ha publicacions que assenyalen al CBD com a tractament efectiu contra la nocicepció en malalts de Parkinson[485]. D'aquesta manera, vam estudiar si els agregats d'α-sinucleïna, característics de la patologia del Parkinson, podien alterar la plasticitat neuronal. Els resultats obtinguts indiquen que l'α-sinucleïna

produeix una reducció de la formació de neurites que, contràriament a l'observat en Aβ, Tau i pTau, no és revertida pel tractament amb CBD.

Finalment, es va avaluar la capacitat del CBD per combatre el deteriorament cognitiu en la patologia de l'Alzheimer. Hi ha molts estudis que defensen la implicació del CBD en prevenir els dèficits cognitius en malalties neurodegeneratives. Alguns exemples són la millora en la memòria de reconeixement social en el model de ratolí transgènic APP/PS1[296] o els efectes beneficiosos del consum de CBD en els símptomes neuropsiquiàtrics de pacients d'Alzheimer[297]. Els nostres resultats obtinguts mitjançant l'assaig NORT (*Novel Object Recognition Test*), indiquen que el CBD podria ser una diana amb molt potencial per tractar el deteriorament cognitiu, demostrant que l'administració de CBD en ratolins 5xFAD revertia les pèrdues de memòria a curt i llarg termini associades a l'Alzheimer.

D'aquesta manera, aquest conjunt d'efectes podria ser explicat degut a la capacitat del CBD de reduir de la formació tant de plaques amiloides com de cabdells neurofibril·lars, un dels inicis potencials de la malaltia d'Alzheimer.

No només el fitocannabinoide CBD ha resultat ser útil com a possible agent terapèutic per tractar diverses malalties neurodegeneratives. El Dronabinol, un compost actiu amb una estructura idèntica a la del Δ^9-THC, però d'origen sintètic ha estat aprovat per l'ús en humans sota el nom de Marinol. També ha estat aprovat el Sativex, una preparació 1:1 de Δ^9-THC i cannabidiol, i una preparació de cannabidiol pur en forma d'oli vegetal. Aquests fàrmacs estan indicats pel tractament de l'esclerosi múltiple, ja que millora els símptomes relacionats amb la rigidesa muscular i els espasmes, però també s'utilitzen en pacients amb càncer que estan sent tractats amb quimioteràpia, degut a les seves propietats antiemètiques i orexigèniques[486].

El descobriment del fet que el bloqueig del receptor CB_1 produïa una pèrdua de pes corporal, reduint la ingesta d'aliments, va portar al fet que l'antagonista del CB_1R, rimonabant, capaç de creuar la barrera hematoencefàlica, fos aprovat per tractar l'obesitat. Malauradament, aquest compost va ser retirat poc després degut a efectes secundaris severs relacionats amb alteracions a nivell del Sistema Nerviós Central[487]. Tot i que es va hipotetitzar que els antagonistes de CB_1R que fossin incapaços de creuar la barrera hematoencefàlica podrien revertir aquests efectes secundaris, hi ha evidències que demostren que aquests efectes antiorexigènics dels antagonistes de CB_1R són, almenys en part, deguts al bloqueig dels receptors al Sistema Nerviós Central, on CB_1R es troba àmpliament expressat[488].

La regulació de la gana és un procés molt complex en el qual intervenen molts factors. La grelina però, és una hormona que té un paper protagonista. Al principi de la seva descoberta, es va observar que la grelina a part de tenir la capacitat d'estimular la secreció de l'hormona de creixement des de la glàndula pituïtària, tenia una funció iniciadora de la sensació de gana, regulant així la ingesta d'aliments i l'obtenció d'energia[389]. Consistentment amb aquest rol, la grelina és produïda a les glàndules gàstriques en condicions de dejú o de restricció calòrica, on s'allibera al torrent sanguini i circula fins a SNC. Després d'un àpat

o en condicions d'excedent d'energia, els nivells de grelina disminueixen, regulant la sensació de gana i l'homeòstasi energètica i modulant el metabolisme mitjançant l'activació de circuits orexigènics[397].

Centrant la nostra atenció en el rol de la grelina en la ingesta d'aliments i la sensació de gana i les propietats orexigèniques dels cannabinoides, ens vam plantejar una possible interacció entre el receptor CB_1R i el receptor de grelina, GHS-R1a. El primer objectiu de l'article "**Ghrelin and Cannabinoid Functional Interactions Mediated by Ghrelin/CB_1 Receptor Heteromers That Are Upregulated in the Striatum From Offspring of Mice Under a High-Fat Diet**" va ser demostrar l'existència d'aquest heteròmer i caracteritzar les conseqüències funcionals d'aquesta interacció. Per mitjà de les tècniques de BRET i PLA, es va demostrar que CB_1R i GHS-R1a interaccionen formant complexos heteromèrics i ho fan en condicions fisiològiques. A nivell funcional, la interacció de CB_1R i GHS-R1a resulta en un bloqueig del receptor cannabinoide en termes de la senyalització mediada per Gi. Aquest bloqueig succeeix simplement per coexpressió, no requereix l'activació del receptor GHS-R1a. D'aquesta manera, les cèl·lules que expressen l'heteròmer CB_1R-GHS-R1a no són reactives a cannabinoides o inclús endocannabinoides, a no ser que hi hagi un *pool* de receptors CB_1 a la superfície cel·lular que no interacciona amb GHS-R1a.

Diversos cannabinoides són capaços de creuar la barrera hematoencefàlica, arribant als circuits de recompensa on actua la grelina[135]. Els receptors de grelina en aquests circuits regulen el control de la ingesta calòrica. Els nostres resultats indiquen un bloqueig de CB_1R en termes de la senyalització a través de Gi, però una potenciació en la via de senyalització del calci intracel·lular. El fet que a baixes dosis d'agonistes de CB_1R (1 nM) hi hagi una major potenciació de la funcionalitat de GHS-R1a és fisiològicament rellevant. Aquest efecte ja s'havia observat en humans i ratolins, on baixes dosis de Δ^9-THC estaven relacionades amb hiperfàgia, mentre que altes dosis la reduïen[489]. Els nivells d'endocannabinoides són importants a l'hora de controlar els *inputs* del sistema de recompensa relacionats amb la ingesta calòrica, sobretot en la part hedònica del menjar[490]. S'ha de tenir molta cura a l'hora de fer consideracions sobre les interaccions entre CB_1R i GHS-R1a en neurones. No totes les neurones expressen ambdós receptors i en cas que un tipus neuronal expressi l'heteròmer CB_1R-GHS-R1a, també expressa heteròmers amb altres receptors en els quals participa CB_1R.

Com que la incidència de l'obesitat és més alta en famílies amb un historial de sobrepès, vam raonar que l'expressió de l'heteròmer podria veure's alterada en les cries de ratolines alimentades amb una dieta alta en greixos, ja que tenien més risc de patir obesitat. Un increment de l'expressió de l'heteròmer CB_1R-GHS-R1a podria estar implicat en la predisposició a l'obesitat en la descendència de pares obesos[491]. Els resultats obtinguts que demostren la sobreexpressió de l'heteròmer CB_1R-GHS-R1a en cries de ratolines alimentades amb dieta alta en greixos indiquen que, ja des del naixement, la funció del CB_1R es veu alterada. A més, vam observar que el nombre d'heteròmers es veia incrementat quan s'activaven tant el receptor CB_1 amb el seu agonista, ACEA 100 nM, o el receptor GHS-R1a amb grelina 100 nM. La sobreexpressió induïda per ACEA en

neurones no es va veure reflectida en cèl·lules HEK-293T transfectades, on el tractament amb ACEA no alterava la corba de saturació de BRET.

És important destacar que l'activació del receptor CB_1 produïda pel tractament amb ACEA observada en cultius primaris, es va veure completament bloquejada en cèl·lules HEK-293T transfectades que expressaven l'heteròmer CB_1R-GHS-R1a. Aquests resultats demostren que no totes les neurones d'estriat expressen l'heteròmer i que segurament el CB_1R es trobi formant complexos heteromèrics amb altres receptors que no bloquegen la seva senyalització o simplement formant homodímers.

En l'anomenada "alimentació hedònica", la dopamina juga un paper clau en els circuits de recompensa[490]. Així doncs, els receptors de dopamina han de ser considerats en aquest escenari. S'ha descrit que el CB_1R interacciona amb el receptor D_2 de dopamina[60]. La interacció de CB_1R i D_2R resulta en un antagonisme funcional, descrit tant en el bloqueig de D_2R per agonistes de CB_1R, com pel canvi d'acoblament de proteïna G que pateixen els receptors quan formen l'heteròmer. Quan es troben aïllats s'acoblen a una Gi, mentre que quan formen complexes canvien l'acoblament a una Gs.

En conclusió, els beneficis dels cannabinoides en una població de neurones que expressen l'heteròmer CB_1R-GHS-R1a, es veuen suprimits pel bloqueig produït pel receptor GHS-R1a, impedint el seu efecte en altres sistemes com el dopaminèrgic. D'altra banda, la coactivació de l'heteròmer CB_1R-GHS-R1a per grelina i cannabinoides produeix una resposta més robusta en la via mediada per Gq. Aquest efecte en la concentració dels nivells citosòlics de calci ha de ser rellevant, ja que l'ió Ca^{2+} regula gairebé tots els processos de la fisiologia neuronal. Els nostres resultats suggereixen un elevat potencial dels antagonistes del receptor GHS-R1a, obtenint un doble benefici: reduir la ingesta calòrica i revertir els efectes nocius de la dieta alta en greixos pel que fa a la funcionalitat de CB_1R en neurones d'estriat. És molt probable que l'heteròmer CB_1R-GHS-R1a es trobi en subpoblacions de neurones, per tant, per entendre la relació funcional entre ambdós receptors, el següent pas seria una descripció acurada a nivell anatòmic i cel·lular, de les zones i les neurones on els dos receptors es troben coexpressats.

Tot i que el receptor CB_1 ha estat durant molts anys l'objectiu principal de la investigació en cannabinoides, els efectes psicoactius resultants de l'activació del receptor i els efectes secundaris de l'antagonista de CB_1R, rimonabant, han fet canviar l'objectiu d'estudi, centrant l'atenció en el receptor CB_2[492]. La seva expressió a nivell de SNC i la seva sobreexpressió en cèl·lules glials activades han fet que el CB_2R hagi estat proposat com a diana terapèutica per tractar la neuroinflamació, reduir la neurotoxicitat induïda per estrès oxidatiu i potenciar la neurogènesi/gliogènesi[493].

Com que el receptor GHS-R1a s'expressa en hipotàlem i molts dels fitocannabinoides coneguts són capaços de creuar la barrera hematoencefàlica produint diversos efectes, entre ells el de la inducció de la gana, ens vam plantejar que potser el CB_2R també podria interaccionar amb aquest sistema orexinèrgic. En l'article "**Identification of the Ghrelin and Cannabinoid**

CB_2 Receptor Heteromer Functionality and Marked Upregulation in Striatal Neurons from Offspring of Mice under a High-Fat Diet" s'ha descobert l'existència de complexos heteromèrics formats per GHS-R1a i CB_2R en neurones d'estriat. A més, s'ha demostrat que el receptor GHS-R1a modula l'efecte dels cannabinoides al cervell.

Des d'un punt de vista molecular, els nostres resultats suggereixen que els receptors GHS-R1a i CB_2R formen una estructura tetramèrica que pot acoblar-se almenys, a una proteïna Gi i una Gq. Aquests resultats són molt semblants als reportats en el primer model estructural de dos GPCR que formen complexos heteromèrics. Els receptors d'adenosina A_1 i A_{2A}, interaccionen formant un tetràmer de dos homodímers, capaços d'acoblar dues proteïnes G diferents, una Gs i una Gi. En aquest exemple, l'activació d'un receptor bloqueja l'activació de l'altre. Tot i això, en el cas de l'heteròmer CB_2R-GHS-R1a, la senyalització a través de CB_2R es troba bloquejada inclús en absència de grelina. Indiferentment de la presència de l'hormona, el CB_2R no pot ser activat si es troba interaccionant amb GHS-R1a. L'activació del receptor CB_2 és només possible en presència de l'antagonista selectiu de GHS-R1a, YIL 781.

Tot i que aquest comportament no és el més típic i no es troba en gaires dels heteròmers de GPCR identificats, s'ha descrit que la mera presència del receptor A_{2B} en la formació de l'heteròmer $A_{2A}R$-$A_{2B}R$, redueix la funció i l'afinitat dels agonistes del receptor A_{2A}[494]. Actualment, l'única hipòtesi raonable per explicar el rol fisiològic tant de l'heteròmer CB_2R-GHS-R1a com el de l'$A_{2A}R$-$A_{2B}R$ és que es formen per regular negativament la funcionalitat d'un dels dos receptors. La formació de complexos heteromèrics entre GPCR és una manera d'aconseguir diversitat funcional. Els heteròmers són unitats funcionals que es comporten diferent dels receptors expressats individualment. Hi ha pocs heteròmers descrits en què intervingui el receptor GHS-R1a. Se sap que GHS-R1a interacciona amb els receptors de dopamina D_1 i D_2, així com amb els receptors de secretina[495]. També s'ha descrit que la cocaïna interacciona amb el receptor sigma-1, modulant la interacció entre D_1-GHS-R1a en neurones hipotalàmiques per suprimir la sensació de gana[496].

Els resultats en aquest article indiquen que la funcionalitat de CB_2R es veu alterada en presència de l'heteròmer CB_2R-GHS-R1a. Aquest descobriment és complementa amb un altre resultat publicat en aquest article; l'expressió de l'heteròmer es veu alterada en la progènie de ratolines obeses en cultius primaris de neurona d'estriat. L'increment de l'expressió de CB_2R-GHS-R1a en mostres de fetus de mares alimentades amb dieta alta en greixos podria explicar alguns dels efectes relacionats amb l'obesitat. Per una banda, la inactivació del gen codificant pel receptor GHS-R1a porta a una millora de la funció de la grelina, però també indueix, amb l'edat, obesitat. D'altra banda, en pacients obesos, observem un procés de neuroinflamació, que mitjançant ajustos en la dieta, es veu reduïda tant en les mares com en la progènie. Aquests resultats són rellevants en quant la proporció d'obesitat infantil és elevada en infants amb pares obesos. La sobreexpressió de l'heteròmer CB_2R-GHS-R1a en ratolins nounats de mares alimentades amb dieta alta en greixos indiquen que ja als primers dies de vida, aquests animals ja tenen afectada la funcionalitat de CB_2R. Els resultats suggereixen que els antagonistes de GHS-R1a podrien tenir un

doble benefici: reduir la ingesta calòrica i reduir els efectes nocius de la dieta alta en greixos sobre la funcionalitat de CB_2R, permetent els beneficis dels cannabinoides perduts pel bloqueig produït per GHS-R1a.

Els receptors cannabinoides es troben implicats en un gran nombre de processos fisiològics, i com hem vist, les seves interaccions proteïna-proteïna amb altres GPCR estan estretament relacionades amb patologies neuroinflamatòries. Recentment, s'ha proposat el receptor GPR55 com a possible tercer receptor cannabinoide. Aquesta hipòtesi se sustenta en què el receptor GPR55 és activat per diferents cannabinoides com el Δ^9-THC, la metanandamida i el JWH015[215]. A més, evidències recents de què tant el CB_1R com el CB_2R interaccionen amb el GPR55 formant complexos heteromèrics, així com la seva àmplia expressió tant a nivell de Sistema Nerviós Central com Perifèric, posen de manifest la importància del GPR55 en diversos processos cel·lulars i patologies, proposant-lo com a una prometedora diana per tractar la inflamació.

L'article "**Expression of GPR55 and either cannabinoid CB_1 or CB_2 heteroreceptor complexes in the caudate, putamen, and accumbens nuclei of control, parkinsonian, and dyskinetic non-human primates**" es va centrar a determinar si la malaltia de Parkinson es correlaciona amb una expressió alterada dels heteròmers CB_1R-GPR55 i/o CB_2R-GPR55. Els resultats obtinguts indiquen que l'expressió dels dos receptors es va veure significativament incrementada en micos tractats amb MPTP, model de la malaltia de Parkinson, tant en putamen, nucli accumbens com en caudat. És interessant destacar la disrupció dels heteròmers en animals Parkinsonians discinètics, a conseqüència d'un tractament crònic amb Levodopa. Aquesta alteració també havia estat detectada en l'heterotrímer $A_{2A}R$-D_2R-CB_1R[497]. Contràriament, també s'han descrit casos d'un increment d'expressió d'heteròmers en animals discinètics. Un exemple és l'heteròmer D_1R-D_3R, on es pot veure una millora de la discinèsia gràcies al bloqueig del receptor D_3 en macacos discinètics[498].

En un article recent, es va detectar l'expressió de l'heteròmer CB_1R-GPR55 en ganglis basals, en interneurones i en neurones de projecció en mostres de *Macaca fascicularis.* Usant un *tracer* dirigit a les subdivisions internes o externes del globus pallidus i mitjançant immunohistoquímica, es va demostrar que els heteròmers s'expressaven en les neurones de projecció estriatofugals. També es va detectar expressió en interneurones positives per òxid nítric i calretinina. En contraposició, les neurones colinèrgiques no expressaven l'heteròmer CB_1R-GPR55[499].

A l'hora de dissenyar fàrmacs, el receptor CB_1 es va considerar, en un principi, una millor diana terapèutica que CB_2R, a causa de la seva elevada expressió en neurones. Els nostres resultats indiquen que per contrarestar l'increment del receptor associat a la malaltia de Parkinson, la millor aproximació era emprar antagonistes de CB_1R o GPR55. Malauradament, la poca informació sobre fàrmacs que actuïn sobre GPR55 i els greus efectes secundaris del rimonabant, juntament amb els efectes psicoactius relacionats amb l'activació de CB_1R, ens van dirigir a centrar-nos en el CB_2R[492].

Els beneficis del CB_2R com a diana farmacològica en la malaltia del Parkinson es basen en la seva sobreexpressió en glia activada, que es dona simultàniament amb la neurodegeneració dopaminèrgica. Els nostres resultats obren les portes a un tractament no dopaminèrgic en pacients de Parkinson. Tot i que la presència de CB_2R en neurones és menys abundant que la de CB_1R, es pot trobar expressat de forma significativa en l'hipocamp, el còrtex, el cerebel i el globus pallidus[203]. Consistentment amb la neuroinflamació derivada del Parkinson, és molt possible que l'increment de l'expressió de l'heteròmer CB_2R-GPR55 sigui degut a l'activació de la micròglia. Els cannabinoides són agents reguladors de la neuroinflamació associada a malalties neurodegeneratives[500]. És per aquest motiu que buscar estratègies per polaritzar el fenotip M1 proinflamatori cap a un fenotip M2 neuroprotector és una prioritat, i s'ha vist que els cannabinoides tenen aquesta capacitat.

Des de fa anys hi ha literatura que indica que el GPR55 podria ser una diana terapèutica per tractar el Parkinson. L'estudi comentat anteriorment sobre la identificació de l'heteròmer CB_1R-GPR55 en neurones d'estriat també va concloure que l'heteròmer s'expressava tant en superfície cel·lular com en estructures intracel·lulars. En aquesta línia, s'ha descrit que el CB_1R podria estar present en mitocòndries i regular la funció mitocondrial per mitjà de cannabinoides[501]. Detectar els heteròmers CB_1R-GPR55 i CB_2R-GPR55 no és fàcil, però val la pena l'esforç per tal de veure si els cannabinoides poden afectar la producció d'energia neuronal a través d'aquests heteròmers i si les alteracions de la seva expressió en malalts de Parkinson es correlaciona amb canvis en l'expressió de l'heteròmer a nivell mitocondrial. L'alteració de la funcionalitat mitocondrial es perfila cada vegada més com a responsable de casos esporàdics de Parkinson[502]. Per tant, centrar-se en receptors intracel·lulars utilitzant cannabinoides permeables a la membrana plasmàtica, podria ser una molt bona estratègia per revertir el mal funcionament mitocondrial i prevenir la mort neuronal.

Encara falta molta recerca per descriure de manera acurada la implicació del receptor GPR55 en la patologia del Parkinson i les seves possibilitats com a diana terapèutica. A part d'investigar l'eficàcia dels seus agonistes, antagonistes o moduladors al·lostèrics en models de Parkinson, la seguretat dels lligands de GPR55, hauria de ser demostrada. L'afinitat, la potència i la selectivitat de diferents cannabinoides, tant sintètics com naturals, sobre el receptor GPR55, són inconsistents entre els diferents estudis publicats. Per exemple, mentre alguns reporten activitat agonista del rimonabant, utilitzant la β-arrestina com a *reporter*, altres indiquen que es comporta com un antagonista en assajos de mobilització intracel·lular de calci[503,215].

En conclusió, els resultats presentats en aquesta Tesi Doctoral aporten noves evidències del rol neuroprotector del Sistema Cannabinoide vers diverses patologies del Sistema Nerviós Central (Figura 18). El fet que el receptor CB_2 es trobi sobreexpressat en episodis de neuroinflamació posa de manifest la seva importància a l'hora d'oferir una resposta neuroprotectora. Aquest efecte l'hem observat en els resultats que es presenten en aquesta Tesi Doctoral en diferents models animals i en diferents patologies.

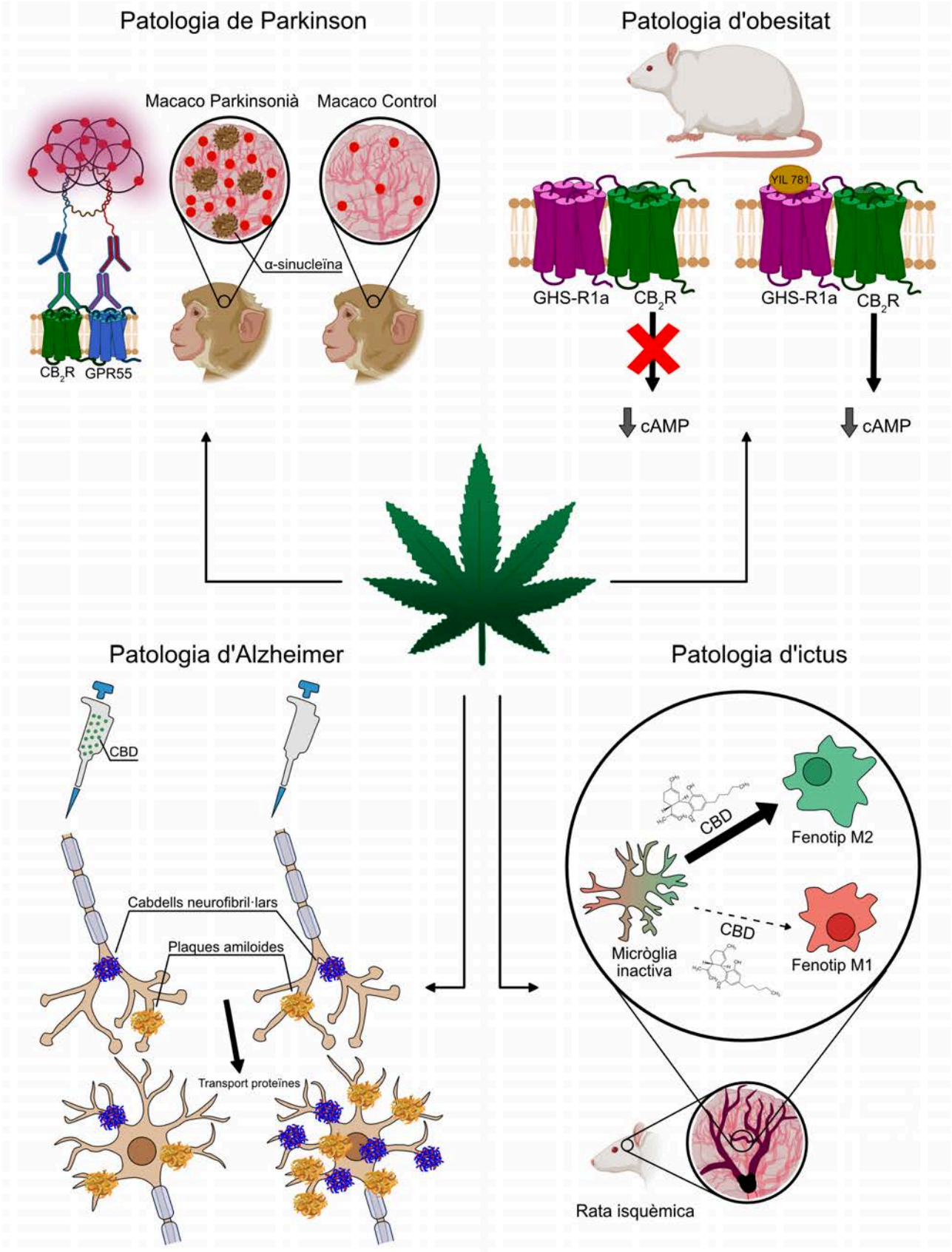

Figura 18: Resum de la implicació del Sistema Cannabinoide en diferents malalties a nivell de Sistema Nerviós Central

Tot i que el CB_2R és el receptor cannabinoide associat més directament amb la neuroprotecció, també hem observat que el CB_1R pot tenir un paper important. En la patologia de l'Alzheimer, els assaigs de qPCR indiquen que en ratolins 5xFAD l'expressió de CB_2R pot incrementar-se fins a gairebé 10 vegades, mentre que la de CB_1R es duplica.

El CBD, el segon component més abundant de la planta del cànnabis també juga un paper important en el tractament de les patologies amb un component inflamatori. Els resultats presentats en aquesta Tesi Doctoral demostren que el CBD pot modular el fenotip de la micròglia activada, a favor d'un fenotip neuroprotector en detriment d'un fenotip proinflamatori. Potser un dels efectes més atractius del CBD és la seva capacitat per inhibir el transport axonal de proteïnes associades a la patologia de l'Alzheimer i el Parkinson entre neurones, limitant l'afectació a neurones veïnes i alentint el progrés de la malaltia. Els dipòsits d'Aβ i pTau són tòxics per les neurones i mitjançant la tècnica d'immunocitoquímica, hem observat que afecten també a la formació de neurites. Sorprenentment, el tractament amb CBD és capaç de recuperar gairebé els nivells basals de neurites que presenta una neurona sana, revertint la pèrdua patològica d'aquestes neurites. Efectes semblants s'han obtingut amb la proteïna α-sinucleïna, que en la patologia de Parkinson es troba en forma d'agregats formant dipòsits tòxics per les neurones.

Tot i que fa molts anys que el Sistema Cannabinoide s'investiga, encara queda molt per estudiar dintre aquest camp. Els resultats d'aquesta Tesi Doctoral aporten noves dades en aquest trencaclosques per poder entendre amb més detall el funcionament d'aquest sistema tan complex, i poder explotar-lo per tractar malalties tan devastadores com les que afecten al Sistema Nerviós Central.

CONCLUSIONS

5. Conclusions

Conclusió 1. S'ha demostrat la sobreexpressió de l'heteròmer CB_2R-NMDAR en neurones model animal $APP_{Sw/Ind}$ de la patologia d'Alzheimer i en micròglia activada. Aquest heteròmer presenta un fenomen de *crosstalk* negatiu on la senyalització del NMDAR es veu truncada pel tractament amb agonistes cannabinoides en neurones d'hipocamp del model animal $APP_{Sw/Ind}$ de la patologia d'Alzheimer.

Conclusió 2. El receptor NMDA interacciona formant complexos heteromèrics amb els sensors de calci calmodulina, calneuron-1 i NCS1 però no amb caldendrina. La expressió de la calmodulina és necessària per l'activació del receptor NMDA tant en neurones com en micròglia i la expressió del NCS1 únicament en neurones. El tractament amb les proteïnes α-sinucleïna, Tau i pTau bloquejava la senyalització de les MAPK en el model animal $APP_{Sw/Ind}$ de la malaltia d'Alzheimer.

Conclusió 3. Els cannabinoides CBD i CBG afavoreixen la formació d'heteròmers CB_2R-$5HT_{1A}R$ i bloquegen l'efecte induït per la serotonina en la via mediada per les β-arrestines, mentre que potencien el seu efecte en la via de l'AMPc. El tractament amb CBD i CBG redueix l'expressió de l'heteròmer CB_2R-$5HT_{1A}R$ en un model de deprivació d'oxigen i glucosa com en un model animal d'isquèmia amb hipòxia. El CBD és capaç de polaritzar la micròglia activada a favor d'un fenotip neuroprotector M2 i en detriment d'un fenotip proinflamatori M1 i induïr un augment en l'activació tant d'astròcits com d'oligodendròcits en seccions de cervell del model animal d'ictus isquèmic. Demostrant el potencial neuroprotector del CBD en el procés d'isquèmia amb hipòxia.

Conclusió 4. El CBD és capaç de disminuir el transport axonal de les proteïnes Aβ, α-sinucleïna, Tau, i pTau. El CBD polaritzar la micròglia activada cap un fenotip neuroprotector M2 i potencia l'activació d'astròcits com d'oligodendròcits en seccions de cervell del model animal d'Alzheimer. El cannabidiol fa disminuir, tant en neurones corticals com d'hipocamp, els nivells d'expressió del mRNA dels receptors CB_1 i CB_2 sobreexpresats en la malaltia de l'Alzheimer.La formació de neurites es veu disminuida amb el tractament amb les proteïnes Aβ, Tau, i pTau i aquest efecte es veu revertit pel tractament amb CBD. El tractament amb CBD resulta en una millora cognitiva significativa de la memòria de reconeixement d'objectes en el model animal 5xFAD de la malaltia.

Conclusió 5. La formació de l'heteròmer CB_1R-GHS-R1a resulta en un bloqueig del receptor CB_1 i en una potenciació de la funcionalitat de GHS-R1a a través de la proteïna Gq. Aquesta potenciació és major a baixes concentracions d'ACEA. La interacció entre els receptors CB_2 i GHS-R1a resulta en un bloqueig del receptor CB_2 que es veu eliminat en presència d'antagonistes del receptor GHS-R1a. L'heteròmer CB_1R-GHS-R1a i el CB_2R-GHS-R1a s'expressen en cultius primaris de neurones i la seva expressió es veu incrementada en la progènie de ratolines alimentades amb una dieta alta en greixos.

Conclusió 6. Els heteròmers CB_1R-GPR55 i CB_2R-GPR55 es troben sobreexpressats en neurones d'estriat de seccions de cervells de *Macaca fascicularis* Parkinsonians. Aquest increment dels nivells d'expressió d'ambdós heteròmers es veu revertit fins als nivells del grup control en macacos Parkinsonians discinètics.

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ANNEXOS

7. Annexos

7.1 Col·laboracions

En el transcurs de la present Tesi Doctoral s'ha col·laborat en l'elaboració dels següents articles:

7.1.1 Irene Reyes-Resina, Hanan Awad Alkozi, Anna Del Ser-Badia, Juan Sánchez-Naves, **Jaume Lillo**, Jasmina Jiménez, Jesús Pintor, Gemma Navarro, Rafael Franco. **Expression of Melatonin and Dopamine D_3 Receptor Heteromers in Eye Ciliary Body Epithelial Cells and Negative Correlation with Ocular Hypertension.**

Article publicat a la revista *Cells*. Gener 2020.

7.1.2 Eva Martínez-Pinilla, Alberto J Rico, Rafael Rivas-Santisteban, **Jaume Lillo**, Elvira Roda, Gemma Navarro, Rafael Franco, José Luis Lanciego. **Expression of cannabinoid CB_1 R-GPR55 heteromers in neuronal subtypes of the Macaca fascicularis striatum.**

Article publicat a la revista *Frontiers in molecular neuroscience*. Agost 2020.

7.1.3 Izarbe Aísa-Marín, M José López-Iniesta, Santiago Milla, **Jaume Lillo**, Gemma Navarro, Pedro de la Villa, Gemma Marfany. **Nr2e3 functional domain ablation by CRISPR-Cas9D10A identifies a new isoform and generates retinitis pigmentosa and enhanced S-cone syndrome models.**

Article publicat a la revista *Neurobiology of disease*. Desembre 2020.

7.1.4 Cristina Miralpeix, Ana Cristina Reguera, Anna Fosch, Maria Casas, **Jaume Lillo**, Gemma Navarro, Rafael Franco, Josefina Casas, Stephen P H Alexander, Núria Casals, Rosalía Rodríguez-Rodríguez. **Carnitine palmitoyltransferase 1C negatively regulates the endocannabinoid hydrolase ABHD6 in mice, depending on nutritional status.**

Article publicat a la revista *British journal of pharmacology*. Abril 2021.

7.1.5 Rafael Rivas-Santisteban, **Jaume Lillo**, Ana Muñoz, Ana I Rodríguez-Pérez, José Luís Labandeira-García, Gemma Navarro, Rafael Franco. **Novel Interactions Involving the Mas Receptor Show Potential of the Renin-Angiotensin system in the Regulation of Microglia Activation: Altered Expression in Parkinsonism and Dyskinesia.**

Article publicat a la revista *Neurotherapeutics.* Abril 2021.

7.1.6 Mireia Casanovas, Irene Reyes-Resina, Alejandro Lillo, **Jaume Lillo**, Raul López-Arnau, Jorge Camarasa, Elena Escubedo, Gemma Navarro, Rafael Franco. **Methamphetamine Blocks Adenosine A_{2A} Receptor Activation via Sigma 1 and Cannabinoid CB_1 Receptors.**

Article publicat a la revista *International journal of molecular sciences*. Març 2021.

7.1.7 Rafael Franco, Rafael Rivas-Santisteban, **Jaume Lillo**, Jordi Camps, Gemma Navarro, Irene Reyes-Resina. **5-Hydroxytryptamine, Glutamate, and ATP: Much More Than Neurotransmitters.**

Article publicat a la revista *Frontiers in cell and developmental biology*. Abril 2021.

7.1.8 Iu Raïch, Rafael Rivas-Santisteban, Alejandro Lillo, **Jaume Lillo**, Irene Reyes-Resina, Xavier Nadal, Carlos Ferreiro-Vera, Verónica Sánchez de Medina, Maria Majellaro, Eddy Sotelo, Gemma Navarro, Rafael Franco. **Similarities and differences upon binding of naturally occurring Δ^9-tetrahydrocannabinol-derivatives to cannabinoid CB_1 and CB_2 receptors.**

Article publicat a la revista *Pharmacological research*. Desembre 2021.

7.1.9 Alejandro Lillo, Iu Raïch, **Jaume Lillo**, Catalina Pérez-Olives, Gemma Navarro, Rafael Franco. **Expression of the Adenosine A_{2A}-A_3 Receptor Heteromer in Different Brain Regions and Marked Upregulation in the Microglia of the Transgenic $APP_{Sw,Ind}$ Alzheimer's Disease Model.**

Article publicat a la revista *Biomedicines*. Gener 2022.

7.1.10 Iu Raïch, Joan Biel Rebassa, **Jaume Lillo**, Arnau Cordomí, Rafael Rivas-Santisteban, Alejandro Lillo, Irene Reyes-Resina, Rafael Franco, Gemma Navarro. **Antagonization of OX_1 Receptor Potentiates CB_2 Receptor Function in Microglia from $APP_{Sw/Ind}$ Mice Model.**

Article publicat a la revista *International journal of molecular sciences*. Octubre 2022.

7.1.11 Rafael Rivas-Santisteban, **Jaume Lillo**, Iu Raïch, Ana Muñoz, Alejandro Lillo, Ana I Rodríguez-Pérez, José L Labandeira-García, Gemma Navarro, Rafael Franco. **The cannabinoid CB_1 receptor interacts with the angiotensin AT_2 receptor. Overexpression of AT_2-CB_1 receptor heteromers in the striatum of 6-hydroxydopamine hemilesioned rats.**

Article publicat a la revista *Experimental Neurology*. Gener 2023.

6.2 Participació en articles de revisió

Mitjançant l'ús d'alguns dels resultats experimentals obtinguts en aquesta Tesis Doctoral s'han escrit les següents revisions:

6.2.1 Rafael Franco, David Aguinaga, Jasmina Jiménez, **Jaume Lillo**, Eva Martínez-Pinilla, Gemma Navarro. **Biased receptor functionality versus biased agonism in G-protein-coupled receptors**.

Revisió publicada a la revista *Biomolecular concepts*. Desembre 2018.

6.2.2 Catalina Pérez-Olives, Rafael Rivas-Santisteban, **Jaume Lillo**, Gemma Navarro, Rafael Franco. **Recent Advances in the Potential of Cannabinoids for Neuroprotection in Alzheimer's, Parkinson's, and Huntington's Diseases.**

Revisió publicada a la revista *Advances in experimental medicine and biology*. Desembre 2021.