Fundació
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Neurodegenerative diseases

CANNABINOID RECEPTOR HETEROMERIC COMPLEXES AS THERAPEUTIC TARGETS IN PARKINSON'S DISEASE

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1. Summary

The aim of the project was to assess the potential of three G-protein-coupled receptors, expressed in the striatum, to form heteromers and to be targets of Parkinson's disease and, possibly, to propose a cannabinoid-like drug (heteromer-selective agonist/antagonist, etc.) that could enter into clinical trials for the disease. First, we investigated the potential heteromerization and physiological role of cannabinoid CB_1 and CB_2 receptors and of GPR55, which was first described as a third cannabinoid receptor but was deorphanized as the receptor for L-*a*-lysophosphatidylcholine. Our results demonstrate that GPR55 may form heteromers with either CB1 or CB2 receptors. The resulting pharmacology of these heteromers is complex and our results do not fit with L-*a*-lysophosphatidylcholine being the endogenous agonist of GPR55. Upon a complete characterization of individual receptor and heteromeric complexes from the point of view of binding affinity and signaling, also taking into account what is known as biased agonism, a phytocannabinoid, cannabidiol (CBD) was selected. At nanomolar concentrations the compound acted as negative allosteric receptor of the CB_2 receptor. Another relevant finding was related to the expression of heteromers in activated microglia thus arising as therapeutic targets for neuroprotection, i.e. to skew cells to the neuroprotective (M2) phenotype.
In the model that more closely mimics the human pathology, *Macaca fascicularis* treated with 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), receptor heteromers were found with differential expression when control animals and non-dyskinetic and dyskinetic MPTP-treated animals were compared. Cannabidiol (CBD) as well as cannabigerol (CBG), another phytocannabinoid (characterized in the project framework), were tested in MPTP-treated animals and the results were quite noteworthy. On the one hand, CBD (not CBG) was able to improve motor scores of parkinsonian animals. On the other hand, CBD (not CBG) was also able to improve dyskinesia in parkinsonian animals chronically treated with levodopa, which in the long run produces dyskinesia in the human patient.

Taken together these results show that allosteric modulators of cannabinoid receptors may be useful in the therapy of Parkinson's disease and that cannabinoid-receptor-containing heteromers in microglia are targets for neuroprotection.

2. Excerpt of Results

In vitro assays

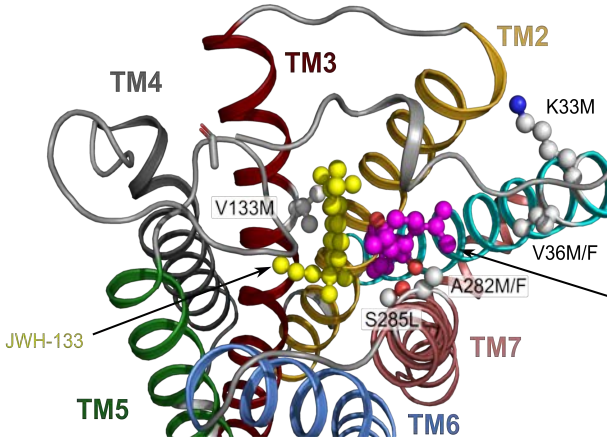
Following ad hoc cloning and procurement of the plasmids, it was demonstrated, in a heterologous expression system, that the GPR55 receptor can form heteromers with CB1 (CB1R) or CB2 (CB2R) receptors. In the last period of the first year, the trimer formed by the CB1, CB2 and GPR55 receptors was identified by means of SRET (Sequential BRET-FRET Technique). The formation of tetrameric complexes of CB1R and CB2R was also demonstrated, but not for other combinations of receptors under study.

An interaction between CB2R with the orphan receptor GPR18 was demonstrated, which, based on recent results, is important for the regulation of the endocannabinoid system. A lack of interaction between CB1R and GPR18 was also demonstrated. We have analyzed the functional consequences of this new CB2R-GPR18 complex and they may help to a better knowledge of the intricacies of the endocannabinoid system.
The method of obtaining primary cultures was refined and the expression of receptors in both cultures and samples from neurological tissues was quantified (using the technique: in situ proximity ligation assay - PLA-). Previously, anti-CB1R, anti-CB2R and anti-GPR55 antibodies were validated to perform immunohistochemistry and immunocytochemistry and PLA experiments. The conclusion is that heteromers are found in all conditions although (as indicated below) there is differential expression in parkinsonian conditions and/or in dyskinesia induced by "therapy" with levodopa.
We designed and synthesized a selective compound for CB2R that is conjugated to a fluorophore and that allowed to perform binding assays (binding) in living cells and in homogeneous fashion (HTRF). The HTRF assays made it possible to detect a second affinity state of the CB2R receptor that had not been previously described. It was confirmed, using minigenes that affect GPCR-G protein coupling, that CB1R and CB2R are coupled to Gi protein in any heteromeric context of those identified during the Project. We obtained evidence that the atypical results that have been found for some of the compounds used are not due to a multiple or partial coupling to Gs protein, but to an allosteric center. We discovered that tetrahydrocannabinol (THC) is not capable of coupling CB1R to Gi and this could be one of the reasons for its psychotropic effects.
One of the relevant results, both unexpected and positive (*in vitro* and *in vivo*), was the discovery that cannabidiol (CBD) behaves as an allosteric modulator of CB2R at nM

concentrations (it can go to the orthosteric center at concentrations in the μM range). An allosteric center for CBD in CB1R was reported in 2015 in another laboratory. The existence of these centers for CBD, "new" since they had not been described when the present Project was written, was taken into account in the functionality assays.
From having characterized the signaling of the heteromer, even studying the biased agonism in CB1R, CB2R or CB1-CB2Hets, the original strategy was modified; in particular based on the unreliability of the GPR55 receptor agonists and the development of new ligands by the group of Dr. Nagerovic of the CSIC of Madrid that provided monovalent and bivalent CB2R ligands. In radioactive binding assays none of the monovalent compounds was able to compete for receptor binding in isolated membranes; however, using the method developed by our laboratory, there was a binding of both monovalent and bivalent ligands (structures are omitted because there is a possibility of a patent application). The bivalent ligands modulated the functionality of CB2R in a multifactorial manner, and the most reasonable hypothesis is that it is due to a bitopic-like interaction. All the data have helped to delineate the amino acids that interact with the pharmacophore of the bivalent ligand.

The signaling experiments were carried out in cells expressing the CB2R (full-length / "wild type") or a receptor with point mutations at sites of potential binding of molecules that act allosterically and/or are bitopic. The receptor mutants were: Mut 1 K3.28 ---- Alanine; Mut 3 S6.58 ---- Alanine and Mut 4 S7.39 ---- Alanine. The results showed that mutants 1 and 4 give differential information that can help map the residues involved in the binding. It should be noted that CB2R forms homodimers (data generated throughout this Project) and that while canonical agonists such as monovalent ligands bind to a single molecule of CB2R, bivalent ligands may be halfway between two molecules of CB2R that form a dimer. Towards the end of the Project the model of the figure was generated (collaboration with L. Pardo and A. Cordomí, UAB):

Molecular model of the CB2 receptor in complex with the agonist JWH-133 and cannabidiol (solid spheres in yellow and magenta, respectively). Residues mutated to Alanine are also indicated as solid spheres, and the modifications they induce are shown by the structural differences of the helices (transmembrane domains, in color). The model is being refined based on the data from the elucidation of the structure of CB2R (Li et al., Cell, January 2019, 10.1016 / j.cell.2018.12.011)



Assays in *Macaca fascicularis*

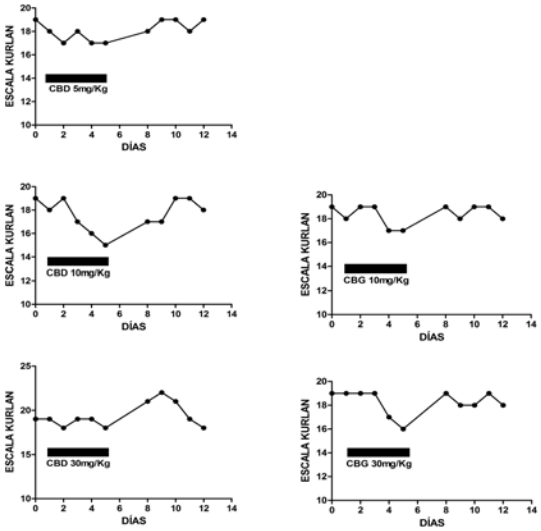
Dopaminergic depletion was induced using MPTP in a total of 9 primates. The achieved parkinsonian status was stable and non-reversible with Kurlan scale score ranging between 16 and 24. Dopaminergic depletion in 3 animals, estimated by microPET neuroimage studies using ^{11}C-dihydrotetrabenazine, was within 72 and 81%. Some animals needed a longer treatment with MPTP to acquire an irreversible parkinsonian state. Parkinsonian animals were treated (orally) with levodopa (under a chronic regime). Animals treated with levodopa were further stratified into dyskinetic and non-dyskinetic.

Immunological based assays were performed in brains obtained from perfused animals. To identify projecting neurons animals were subjected to stereotaxic surgery by injecting a retrograde tracer (BDA 3 kDa) in the external and internal subdivisions of the globus pallidus.

After completing the histological processing, it was possible to demonstrate the accuracy of the tracer deposits made by stereotactic surgery, as well as the presence of CB1-GPR55 and CB2-GPR55 heteromers in the brain areas of interest (caudate and putamen nuclei), as well as in the core and shell subdivisions of the nucleus accumbens. We have also obtained diverse data about its subcellular distribution by electron microscopy, data at the moment only referring to individual receptors and not in heteromeric configuration. The quantification performed has focused on 3 main parameters, namely (i) total density of heteromers by territory, (ii) average neuronal density per single cell and (iii) percentage of cells per territory with or without expression of heteromers. The characterization of the different cell types where these

heteromers are expressed (projection neurons and different types of interneurons in the areas of interest) is also completed. The data show a higher density of CB1-GPR55 heteromers than of CB2-GPR55 in all the subdivisions analyzed. Considering each heteromer individually, its highest density has been found in the shell of the nucleus accumbens, followed by the core of this nucleus. The post-commissural territories of putamen and caudate presented a higher density of both types of heteromers by comparison with the precommissural areas of both divisions. In parkinsonian animals there is an increase in the density of heteromeric endocannabinoid receptors in the caudate and putamen nuclei, preferably in their post-commissural territories, while both subdivisions of the nucleus accumbens reflected minor changes that did not reach statistical significance (taking into account that the dopaminergic innervation of the nucleus accumbens comes mostly from the ventral tegmental area, whose dopaminergic neurons are much less vulnerable to the neurodegeneration induced by MPTP, that is, it is a more preserved innervation and therefore represents a lesser impact on the referring to the number of quantized heteromers). All these upward changes in the territorial density of endocannabinoid receptors were normalized to levels observed in control animals after chronic treatment with levodopa. In order to validate the results obtained in an independent manner, all the staining carried out with CB1-GPR55 and CB2-GPR55 heteromers was repeated, proceeding to send the histological samples to Dr. Franco's laboratory at the University of Barcelona (analyzed there on a double-blind basis).

Finally, animals were treated with CBD and CBG. Due to space limitation, only the figure resulting from one of these experiments is included in this report. It summarizes the effect of CBD (at different concentrations) on the motor scores of the parkinsonian model. The panel corresponding to 10 mg/Kg (oral administration) led to a sustained reduction in motor scores (i.e. antiparkinsonian effect) that lasted for some days after treatment cessation. This result could support a clinical trial for using CBD (a very safe compound) in the therapy of patients suffering from Parkinson’s disease (see below).



3. Relevance and future prospects

This project provides evidence of cannabinoid receptors and cannabinoid receptors-containing heteromers in the striatum of models of Parkinson's disease and that there is variation of expression in different phases of the disease. The presence of receptors in the areas of interest and in vitro results indicate that they are therapeutic targets of this disease.

From a more therapeutic perspective, CBD, which is a natural product that modulates the effect of endogenous cannabinoids, is effective in the Parkinson's model closest to humans: the *Macaca fascicularis* primate treated with the neurotoxic 1-methyl -4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).

From the point of view of the human disease, the results in the first years of development of the project culminated in a fairly relevant result that leads to the presentation of clinical trials with Parkinson's patients. The intervention would consist of testing whether the CBD has beneficial effects both at the motor level and to reduce / eliminate dyskinesias caused as a result of the therapy with levodopa. Based on our data, several concentrations (in mg / Kg) of the compound should be tested. It is noteworthy that the compound is safe since safety is essential for the approval of a new drug by the US Food and Drug Administration (FDA). Effectively, the FDA has approved Epidiolex® (CBD preparation in sesame oil; https://www.epidiolex.com/) for

the treatment of childhood epilepsy. It is noteworthy that there is a proposal to conduct clinical trials for the use of CBD in neonates in cases of hypoxia.

Our data also allow the design of new compounds (synthesis) that are more potent than CBD. In this sense and after the recent elucidations of the three-dimensional structures of the cannabinoid receptors, we have located the probable zone of the allosteric union of the CBD. This will allow the design, in collaboration with Dr. Jagerovic of the CSIC (Institute of Medical Chemistry of Madrid), of novel synthetic compounds with therapeutic potential.

In summary, the results of the project allow us to test a new (first-in-class) treatment to fight Parkinson's and provide information to design new molecules that, through cannabinoid receptors, can combat Parkinson's and/or neurodegeneration associated with this or other neurodegenerative diseases.

4. Literature and theses produced

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PhD Theses (all with "*European mention*")

Author: Iñigo Etayo Labiano Year: 2018 Title: Interrelaciones moleculares y funcionales entre los receptores cannabinoides; CB1, CB2 y GPR55 Supervisors: Rafael Franco & Gemma Navarro

Author: Irene Reyes Resina Year: 2018 Title: GPR55, CB1 and CB2 receptor heteromers. From cell signalling to pharmacology Supervisors: Rafael Franco & Gemma Navarro

Author: Edgar Angelats Canals Year: 2018 Title: Relevant molecular and functional G-protein coupled receptors interactions in neuroinflammation and addiction. Supervisors: Gemma Navarro & Rafael Franco