

## Review

# Usefulness of identifying G-protein-coupled receptor dimers for diagnosis and therapy of neurodegenerative diseases and of gliomas

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**Summary.** Immunochemical detection of G-protein-coupled receptors (GPCRs) in cells and tissues was a technical challenge for years. After the discovery of formation of GPCR dimers/trimers/tetramers in transfected cells, a most recent challenge has been to confirm receptor-receptor interactions in natural sources. The occurrence of dimers or higher order oligomers is important from a therapeutic point of view, mainly because their physiology/pharmacology is different from those of individual receptors. On the one hand, pathophysiological factors need to count more on GPCR dimers than on individual receptors. On the other hand, the expression of dimers, trimers, etc. may change in pathological conditions and/or along the course of a disease. This review will focus on G-protein-coupled receptor dimers, on how to detect them by novel histological techniques and on how the detection may be used in diagnosis and therapy of ailments of the central nervous system, for instance in neurodegenerative diseases and gliomas.

**Key words:** CB<sub>1</sub>, CB<sub>2</sub>, Cannabinoid receptor, GPR55, Adenosine receptor, Dopamine receptor, D<sub>1</sub>, D<sub>2</sub>, D<sub>3</sub>, Heteroreceptor complex, Proximity ligation assay (PLA)

## Introduction

G-protein-coupled receptors (GPCRs) constitute the largest family of receptors in the mammalian genome. They receive the name because they couple to heterotrimeric G proteins that mediate signal transduction. They are also known as 7TM receptors as they have 7 heptahelical domains that span the plasma membrane and that separate the N-terminal extracellular and the C-terminal cytoplasmic domains (Prazeres and Martins, 2015). Studied for decades as monomeric structures it is now well established that GPCRs are able to form homo- and/or, heterodimers (Borroto-Escuela et al., 2014). They are even able to be expressed as higher-order structures (Cordomí et al., 2015). The most reliable structural model to date consists of four GPCRs and two G proteins. To be more precise, the macromolecular complex contains two adenosine A<sub>1</sub>, two adenosine A<sub>2A</sub> receptors and one G<sub>s</sub> protein and one G<sub>i</sub> protein (Navarro et al., 2016) (Fig. 1). One of the limitations of the research of GPCRs dimers is that direct interaction can almost exclusively be detected by energy transfer techniques, which require a heterologous cell model expressing constructs of fusion proteins consisting of receptors and donor or acceptor of energy transfer assays (Gomes et al., 2016). Then, the demonstration of dimers in natural sources has been a challenge that has led to novel technological approaches. One of them is functional-like and consists of identifying

the so-called heteromer signature, for instance the so-called cross-antagonism, by which the selective antagonist of one receptor also antagonizes the signal originating at the partner receptor in the heterodimer (Guidolin et al., 2015; Franco et al., 2016). The other one is histological in nature and will receive the focus in this review: *in situ* proximity ligation (Borroto-Escuela et al., 2016).

### Description of the *in situ* proximity ligation (PLA) assay

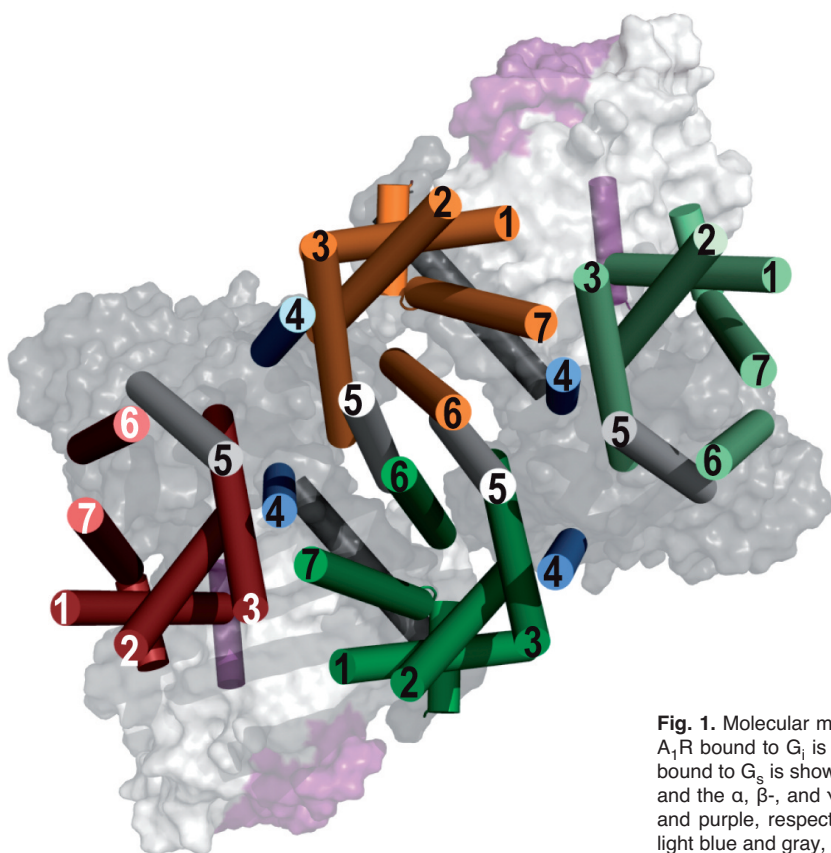
#### The beginnings

The PLA technique was first described in 2002, and it was used to detect zeptomol ( $40 \times 10^{-21}$  mol) amounts (in *inter alia* body fluids or cell culture media) of the homodimer of the platelet-derived growth factor B-chain (PDGF-BB). At that point of time the developers indicated that it was an homogeneous assay that “could be performed without washes or separations, and the mechanism could be generalized to other forms of protein analysis” (Fredriksson et al., 2002).

As the assay could be performed *in situ*, it was easily adapted to the study of localization and distribution of

proteins in tissues. Another possibility was to use the PLA for solid phase protein detection in the form of protein arrays. Overall, PLA was presented as a useful tool for proteomic analyses, and as able to solve some issues related to protein detection in high-throughput, high-performance protein analyses (Gullberg et al., 2003).

PLA was also considered for genome analyses. The idea was to look for gene sequence variants which could be relevant for diseases. PLA was seen as a tool with potential to identify gene usage in healthy and diseased tissues and, therefore, useful for diagnosis of diseases for which biopsies could be made. In 2005, PLA creators defined it as “a technology to convert hard analytical problems of detecting, quantifying, and localizing proteins, to the corresponding more tractable DNA analyses” (Gustafsdottir et al., 2005). The potential clinical usefulness of PLA included, i) detection of polymorphisms or mutations in nucleic acid sequences (Landegren et al., 2003, 2004), ii) quantification of protein expression and co-expression levels in body fluids such as in plasma but qualitatively and quantitatively expanding the clinical chemistry parameters useful for diagnosis of a huge variety of ailments (from cancer to infection and inflammation) or



**Fig. 1.** Molecular model of the  $A_1R$ - $A_{2A}R$  tetramer in complex with  $G_i$  and  $G_s$ .  $A_1R$  bound to  $G_i$  is shown in red,  $G_i$ -unbound  $A_1R$  is shown in orange,  $A_{2A}R$  bound to  $G_s$  is shown in dark green,  $G_s$ -unbound  $A_{2A}R$  is shown in light green, and the  $\alpha$ ,  $\beta$ -, and  $\gamma$ -subunits of  $G_i$  and  $G_s$  are shown in dark gray, light gray, and purple, respectively. Transmembrane helices 4 and 5 are highlighted in light blue and gray, respectively. Taken from (Navarro et al., 2016).

## Receptor heteromers in diagnosis and therapy

for following up the therapies (for instance recovery of immune-related responses), iii) detection and quantification of molecule-to-molecule interactions, for instance as a response to medication aimed at inhibiting protein-protein or ligand-receptor interactions, (as Avastin -bevacizumab-, that was designed to inhibit tumor angiogenesis) and iv) *in situ* analysis of expression levels and cellular and subcellular localization of interacting proteins. Since its development, PLA has been used for cancer diagnosis as a technique to identify biomarkers in malignancies such as in pancreas (Chang et al., 2009), breast (Poulard et al., 2014) or colon (Ghanipour et al., 2016) cancers. Due to the demonstration of direct GPCR-GPCR interaction, PLA has been adapted to identify GPCR dimers in natural sources (see below). To our knowledge pioneering articles using PLA for this specific purpose are those of (Borroto-Escuela et al., 2011; Trifilieff et al., 2011).

### The technical principle

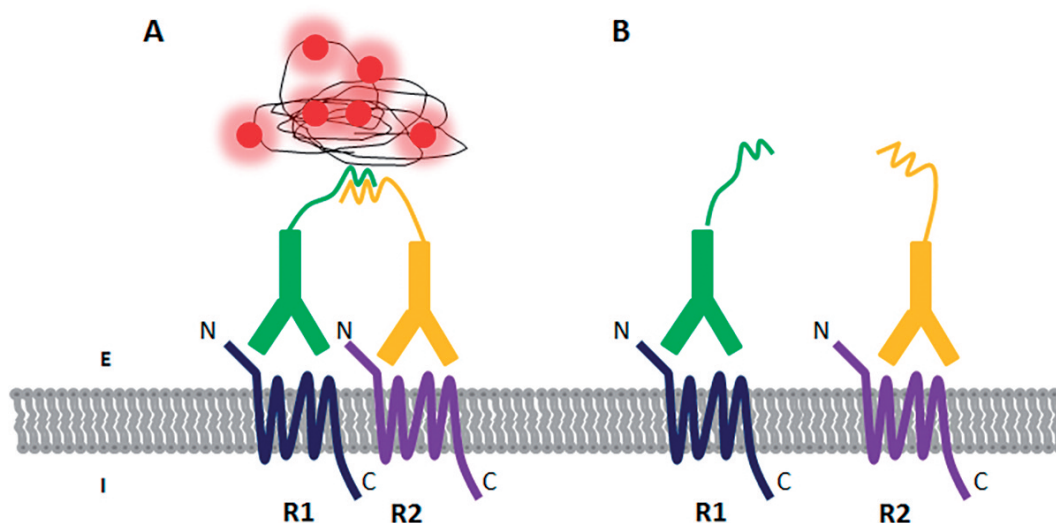
Detection of the presence/absence of receptor-receptor molecular interaction in the sample may be detected by using the Duolink II *in situ* PLA detection kit (Duolink® In Situ Detection Reagents Red, DUO92008, developed by Olink Bioscience, Uppsala, Sweden; and now distributed by Sigma-Aldrich as Duolink® using PLA® Technology). More details on the PLA protocol can be found elsewhere (Borroto-Escuela et al., 2016). A scheme of the technique is shown in Fig. 2.

In the case of using brain sections, brains must be obtained from animals perfused with a fixation solution or may (in small pieces) be fixed in 4% paraformaldehyde (for 24 h at 4°C). PBS washed samples may be cryo-preserved in a 30 % sucrose solution (48 h at 4°C) and stored at -20°C until

sectioning. 30 µm thick sections are cut using a cryostat. Sections are mounted on glass Superfrost™ Plus glass slides. Samples are washed with PBS containing 20 mM glycine (PBS-Gly), permeabilized with PBS containing 0.05% Triton X-100 for 15 min and washed with PBS-Gly. Blocking is performed by incubation for 1 h at 37°C with blocking solution (included in the PLA kit), and then specific antibodies against R1 and R2 receptors are incubated overnight at 4°C. Samples are then incubated (1 h at 37°C) with secondary antibodies containing the plus or the minus PLA probe (Duolink II PLA probe plus or minus). Sections are subsequently incubated with the ligation solution (1 h, 37°C) followed by the amplification solution (100 min, 37°C) (both included in the PLA kit). Finally, samples are mounted using Mowiol (475904, Calbiochem, Merck KGaA, Darmstadt, Germany). Nuclei are stained with Hoechst (1/200). To check for nonspecific labeling negative controls must be performed; among the different possibilities the most used is the omission of one of the primary antibodies. If possible a good option is to analyze sections from animals that are KO for one of the receptors.

Samples are observed in a confocal microscope equipped with an apochromatic 63x oil-immersion objective. For each field of view a stack of two channels (one per staining) and 10-20 Z stacks with a step size of 1 µm are acquired. A long pass filter of UV laser at 406-501 nm is used to visualize the emission from the laser at 364 nm and color coded in blue. The emission following excitation with the laser at 561 nm is filtered through a band pass filter of 576-648 nm and color coded in red to ensure appropriate visualization of the labeled elements and to avoid false positive results (Navarro et al., 2018).

Images taken from the fluorescence microscope must be analyzed, if possible by double blind procedure.



**Fig. 2.** Scheme of the PLA technique. Specific antibodies (green and yellow) linked to the plus and minus polynucleotide probes are specific for the two GPCRs (R1 and R2; blue and purple, respectively). If the two GPCRs are in close proximity the plus and minus probes will hybridize and the DNA amplification may take place (A), yielding (by using the appropriate reagent) a red fluorescent signal. If the GPCRs do not form an heteromer, no red spots/cluster are observed in the microscope (B). E: extracellular side, I: intracellular side; N: N-terminal end, C: C-terminal end.

We have tested an *ad hoc* macro and a devoted software developed by the commercial supplier: Duolink Image tool software (DUO90806, Sigma-Olink). The commercially available software is of election as it is user friendly and the parameters (size of cell, size of nucleus, etc.) may be easily modified. Apart from the total red fluorescence of the observation field, the most straightforward parameters are the number of cells that express red dots/clusters and the number of clusters per cell (to be more precise: number of clusters per cell expressing clusters, i.e. cells with no clusters do not count for calculation of the ratio).

### Use of PLA to study, in rodent and primate models of neurodegeneration, the expression of GPCR dimers involved in neurotransmission/neuroregulation

Parkinson's and Alzheimer's are the two main neurodegenerative diseases in modern Societies in which life expectancy is very high. Indeed, the main risk factor in non-familial cases of the two diseases is age (Lindsay et al., 2002; Collier et al., 2011). Furthermore, in the case of Parkinson's disease, the death of neurons in the substantia nigra occurs progressively and takes years to lead to clinical symptoms. It is estimated that the main characteristics of the disease, tremor, bradykinesia, etc., start to be manifested when circa 70% of dopaminergic neurons of the *substantia nigra* have disappeared (Heim et al., 2002; Hornykiewicz, 2006; Mahlknecht and Poewe, 2013; Mahlknecht et al., 2015).

Among the first GPCR dimers to be identified were those formed by adenosine  $A_1$  and dopamine  $D_1$  and of adenosine  $A_{2A}$  and dopamine  $D_2$  receptors (Gines et al., 2000; Hillion et al., 2002; Canals et al., 2003; Ciruela et al., 2004). They are involved in motor control and, consequently they must be taken into account for pathology mechanisms and also for therapy in Parkinson's disease. On the one hand, the first pair is found in the so-called direct pathway of motor control and the second pair of receptors is mainly found in the indirect pathway. Parkinson's disease indeed causes an alteration in the balance between direct and indirect pathways. On the other hand, dopamine replacement therapies (e.g. with levodopa, the most used medication (Birkmayer and Hornykiewicz, 1962, 1964)), target dopamine receptors both expressed as monomers and as heteromers ( $A_1$ - $D_1$ ,  $A_{2A}$ - $D_2$ , etc.).

The main problem of detecting how the expression of dimers change in neurodegenerative diseases is the lack of suitable neurological samples. Obviously, those samples must be obtained post-mortem (Braak and Braak, 1991; Goedert et al., 2012) and it is very reasonable to assume that not only the disease but the medications(s) are affecting GPCR-dimer expression. For such diseases, the only possibility is to detect dimers in animal models of the neurological disease. For Alzheimer's disease, there are mainly transgenic models (Franco and Cedazo-Minguez, 2014; Medina and Avila, 2014) whereas for Parkinson's disease there are rodent,

but more importantly, non-human primate models (Mendez and Finn, 1975; Burns et al., 1983; Snyder and D'Amato, 1986; Williams, 1986; Van Kampen et al., 2015).

It should be noted that adenosine receptors in both the direct and indirect pathways mediate a negative control on dopaminergic transmission. This effect of adenosine which seems, at least in part, to be mediated by adenosine-dopamine receptor heteromers, led to the proposal of adenosine receptor antagonists in the therapy of Parkinson's disease. The first adenosine receptor antagonist (istradefylline) has been approved in Japan for use with dopaminergic drugs in parkinsonian patients (Kondo et al., 2015). It is also of interest that patients using levodopa as dopamine-replacement may develop involuntary movements known as dyskinesias (Rinne, 1981; Chase, 1998; Vijayakumar and Jankovic, 2016).

In both rodent and non-human primate models of Parkinson's disease we have addressed the expression of adenosine-dopamine receptor heteromers. When possible we have also studied the formation of trimers formed by  $A_{2A}$ - $D_2$  plus cannabinoid  $CB_1$  receptors, which are the most abundant GPCRs in the CNS (Carriba et al., 2007, 2008; Navarro et al., 2008; Urigüen et al., 2009). In non-human primates, receptor heteromers were robustly expressed in the caudate nucleus of both naïve monkeys and monkeys treated with a neurotoxin addressed to produce the death of nigral dopaminergic neurons. However, the heteromers were virtually absent in animals chronically treated with levodopa and showing dyskinesia. At present, it is not known whether the disruption of heteromerization is a cause or consequence of dyskinesia. But what it is clear is that any adenosinergic or dopaminergic medication is targeting  $A_{2A}$ - $D_2$  receptor heteromers in parkinsonian animals but not in animals rendered dyskinetic by chronic levodopa treatment. Those results were deduced from PLA assays and from the presence/absence of heteromer signature in radioligand binding assays using membranes from caudate samples freshly obtained (Fig. 3) (Bonaventura et al., 2014). To our knowledge this was the first report showing alteration in heteromer expression in the course of a neurodegenerative disease.

Similar results were obtained in a rodent model of the disease, namely rats rendered parkinsonian by unilateral 6-hydroxydopamine (6-OHDA) lesion. The heteromer ( $A_{2A}$ - $D_1$ - $CB_1$  hetero-oligomer) signature was first detected by means of radioligand binding assays to freshly obtained striatal membranes. It was present in naïve and in parkinsonian animals. As in the primate model, a chronic treatment with levodopa led to the disappearance of the heteromer signature. In fact the drug used in patients (levodopa (Birkmayer and Hornykiewicz, 1962)) annihilated the molecular cross-talk established when the three receptors are directly interacting; hence levodopa leads to a structural and/or functional disruption of the receptor heteromer (Pinna et al., 2014).

Unlike the other three existing dopamine receptors

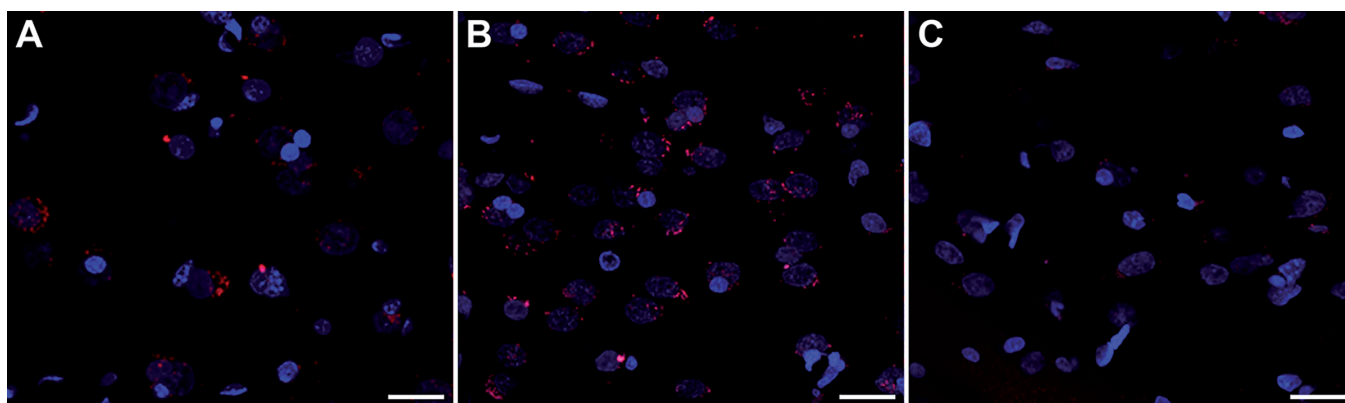


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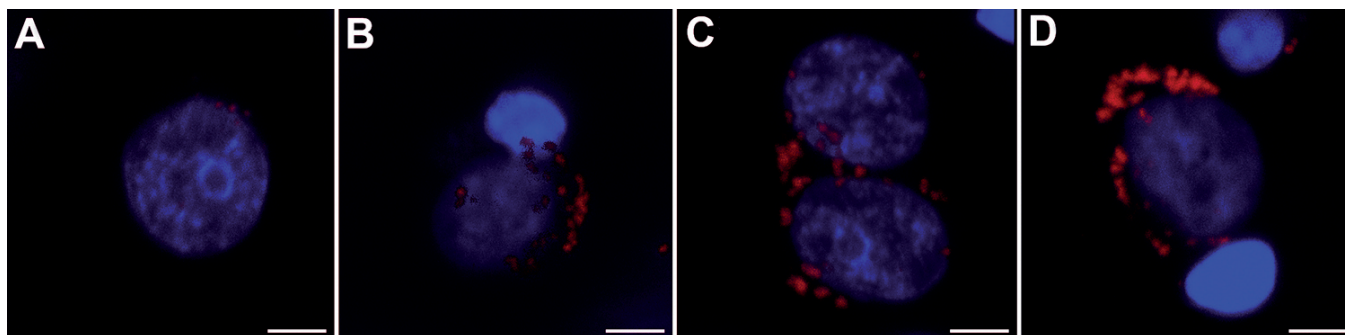
(D<sub>3</sub>, D<sub>4</sub> and D<sub>1</sub>), D<sub>1</sub> and D<sub>2</sub> have been extensively studied. However, the D<sub>3</sub> receptor is gaining momentum due to its possible relationship with dyskinesia. In fact, it is one of the targets that are being explored to combat levodopa-induced abnormal movements (Joyce, 2001; Bézard et al., 2003; Berthet and Bezard, 2009; Farré et al., 2015). First of all, it should be noted that D<sub>1</sub> and D<sub>3</sub> receptors, when expressed in the same cell/neuron, may directly interact (proposed in Fuxe et al. (1983)) and first shown in Marcellino et al. (2008). In both rodent and monkey models of Parkinson's disease the striatal expression of D<sub>1</sub>-D<sub>3</sub> receptor heteromers detected by PLA increased in rats rendered dyskinetic by chronic treatment with levodopa. Again, similar results were obtained when samples from dyskinetic non-human primates were analyzed (Fig. 4) (Farré et al., 2015). Interestingly, activation of D<sub>3</sub> receptors led in dyskinetic, but not in levodopa-treated or in lesioned rats, to a higher dopamine sensitivity. Aberrant activation of the

direct pathway and lack of right/left lateralization together with weak signaling via the indirect pathway may result in a motor dyskinesia-associated imbalance in the direct/indirect pathway. Therefore, there is a correlation between levodopa activating both receptors in dyskinetic patients and a lack of imbalance (i.e. a higher right/left striatal balance) in D<sub>1</sub> receptor-mediated neurotransmission. A recent report shows that mice lacking the D<sub>3</sub> receptor manifested a reduced dyskinesia without affecting the antiparkinsonian efficacy of levodopa (Solís et al., 2017). Overall, the stronger D<sub>1</sub> receptor-mediated neurotransmission in dyskinesia seems to be mediated by D<sub>1</sub>-D<sub>3</sub> receptor heteromers which arise as targets to combat this side effect of medication.

It should be noted that GPCRs may establish interactions with ionotropic glutamate receptors and that levodopa-induced dyskinesia correlates with changes in synaptic D<sub>1</sub>-NMDA glutamate receptor complexes



**Fig. 3.** A<sub>2A</sub>-CB<sub>1</sub> receptor heteromer expression in the monkey striatum. *In situ* proximity ligation assays (PLA) were performed using slices of the caudate nucleus of naive (A), MPTP-lesioned (B) or L-DOPA-treated dyskinetic (C) *Macaca fascicularis* monkeys, and primary antibodies for A<sub>2A</sub> and CB<sub>1</sub> receptors. Confocal microscopy images are shown (superimposed sections) in which heteromers appear as red clusters. Cell nuclei were stained with DAPI (blue). Modified from (Bonaventura et al., 2014). Scale bars: 20 μm.



**Fig. 4.** D<sub>1</sub>-D<sub>3</sub> receptor heteromer expression in monkey striatum. *In situ* proximity ligation assays were performed using caudate sections from *Macaca fascicularis*, untreated (B), treated with MPTP (C), and treated with MPTP and rendered dyskinetic by chronic L-DOPA administration (D). In A, negative controls were performed using only the anti-D<sub>1</sub> receptor primary antibody. Confocal microscopy images (superimposed sections) are shown; heteromers appear as red clusters surrounding DAPI-stained nuclei in blue. Modified from (Farré et al., 2015). Scale bars: 5 μm.

(Fiorentini et al., 2008). PLA has confirmed the occurrence of these macromolecular complexes in the central nervous system (Rodríguez-Ruiz et al., 2017).

Very recent results have provided evidence concerning the expression of another heteromer, formed by cannabinoid CB<sub>1</sub> and CB<sub>2</sub> receptors, in non-neuronal (i.e. glial) cells. Activation of microglial cells, which are the main mediators of neuroinflammation, may lead to two main phenotypes: neuroprotective or neuro-inflammatory (see Franco and Fernández-Suárez (2015) for review). Unlike CB<sub>1</sub>, which is expressed in resting microglia and mildly upregulates upon activation, CB<sub>2</sub> receptors and CB<sub>1</sub>-CB<sub>2</sub> heteroreceptor complexes are markedly upregulated in microglial cells treated with interferon-gamma and also in striatal sections from hemilesioned parkinsonian rats. Furthermore, a high number of activated microglia was found in lesioned and in levodopa treated rats (dyskinetic or non-dyskinetic) but not in control animals. Cannabinoid receptor heteromers in activated cells are robustly coupled to the signaling machinery and evidence supports a neuroprotective role. Interestingly, PLA showed that the percentage of striatal microglial cells showing red clusters corresponding to heteromers was similar (<10%) in naïve animals, in the control hemisphere of lesioned animals, and on the lesioned hemisphere of non-dyskinetic levodopa treated animals. In contrast, heteromer expression increased to 34% in dyskinetic animals thus showing a correlation between abnormal movements and CB<sub>1</sub>-CB<sub>2</sub> receptor heteromer expression. It remains to be established whether activation of overexpressed cannabinoid receptor heteromers in dyskinetic animals is beneficial or detrimental. In any case these heteromers expressed in microglial cells constitute a target to be explored to combat dyskinetic states (Navarro et al., 2018).

### Potential of PLA to detect heteroreceptor complexes in gliomas

Gliomas constitute a set of tumorous diseases with devastating effects and in urgent need of more therapeutic tools (Ahmed and Chinnaiyan, 2014; Kamran et al., 2016). One of the strategies to expand the therapeutic arsenal is to advance in phenotypic characterization. Among the GPCR superfamily, cannabinoid receptors appear as important for glioma fate. Some low grade pediatric gliomas may undergo spontaneous involution after surgical removal of a high percentage of the tumor mass. In results reported by (Sredni et al., 2016) the gene encoding for CB<sub>1</sub> receptor is overexpressed in those tumors that are likely to undergo involution. The authors concluded that the endocannabinoid system is, in those cases, involved in tumor regression. Equally relevant (in our opinion) is the finding of a polymorphism, G1359A, in the gene of the receptor that is associated to glioma patients in Spain (Núñez et al., 2010).

In experiments performed using glioma cells from

patients it is confirmed that CB<sub>1</sub> receptor may have a key role. In fact expression of the receptor, whose activation impacts on a cancer-relevant molecule, STAT3, correlates with the effect of a selective receptor antagonist (Ciaglia et al., 2015). These results provide evidence on the potential of receptor antagonist (in multimodal anti-glioma therapy) but only if the expression of the CB<sub>1</sub> receptors in glioma is significant.

As expected, due to its higher expression in glial than in neuronal cells, gliomas may also express the CB<sub>2</sub> receptor and, therefore, any potential anti-tumor action of cannabinoid receptor ligands should take into account the two receptors (Ellert-Miklaszewska et al., 2013; Zogopoulos et al., 2015). On the one hand, (De Jesús et al., 2010) have reported that the expression of the two proteins in human gliomas samples is diverse, i.e. gliomas having more CB<sub>2</sub> receptors have (relatively) less CB<sub>1</sub> receptors and vice versa. On the other hand, astrocytoma cells expressing different proportions of cannabinoid receptors show different susceptibility to undergo cannabinoid-induced apoptosis (Cudaback and Stella, 2007; Cudaback et al., 2010).

GPR55 is a recent orphanized receptor whose endogenous ligand is lysophosphatidylinositol. GPR55 has been associated to cancer development and progression although the underlying mechanism is poorly known (see (Hu et al., 2011; Falasca and Ferro, 2016) for review). Taking three ovarian cell lines as a model (Hofmann et al., 2015) has provided evidence suggesting that lysophosphatidylinositol mediates cell-induced angiogenesis. In cell lines from two unrelated cancer types, GPR55 activation results in calcium mobilization and activation of Akt and MAP kinases. Furthermore, authors discovered an autocrine loop by which the trigger of GPR55 activation is lysophosphatidylinositol produced by phospholipase A2 and released to the extracellular milieu (Piñeiro et al., 2011). Another example showing (by indirect evidence) a link between GPR55 and cancer is upregulated in human squamous cell carcinomas and has a potential role in driving skin carcinogenesis (Andradas et al., 2011).

Interestingly, GPR55 may form heteromers with CB<sub>1</sub> (Martínez-Pinilla et al., 2014) or with CB<sub>2</sub> (Balenga et al., 2014; Moreno et al., 2014) receptors. As above indicated the two receptors may form heteromers that are overexpressed in activated microglia. Accordingly, we speculate that phenotypic characterization, including as novel parameters the expression of CB<sub>1</sub>-CB<sub>2</sub>, CB<sub>1</sub>-GPR55 and/or CB<sub>2</sub>-GPR55 complexes, may help in better classifying the tumor type, in deciding the most appropriate optimal therapeutic approach and in guiding anti-glioma drug discovery efforts.

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*Conflict of interest.* Authors declare no conflict of interest.

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