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

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Abstract

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.

Keywords: CB1, CB2, cannabinoid receptor, GPR55, adenosine receptor, dopamine receptor, D1, D2, D3, Heteroreceptor complex, proximity ligation assay (PLA)

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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_1, two adenosine A_{2A} receptors and one G_s protein and one G_i protein (Navarro et al., 2016) (Figure 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 (40×10^{-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*

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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 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 pancreatic (Chang et al., 2009), breast (Poulard et al., 2014) or colorectal (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 Figure 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 4ºC). PBS washed samples may be cryo-preserved in a 30 % sucrose solution (48 h at 4ºC) 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 37ºC with blocking solution (included in the PLA kit), and then specific antibodies against R1 and R2 receptors are incubated overnight at 4 ºC. Samples are then incubated (1 h at 37ºC) 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, 37ºC) followed by the amplification solution (100 min, 37ºC) (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

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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., 2017).

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

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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 (Figure 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_2-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 (D_3, D_4 and D_5), D_1 and D_2 have been extensively studied. However, the D_3 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_1 and D_3 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_1–D_3 receptor heteromers detected by PLA increased in rats rendered dyskinetic

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by chronic treatment with levodopa. Again, similar results were obtained when samples from dyskinetic non-human primates were analyzed (Figure 4) (Farré et al., 2015). Interestingly, activation of D_3 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_1 receptor-mediated neurotransmission. A recent report shows that mice lacking the D_3 receptor manifested a reduced dyskinesia without affecting the antiparkinsonian efficacy of levodopa (Solís et al., 2017). Overall, the stronger D_1 receptor-mediated neurotransmission in dyskinesia seems to be mediated by D_1-D_3 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_1-NMDA glutamate receptor complexes (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_1 and CB_2 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 neuroinflammatory (see (Franco and Fernández-Suárez, 2015) for review). Unlike CB_1, which is expressed in resting microglia and mildly upregulates upon activation, CB_2 receptors and CB_1-CB_2 heteroreceptor complexes are markedly upregulated in microglial cells treated win 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_1-CB_2 receptor heteromer expression. It remains to be established whether activation of overexpressed cannabinoid receptor heteromers in dyskinetic animals is beneficious or detrimental. In any case these heteromers expressed in microglial cells constitute a target to be explored to combat dyskinetic states (Navarro et al., 2017).

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

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high percentage of the tumor mass. In results reported by (Sredni et al., 2016) the gene encoding for CB1 receptor is overexpressed in those tumors that are likely to undergo involution. The authors conclude 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_1 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_1 receptors in glioma is significant.

As expected, due to its higher expression in glial than in neuronal cells, gliomas may also express the CB_2 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_2 receptors have (relatively) less CB_1 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 deorphanized 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_1 (Martínez-Pinilla et al., 2014) or with CB_2 (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_1-CB_2, CB_1-GPR55 and/or CB_2-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.

References

Ahmed K.A. and Chinnaiyan P. (2014). Applying metabolomics to understand the aggressive phenotype and identify novel therapeutic targets in glioblastoma. *Metabolites* 4, 740-750.

Andradas C., Caffarel M.M., Pérez-Gómez E., Salazar M., Lorente M., Velasco G., Guzmán M. and Sánchez C. (2011). The orphan G protein-coupled receptor GPR55 promotes cancer cell proliferation via ERK. *Oncogene* 30, 245-252.

Balenga N., Martínez-Pinilla E., Kargl J., Schröder R., Peinhaupt M., Platzer W., Bálint Z., Zamarbide M., Dopeso-Reyes I., Ricobaraza A., Pérez-Ortiz J.M., Kostenis E., Waldhoer M., Heinemann A. and Franco R. (2014). Heteromerization of GPR55 and cannabinoid CB2 receptors modulates signaling. *Br. J. Pharmacol.* 1-64.

Berthet A. and Bezard E. (2009). Dopamine receptors and l-dopa-induced dyskinesia. *Parkinsonism. Relat. Disord.* 15, S8-S12.

Bézard E., Ferry S., Mach U., Stark H., Leriche L., Boraud T., Gross C. and Sokoloff P. (2003). Attenuation of levodopa-induced dyskinesia by normalizing dopamine D3 receptor function. *Nat. Med.* 9, 762-767.

Birkmayer W. and Hornykiewicz O. (1962). [The L-dihydroxyphenylalanine (L-DOPA) effect in Parkinson's syndrome in man: On the pathogenesis and treatment of Parkinson akinesis]. *Arch. Psychiatr. Nervenkr. Z Gesamte Neurol. Psychiatr.* 203, 560-574.

Birkmayer W. and Hornykiewicz O. (1964). Additional experimental studies on L-DOPA in Parkinson's syndrome and reserpine parkinsonism. *Arch. Psychiatr. Nervenkr.* 206, 367-381.

Bonaventura J., Rico A.J., Moreno E., Sierra S., Sánchez M., Luquin N., Farré D., Müller C.E., Martínez-Pinilla E., Cortés A., Mallol J., Armentero M-T., Pinna A., Canela E.I., Lluís C., McCormick P.J., Lanciego J.L., Casadó V. and Franco R. (2014). L-DOPA-treatment in primates disrupts the expression of A(2A) adenosine-CB(1) cannabinoid-D(2) dopamine receptor heteromers in the caudate nucleus. *Neuropharmacology* 79, 90-100.

Borroto-Escuela D.O., Brito I., Romero-Fernandez W., Di Palma M., Oflijan J., Skieterska K., Duchou J., Van Craenenbroeck K., Suárez-Boomgaard D., Rivera A., Guidolin D., Agnati L.F. and Fuxe K. (2014). The G protein-coupled receptor heterodimer network (GPCR-HetNet)

HISTOLOGY AND HISTOPATHOLOGY (non-edited manuscript)

and its hub components. *Int. J. Mol. Sci.* 15, 8570-8590.

Borroto-Escuela D.O., Van Craenenbroeck K., Romero-Fernandez W., Guidolin D., Woods A.S., Rivera A., Haegeman G., Agnati L.F., Tarakanov A.O. and Fuxe K. (2011). Dopamine D2 and D4 receptor heteromerization and its allosteric receptor-receptor interactions. *Biochem. Biophys. Res. Commun.* 404, 928-934.

Borroto-Escuela D.O., Hagman B., Woolfenden M., Pinton L., Jiménez-Beristain A., Oflijan J., Narvaez M., Di Palma M., Feltmann K., Sartini S., Ambrogini P., Ciruela F., Cuppini R. and Fuxe K. (2016). In situ proximity ligation assay to study and understand the distribution and balance of GPCR homo- and heteroreceptor complexes in the brain. *Neuromethods* 110, 109-124.

Braak H. and Braak E. (1991). Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathol.* 82, 239-259.

Burns R.S., Chiueh C.C., Markey S.P., Ebert M.H., Jacobowitz D.M. and Kopin I.J. (1983). A primate model of parkinsonism: selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Proc. Natl. Acad. Sci. USA* 80, 4546-4550.

Canals M., Marcellino D., Fanelli F., Ciruela F., De Benedetti P., Goldberg S.R., Neve K., Fuxe K., Agnati L.F., Woods A.S., Ferré S., Lluis C., Bouvier M. and Franco R. (2003). Adenosine A2A-dopamine D2 receptor-receptor heteromerization: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. *J. Biol. Chem.* 278, 46741-46749.

Carriba P., Navarro G., Ciruela F., Ferré S., Casadó V., Agnati L., Cortés A., Mallol J., Fuxe K., Canela E.I.E.I., Lluís C. and Franco R. (2008). Detection of heteromerization of more than two proteins by sequential BRET-FRET. *Nat. Methods* 5, 727-733.

Carriba P., Ortiz O., Patkar K., Justinova Z., Stroik J., Themann A., Müller C., Woods A.S., Hope B.T., Ciruela F., Casadó V., Canela E.I., Lluis C., Goldberg S.R., Moratalla R., Franco R. and Ferré S. (2007). Striatal Adenosine A2A and Cannabinoid CB1 Receptors Form Functional Heteromeric Complexes that Mediate the Motor Effects of Cannabinoids. *Neuropsychopharmacology* 32, 2249–2259.

Chang S.T., Zahn J.M., Horecka J., Kunz P.L., Ford J.M., Fisher G.A., Le Q.T., Chang D.T., Ji H. and Koong A.C. (2009). Identification of a biomarker panel using a multiplex proximity ligation assay improves accuracy of pancreatic cancer diagnosis. *J. Transl. Med* 7, 105.

Chase T.N. (1998). The significance of continuous dopaminergic stimulation in the treatment of Parkinson's disease. *Drugs* 55 Suppl. 1, 1-9.

Ciaglia E., Torelli G., Pisanti S., Picardi P., D'Alessandro A., Laezza C., Maria Malfitano A., Fiore D., Pagano Zottola A.C., Chiara Proto M., Catapano G., Gazzerro P. and Bifulco M. (2015). Cannabinoid receptor CB1 regulates STAT3 activity and its expression dictates the responsiveness to SR141716 treatment in human glioma patients’ cells. *Oncotarget* 6, 15464–15481.

Ciruela F., Burgueño J., Casadó V., Canals M., Marcellino D., Goldberg S.R., Bader M., Fuxe K., Agnati L.F., Lluis C., Franco R., Ferré S. and Woods A.S. (2004). Combining mass spectrometry and pull-down techniques for the study of receptor heteromerization.

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Direct epitope-epitope electrostatic interactions between adenosine A2A and dopamine D2receptors. *Anal Chem.* 76, 5354-5363.

Collier T.J., Kanaan N.M. and Kordower J.H. (2011) Ageing as a primary risk factor for Parkinson's disease: evidence from studies of non-human primates. *Nat. Rev. Neurosci.* 12, 359-366.

Cordomí A., Navarro G., Aymerich M.S. and Franco R. (2015). Structures for G-Protein-Coupled Receptor Tetramers in Complex with G Proteins. *Trends Biochem. Sci.* 40, 548-551.

Cudaback E., Marrs W., Moeller T. and Stella N. (2010). The Expression Level of CB1 and CB2 Receptors Determines Their Efficacy at Inducing Apoptosis in Astrocytomas. *PLoS One* 5:e8702.

Cudaback E. and Stella N. (2007). Targeting Astrocytomas and Invading Immune Cells with Cannabinoids: A Promising Therapeutic Avenue. *Mol. Neurobiol.* 36, 36-44.

Ellert-Miklaszewska A., Ciechomska I. and Kaminska B. (2013). Cannabinoid Signaling in Glioma Cells. In: *Advances in experimental medicine and biology*. pp. 209-220.

Falasca M. and Ferro R. (2016). Role of the lysophosphatidylinositol/GPR55 axis in cancer. *Adv. Biol. Regul.* 60, 88-93.

Farré D., Muñoz A., Moreno E., Reyes-Resina I., Canet-Pons J., Dopeso-Reyes I.G., Rico A.J., Lluís C., Mallol J., Navarro G., Canela E.I., Cortés A., Labandeira-García J.L., Casadó V., Lanciego J.L. and Franco R. (2015). Stronger Dopamine D1 Receptor-Mediated Neurotransmission in Dyskinesia. *Mol. Neurobiol.* 52, 1408-1420.

Fiorentini C., Busi C., Spano P. and Missale C. (2008). Role of receptor heterodimers in the development of l-dopa-induced dyskinesias in the 6-hydroxydopamine rat model of Parkinson's disease. *Parkinsonism Relat. Disord.* 14, S159-S164.

Franco R. and Cedazo-Minguez A. (2014). Successful therapies for Alzheimer's disease: why so many in animal models and none in humans? *Front Pharmacol.* 5, 146.

Franco R. and Fernández-Suárez D. (2015). Alternatively activated microglia and macrophages in the central nervous system. *Prog. Neurobiol.* 131, 65-86.

Franco R., Martínez-Pinilla E., Lanciego J.L.J.L. and Navarro G. (2016) Basic Pharmacological and Structural Evidence for Class A G-Protein-Coupled Receptor Heteromerization. *Front Pharmacol.* 7, 76.

Fredriksson S., Gullberg M., Jarvius J., Olsson C., Pietras K., Gústafsdóttir S.M., Ostman A. and Landegren U. (2002). Protein detection using proximity-dependent DNA ligation assays. *Nat. Biotechnol.* 20, 473-477.

Fuxe K., Agnati L.F., Benfenati F., Celani M., Zini I., Zoli M. and Mutt V. (1983). Evidence for the existence of receptor--receptor interactions in the central nervous system. Studies on the regulation of monoamine receptors by neuropeptides. *J. Neural Transm. Suppl* 18, 165-179.

Ghanipour L., Darmanis S., Landegren U., Glimelius B., Påhlman L. and Birgisson H. (2016). Detection of Biomarkers with Solid-Phase Proximity Ligation Assay in Patients with

HISTOLOGY AND HISTOPATHOLOGY (non-editted manuscript)

Colorectal Cancer. *Transl. Oncol.* 9, 251-255.

Gines S., Hillion J., Torvinen M., Le Crom S., Casado V., Canela E.I., Rondin S., Lew J.Y., Watson S., Zoli M., Agnati L.F., Verniera P., Lluis C., Ferre S., Fuxe K. and Franco R. (2000). Dopamine D1 and adenosine A1 receptors form functionally interacting heteromeric complexes. *Proc. Natl. Acad. Sci.* 97, 8606-8611.

Goedert M., Spillantini M.G., Del Tredici K. and Braak H. (2012). 100 years of Lewy pathology. *Nat. Rev. Neurol.* 9, 13-24.

Gomes I., Ayoub M.A., Fujita W., Jaeger W.C., Pfleger K.D.G. and Devi L.A. (2016). G Protein-Coupled Receptor Heteromers. *Annu. Rev. Pharmacol. Toxicol.* 56, 403-425.

Guidolin D., Agnati L.F., Marcoli M., Borroto-Escuela D.O. and Fuxe K. (2015). G-protein-coupled receptor type A heteromers as an emerging therapeutic target. *Expert Opin. Ther. Targets* 19, 265-283.

Gullberg M., Fredriksson S., Taussig M., Jarvius J., Gustafsdottir S. and Landegren U. (2003). A sense of closeness: protein detection by proximity ligation. *Curr. Opin. Biotechnol.* 14, 82-86.

Gustafsdottir S.M., Schallmeiner E., Fredriksson S., Gullberg M., Söderberg O., Jarvius M., Jarvius J., Howell M. and Landegren U. (2005). Proximity ligation assays for sensitive and specific protein analyses. *Anal Biochem.* 345, 2-9.

Heim C., Sova L., Kurz T., Kolasiewicz W., Schwegler H. and Sontag K.H. (2002). Partial loss of dopaminergic neurons in the substantia nigra, ventrotegmental area and the retrorubral area - Model of the early beginning of Parkinson's symptomatology?. *J. Neural. Transm.* 109, 691-709.

Hillion J., Canals M., Torvinen M., Casado V., Scott R., Terasmaa A., Hansson A., Watson S., Olah M.E., Mallol J., Canela E.I., Zoli M., Agnati L.F., Ibanez C.F., Lluis C., Franco R., Ferre S. and Fuxe K. (2002). Coaggregation, cointernalization, and codesensitization of adenosine A2A receptors and dopamine D2 receptors. *J. Biol. Chem.* 277, 18091–18097.

Hofmann N.A., Yang J., Trauger S.A., Nakayama H., Huang L., Strunk D., Moses M.A., Klagsbrun M., Bischoff J. and Graier W.F. (2015). The GPR 55 agonist, L-α-lysophosphatidylinositol, mediates ovarian carcinoma cell-induced angiogenesis. *Br. J. Pharmacol.* 172, 4107-4118.

Hornykiewicz O. (2006). The discovery of dopamine deficiency in the parkinsonian brain. *J. Neural. Transm. Suppl*, 9-15.

Hu G., Ren G. and Shi Y. (2011). The putative cannabinoid receptor GPR55 promotes cancer cell proliferation. *Oncogene* 30, 139-141.

De Jesús M.L., Hostalot C., Garibi J.M., Sallés J., Meana J.J. and Callado L.F. (2010). Opposite changes in cannabinoid CB1 and CB2 receptor expression in human gliomas. *Neurochem. Int.* 56, 829-833.

Joyce J.N. (2001). Dopamine D3 receptor as a therapeutic target for antipsychotic and antiparkinsonian drugs. *Pharmacol. Ther.* 90, 231-259.

Van Kampen J.M., Baranowski D.C., Robertson H.A., Shaw C.A. and Kay D.G. (2015). The

HISTOLOGY AND HISTOPATHOLOGY
(non-edited manuscript)

Progressive BSSG Rat Model of Parkinson's: Recapitulating Multiple Key Features of the Human Disease. *PLoS One* 10, e0139694.

Kamran N., Calinescu A., Candolfi M., Chandran M., Mineharu Y., Asad A.S., Koschmann C., Nunez F.J., Lowenstein P.R. and Castro M.G. (2016). Recent advances and future of immunotherapy for glioblastoma. *Expert Opin. Biol. Ther.* 16, 1245-1264.

Kondo T., Mizuno Y. and Japanese Istradefylline Study Group (2015). A long-term study of istradefylline safety and efficacy in patients with Parkinson disease. *Clin. Neuropharmacol.* 38, 41-46.

Landegren U., Dahl F., Nilsson M., Fredriksson S., Banér J., Gullberg M., Jarvius J., Gustafsdottir S., Söderberg O., Ericsson O., Stenberg J. and Schallmeiner E. (2003). Padlock and Proximity Probes for *In Situ* and Array-Based Analyses: Tools for the Post-Genomic Era. *Comp. Funct Genomics* 4, 525-530.

Landegren U., Schallmeiner E., Nilsson M., Fredriksson S., Banér J., Gullberg M., Jarvius J., Gustafsdottir S., Dahl F., Söderberg O., Ericsson O. and Stenberg J. (2004). Molecular tools for a molecular medicine: analyzing genes, transcripts and proteins using padlock and proximity probes. *J. Mol. Recognit.* 17, 194-197.

Lindsay J., Laurin D., Verreault R., Hébert R., Helliwell B., Hill G.B. and McDowell I. (2002). Risk factors for Alzheimer's disease: A prospective analysis from the Canadian Study of Health and Aging. *Am. J. Epidemiol.* 156, 445-453.

Mahlknecht P. and Poewe W. (2013). Is there a need to redefine Parkinson's disease? *J. Neural. Transm.* 120, 9-17.

Mahlknecht P., Seppi K. and Poewe W. (2015). The concept of prodromal Parkinson's disease. *J. Parkinsons Dis.* 5, 681-697.

Marcellino D., Ferré S., Casadó V., Cortés A., Le Foll B., Mazzola C., Drago F., Saur O., Stark H., Soriano A., Barnes C., Goldberg S.R., Lluis C., Fuxe K. and Franco R. (2008). Identification of dopamine D1-D3 receptor heteromers: Indications for a role of synergistic D1-D3 receptor interactions in the striatum. *J. Biol. Chem.* 283, 26016-26025.

Martínez-Pinilla E., Reyes-Resina I., Oñatibia-Astibia A., Zamarbide M., Ricobaraza A., Navarro G., Moreno E., Dopeso-Reyes I.G.G., Sierra S., Rico A.J.J., Roda E., Lanciego J.L.L. and Franco R. (2014). CB1 and GPR55 receptors are co-expressed and form heteromers in rat and monkey striatum. *Exp. Neurol.* 261, 44-52.

Medina M. and Avila J. (2014). The need for better AD animal models. *Front Pharmacol.* Oct 24;

Mendez J.S. and Finn B.W. (1975). Use of 6-hydroxydopamine to create lesions in catecholamine neurons in rats. *J. Neurosurg.* 42, 166-173.

Moreno E., Andradas C., Medrano M., Caffarel M.M., Pérez-Gómez E., Blasco-Benito S., Gómez-Cañas M., Pazos M.R., Irving A.J., Lluís C., Canela E.I., Fernández-Ruiz J., Guzmán M., McCormick P.J. and Sánchez C. (2014). Targeting CB2-GPR55 receptor heteromers modulates cancer cell signaling. *J. Biol. Chem.* 289, 21960-21972.

Navarro G., Borroto-Escuela D., Angelats E., Etayo Í., Reyes-Resina I., Pulido-Salgado M.,

HISTOLOGY AND HISTOPATHOLOGY
(non-edited manuscript)

Rodríguez-Pérez A.I., Canela E.I., Saura J., Lanciego J.L., Labandeira-García J.L., Saura C.A., Fuxe K. and Franco R. (2017). Receptor-heteromer mediated regulation of endocannabinoid signaling in activated microglia. Role of CB<inf>1</inf> and CB<inf>2</inf> receptors and relevance for Alzheimer's disease and levodopa-induced dyskinesia. *Brain Behav. Immun*.

Navarro G., Carriba P., Gandía J., Ciruela F., Casadó V., Cortés A., Mallol J., Canela E.I., Lluis C. and Franco R. (2008). Detection of heteromers formed by cannabinoid CB1, dopamine D2, and adenosine A2A G-protein-coupled receptors by combining bimolecular fluorescence complementation and bioluminescence energy transfer. *Sci. World J.* 8, 1088-1097.

Navarro G., Cordomí A., Zelman-Femiak M., Brugarolas M., Moreno E., Aguinaga D., Perez-Benito L., Cortés A., Casadó V., Mallol J., Canela E.I., Lluís C., Pardo L., García-Sáez A.J., McCormick P.J. and Franco R. (2016). Quaternary structure of a G-protein-coupled receptor heterotetramer in complex with Gi and Gs. *BMC Biol.* 14, 26.

Núñez M., Perdomo S., Moreta J., Santos-Briz Á. and González-Sarmiento R. (2010). The G1359A-CNR1 gene polymorphism is associated to glioma in Spanish patients. *Clin. Transl. Oncol.* 12, 825-828.

Pinna A., Bonaventura J., Farré D., Sánchez M., Simola N., Mallol J., Lluís C., Costa G., Baqi Y., Müller C.E., Cortés A., McCormick P., Canela E.I., Martínez-Pinilla E., Lanciego J.L., Casadó V., Armentero M-T. and Franco R. (2014). l-DOPA disrupts adenosine A2A–cannabinoid CB1–dopamine D2 receptor heteromer cross-talk in the striatum of hemiparkinsonian rats: Biochemical and behavioral studies. *Exp. Neurol.* 253, 180-191.

Piñeiro R., Maffucci T. and Falasca M. (2011). The putative cannabinoid receptor GPR55 defines a novel autocrine loop in cancer cell proliferation. *Oncogene* 30, 142-152.

Poulard C., Rambaud J., Le Romancer M. and Corbo L. (2014). Proximity Ligation Assay to Detect and Localize the Interactions of ERα with PI3-K and Src in Breast Cancer Cells and Tumor Samples. In: *Methods in molecular biology (Clifton, N.J.)*. pp. 135-143.

Prazeres D.M.F. and Martins S.A.M. (2015). G protein-coupled receptors: an overview of signaling mechanisms and screening assays. *Methods Mol. Biol.* 1272, 3-19.

Rinne U.K. (1981). Treatment of Parkinson's disease: problems with a progressing disease. *J. Neural. Transm.* 51, 161-174.

Rodríguez-Ruiz M., Moreno E., Moreno-Delgado D., Navarro G., Mallol J., Cortés A., Lluís C., Canela E.I., Casadó V., McCormick P.J. and Franco R. (2017). Heteroreceptor Complexes Formed by Dopamine D1, Histamine H3, and N-Methyl-D-Aspartate Glutamate Receptors as Targets to Prevent Neuronal Death in Alzheimer's Disease. *Mol. Neurobiol.* 54, 4537–4550.

Snyder S.H. and D'Amato R.J. (1986). MPTP: a neurotoxin relevant to the pathophysiology of Parkinson's disease. The 1985 George C. Cotzias lecture. *Neurology* 36, 250-258.

Solís O., Garcia-Montes J.R., González-Granillo A., Xu M. and Moratalla R. (2017). Dopamine D3 Receptor Modulates l-DOPA-Induced Dyskinesia by Targeting D1 Receptor-Mediated Striatal Signaling. *Cereb. Cortex* 27, 435-446.

HISTOLOGY AND HISTOPATHOLOGY
(non-edited manuscript)

Sredni S.T., Huang C-C., Suzuki M., Pundy T., Chou P. and Tomita T. (2016). Spontaneous involution of pediatric low-grade gliomas: high expression of cannabinoid receptor 1 (CNR1) at the time of diagnosis may indicate involvement of the endocannabinoid system. *Child's Nerv. Syst.* 32, 2061-2067.

Trifilieff P., Rives M-L., Urizar E., Piskorowski R., Vishwasrao H., Castrillon J., Schmauss C., Slättman M., Gullberg M. and Javitch J. (2011). Detection of antigen interactions ex vivo by proximity ligation assay: endogenous dopamine D2-adenosine A2A receptor complexes in the striatum. *Biotechniques* 51, 111-118.

Urigüen L., García-Fuster M.J., Callado L.F., Morentin B., La Harpe R., Casadó V., Lluis C., Franco R., García-Sevilla J.A. and Meana J.J. (2009). Immunodensity and mRNA expression of A2A adenosine, D 2 dopamine, and CB1 cannabinoid receptors in postmortem frontal cortex of subjects with schizophrenia: Effect of antipsychotic treatment. *Psychopharmacology (Berl)* 206, 313-324.

Vijayakumar D. and Jankovic J. (2016). Drug-Induced Dyskinesia, Part 1: Treatment of Levodopa-Induced Dyskinesia. *Drugs* 76, 759-777.

Williams A. (1986). MPTP toxicity: clinical features. *J. Neural. Transm. Suppl.* 20, 5-9.

Zogopoulos P., Korkolopoulou P., Patsouris E. and Theocharis S. (2015). The antitumor action of cannabinoids on glioma tumorigenesis. *Histol. Histopathol.* 30, 629-645.

HISTOLOGY AND HISTOPATHOLOGY
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Figure Legends

Figure 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 α, β-, and γ-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).

Figure 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.

Figure 3. A_{2A}-CB_1 receptor heteromer expression in the monkey striatum. In situ proximity ligation assays (PLA) were performed using slices of the caudate nucleus of naïve (A), MPTP-lesioned (B) or L-DOPA-treated dyskinetic (C) *Macaca fascicularis* monkeys, and primary antibodies for A_{2A} and CB_1 receptors. Confocal microscopy images are shown (superimposed sections) in which heteromers appear as red clusters. Cell nuclei were stained with DAPI (blue). Scale bars: 20 µm. Modified from (Bonaventura et al., 2014).

Figure 4. D_1–D_3 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_1 receptor primary antibody. Confocal microscopy images (superimposed sections) are shown; heteromers appear as red clusters surrounding DAPI-stained nuclei in blue. Scale bars: 5 µm. Modified from (Farré et al., 2015).

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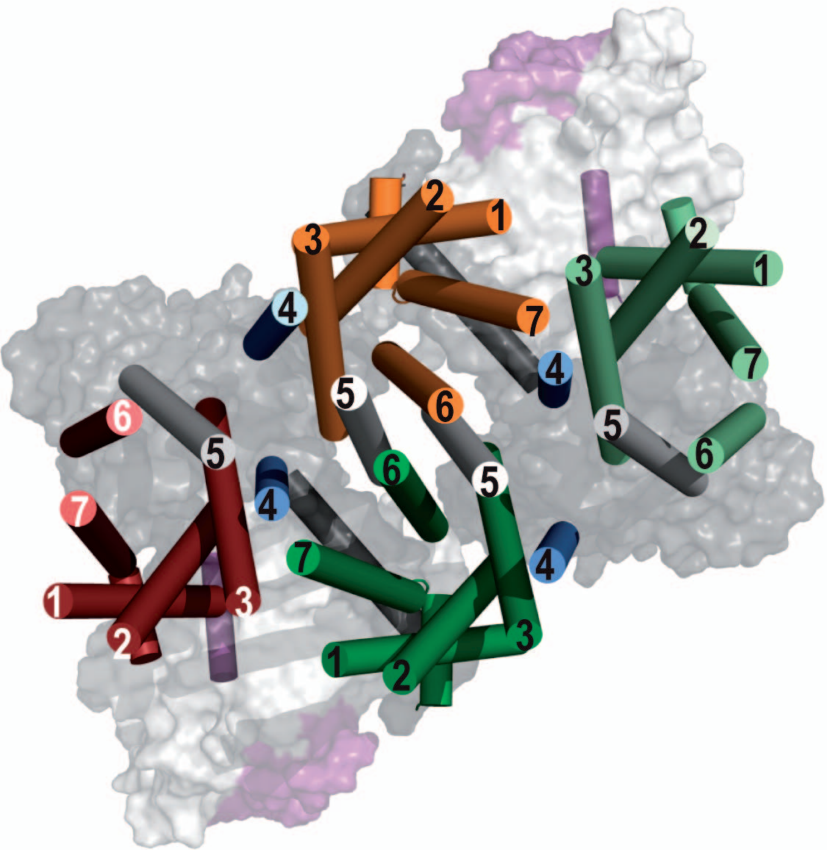

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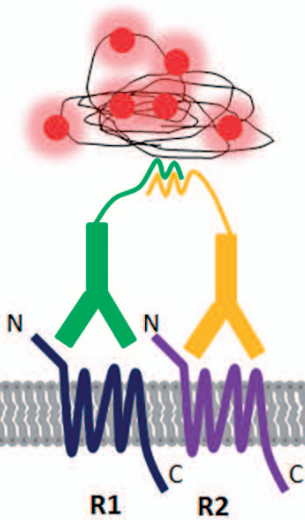
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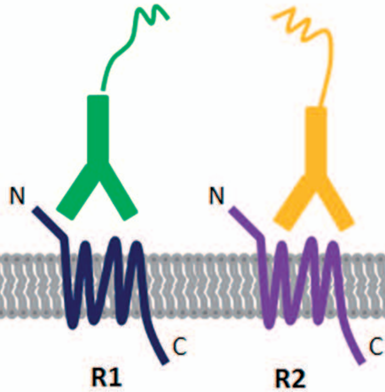
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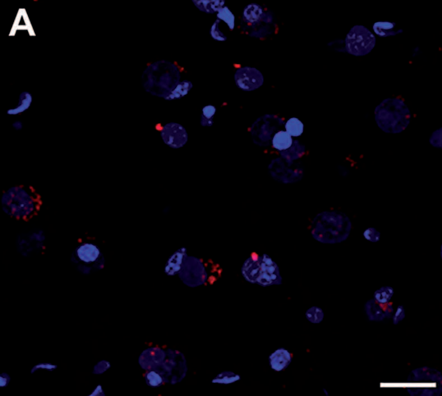
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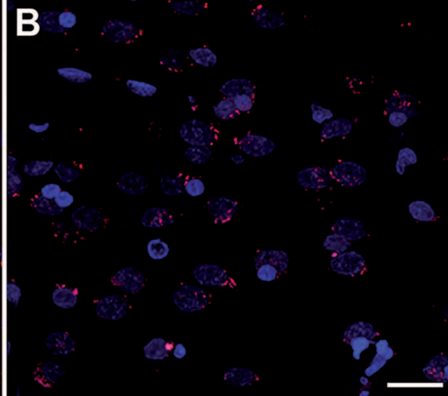
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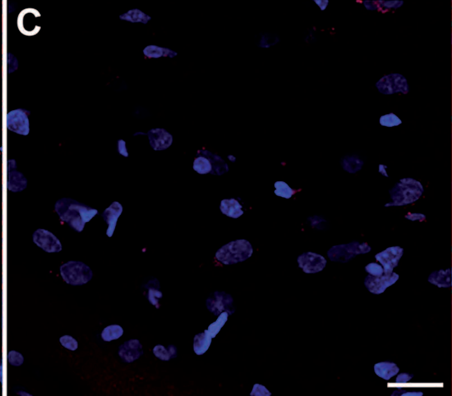
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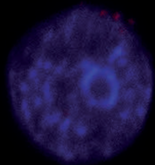
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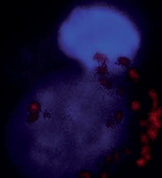
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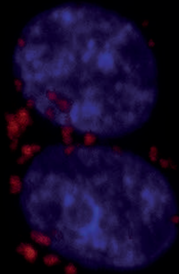
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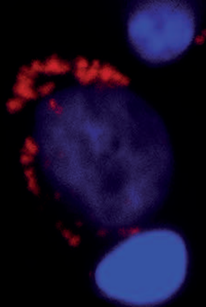
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