

Agonist-independent and -dependent oligomerization of dopamine D₂ receptors by fusion to fluorescent proteins

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Abstract Oligomerization of the short (D_{2S}) and long (D_{2L}) isoforms of the dopamine D₂ receptor was explored in transfected Cos-7 cells by their C-terminal fusion to either an enhanced cyan or enhanced yellow fluorescent protein (ECFP or EYFP) and the fluorescent fusion protein interaction was monitored by a fluorescence resonance energy transfer (FRET) assay. The pharmacological properties of the fluorescent fusion proteins, as measured by both displacement of [³H]nemonapride binding and agonist-mediated stimulation of [³⁵S]GTPγS binding upon co-expression with a G_{oo}Cys³⁵¹Ile protein, were not different from the respective wild-type D_{2S} and D_{2L} receptors. Co-expression of D_{2S}:ECFP+D_{2S}:EYFP in a 1:1 ratio and D_{2L}:ECFP+D_{2L}:EYFP in a 27:1 ratio resulted, respectively, in an increase of 26% and 16% in the EYFP-specific fluorescent signal. These data are consistent with a close proximity of both D_{2S} and D_{2L} receptor pairs of fluorescent fusion proteins in the absence of ligand. The agonist-independent D_{2S} receptor oligomerization could be attenuated by co-expression with either a wild-type, non-fluorescent D_{2S} or D_{2L} receptor subtype, but not with a distinct β₂-adrenoceptor. Incubation with the agonist (–)-norpropylapomorphine dose-dependently (EC₅₀: 0.23 ± 0.06 nM) increased the FRET signal for the co-expression of D_{2S}:ECFP and D_{2S}:EYFP, in support of agonist-dependent D_{2S} receptor oligomerization. In conclusion, our data strongly suggest the occurrence of dopamine D₂ receptor oligomers in intact Cos-7 cells. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: D₂ receptor; Oligomerization; Fluorescence resonance energy transfer; Fluorescent protein; Intact Cos-7 cell

1. Introduction

A growing body of evidence supports the concept that G protein-coupled receptors (GPCRs) may occur in living cells as oligomers generated by the association of two or more molecules of the same GPCR (homo-oligomerization)

or GPCRs belonging to different families (hetero-oligomerization) [1–3]. These include the β₂-adrenoceptor [4], muscarinic m₃ receptor [5], vasopressin V2 receptor [6], yeast Ste2 receptor [7], δ and κ opioid receptors [8,9], thyrotropin-releasing hormone receptor [10] and chemokine CCR5 receptor [11]. Recent evidence also suggests a requirement of hetero-dimerization between the γ-aminobutyric acid (GABA)_{B1} and GABA_{B2} receptors to generate a functional receptor entity with different responses as compared to each receptor which is non-functional if separately expressed in recombinant mammalian systems [12,13]. Earlier experiments on GPCR oligomerization have been based on the co-immunoprecipitation of co-expressed but differently labelled epitope-tagged GPCRs (see [14]). This approach cannot exclude non-specific GPCR interactions which may result from either detergent dissolution of cellular membranes or the use of anti-tag antibodies. Recent reports [4,9,10,15] successfully applied the resonance energy transfer between either two fluorescent molecules (FRET) or one luminescent donor and a fluorescent acceptor molecule to investigate GPCR homo- and hetero-oligomerization.

The D₂ class of dopamine receptors (including D₂, D₃, and D₄ receptors) are GPCRs which couple to the inhibitory G_{i/o} heterotrimeric G proteins. The dopamine D₂ receptor has two splice isoforms; the long variant arising from alternative splicing contains a 29 amino acid insertion in its receptor's third intracellular loop [16]. Receptors of the D₂ class have been proposed to oligomerize both in vivo and in vitro, based on the following lines of evidence: (i) a difference in the binding parameters of labelled benzamide and butyrophenone derivatives led to the hypothesis that [³H]spiperone binds to D₂ receptor dimers whereas [³H]raclopride interacts with receptor monomers [17], and that [³H]raclopride labelled about half the number of dopamine D₂ receptor sites as labelled by [³H]spiperone [18]. These findings have been confirmed by photoaffinity labelling and competition experiments for inhibition of D₂ receptor dimer formation [19]; (ii) oligomeric D₂ and D₃ receptor protein associations have been detected in primate and rodent brain homogenates and in rat GH3 cells stably expressing a D₃ receptor upon immunoprecipitation and immunoblot experiments [20,21] as well as by a truncated receptor approach where an N-terminal portion of a D₂ receptor could functionally associate with a C-terminal portion of a D₃ receptor [22]; (iii) heterodimerization of dopamine D₂ receptor and somatostatin receptor 5 (SSTR5), as demonstrated by a FRET-based assay, yielded an enhanced inhibition of cAMP production [15]; and (iv) indirect evidence of D₂ receptor oligomerization has been obtained by the inhibi-

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Abbreviations: ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; FRET, fluorescence resonance energy transfer; GPCR, G protein-coupled receptor; (–)-NPA, R(–)-norpropylapomorphine; (+)-UH-232, *cis*-(+)-5-methoxy-1-methyl-2-(di-n-propylamino)tetralin

tion of wild-type D₂ receptor function by truncated amino- and carboxy-terminal D₂ receptor portions [23].

In the present study, D₂ receptor oligomerization was investigated by employing a newly developed biophysical method (see [30]), based on energy transfer from two fluorescently labelled proteins in close spatial proximity in living cells. D_{2S} and D_{2L} receptor subtypes were fused to either enhanced cyan or enhanced yellow fluorescent protein (ECFP, EYFP) and the interaction of both fluorescent fusion proteins was monitored by the transfer of energy from one to the other. Upon pharmacological validation of the fluorescent fusion proteins at the radioligand binding and G_α protein activation levels, the occurrence of D_{2S} and D_{2L} receptor oligomers was confirmed in both a ligand-independent and agonist-mediated manner.

2. Materials and methods

2.1. Construction of D₂ receptor fluorescent fusion proteins

The stop codon of the D_{2S} and D_{2L} receptor cDNA [24] was modified by PCR to create a unique *Sma*I restriction site. The entire coding sequence of either ECFP or EYFP was ligated as a *Sma*I-*Not*I fragment in-frame with the modified D_{2S} or D_{2L} receptor cDNA in a pCR3.1 expression vector. DNA sequencing of the entire fusion proteins confirmed the respective constructions.

2.2. Cell culture and transfection

Cos-7 (ATCC CRL-1651) and CHO-K1 (ATCC CRL-9618) cells were grown in respectively Dulbecco's modified Eagle's medium and Ham's F12 nutrient medium, each supplemented with 10% heat-inactivated fetal calf serum. Radioligand binding and fluorescence assays were performed in Cos-7 cells, transiently transfected with 10 µg of the indicated plasmids (1 µg/µl) by using a Bio-Rad gene pulser apparatus (5 × 10⁶ cells, 250 mV, 250 µF). This transient transfection procedure results in a mixed population of Cos-7 cells either containing or not the desired plasmid. [³⁵S]GTPγS binding responses were measured in CHO-K1 cells upon co-transfection with D_{2S}:ECFP, D_{2S}:EYFP, D_{2L}:ECFP and D_{2L}:EYFP fusion protein plasmids and G_αCys³⁵¹Ile protein plasmid [25] using a LipofectAMINE plus kit under conditions indicated by the supplier. Pertussis toxin (100 ng/ml) treatment was carried out in complete culture medium for 16 h before analysis.

2.3. Membrane preparation and radioligand binding assays

Membrane preparations of Cos-7 and CHO-K1 cells were prepared in 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5 as previously described [26]. [³H]Nemonapride (0.1 nM) binding assays were performed on Cos-7 cellular membranes as described previously [26]. 1 µM of (+)-butaclamol was added to determine non-specific binding. Agonist-dependent [³⁵S]GTPγS binding responses were performed to the CHO-K1 membrane preparations described above in 20 mM HEPES, 30 µM GDP, 100 mM NaCl, 3 mM MgCl₂, 0.2 mM ascorbic acid (pH 7.4) under assay conditions described previously [27].

Membrane protein levels were estimated with a dye binding assay using the Bio-Rad protein assay kit and bovine serum albumin as a standard [28].

2.4. FRET assay

Cos-7 cells were transiently transfected by electroporation as described above with 10 µg of plasmid encoding either D_{2S}:ECFP or D_{2S}:EYFP fusion protein alone or both plasmids together, in the absence or presence of non-fluorescent receptor plasmids as indicated, or with 9 µg of D_{2L}:ECFP and 0.33 µg of D_{2L}:EYFP plasmid, and seeded in 96 well plates (5 × 10⁴ cells/well). Fluorescent readings were performed 24–48 h following transfection using a fluorometric image plate reader (FLIPR, Molecular Devices). Cells were washed twice with Hanks' balanced salt solution (pH 7.4) and were incubated in 100 µl of this latter buffer. The excitation argon laser was set at 457 nm. Emission filters correspond to 445–495 nm to detect ECFP emission and 500–540 nm to detect EYFP emission. Fluorescent emission was recorded by a CCD camera for the entire 96 well plate 0.8 s after

excitation and expressed as arbitrary fluorescence units (AFU). When indicated, ligands were applied to the cells and readings were performed immediately upon treatment. The extinction coefficients (*E*_m, cm⁻¹ M⁻¹) are: for ECFP, 26 000 at 433 nm excitation and for EYFP, 84 000 at 514 nm excitation.

2.5. Statistical analysis

Statistical analysis was performed by a two-group comparison procedure using Student's *t*-test.

2.6. Materials

All molecular biology reagents were from Applied Biosystems (Foster City, CA, USA), Clontech (Palo Alto, CA, USA) and Invitrogen (La Jolla, CA, USA). The cell lines were from the American Type Cell Culture (Rockville, MD, USA). All cell culture reagents were from Invitrogen Life Technologies (Paisley, UK). [³H]Nemonapride (85 Ci/mmol) and [³⁵S]GTPγS (1000 Ci/mmol) were, respectively, from New England Nuclear (Boston, MA, USA) and Amersham Pharmacia Biotech (Les Ulis, France). Dopamine and (–)-norpropylapomorphine ((–)-NPA) were from RBI-Sigma (St. Louis, MO, USA). *cis*-(+)-5-Methoxy-1-methyl-2-(di-*n*-propylamino)tetralin ((+)-UH-232) was from Tocris (Bristol, UK).

3. Results

3.1. Pharmacological properties of D₂ receptor fluorescent fusion proteins

Cos-7 cells expressed between 8.5 and 15.6 pmol/mg protein of [³H]nemonapride binding sites when transformed with the D_{2S} receptor ECFP and EYFP fusion proteins and between 8.5 and 14.9 pmol/mg protein for the D_{2L} receptor ECFP and EYFP fusion proteins (Table 1). Inhibition of [³H]nemonapride binding by a series of dopaminergic ligands including the agonists (–)-NPA, bromocriptine, the partial agonist (+)-UH-232 and the putative antagonist nemonapride, yielded the same inhibition constants for the wild-type D_{2S}, D_{2S}:ECFP and D_{2S}:EYFP fusion proteins as well as for the wild-type D_{2L}, D_{2L}:ECFP and D_{2L}:EYFP fusion proteins (Table 1). We also investigated the ability of these fusion proteins to activate a recombinant G_αCys³⁵¹Ile protein by monitoring [³⁵S]GTPγS binding. In the absence of recombinant G_αCys³⁵¹Ile protein, neither D_{2S} nor D_{2L} receptors were able to stimulate [³⁵S]GTPγS binding upon stimulation by (–)-NPA (10 µM) in CHO-K1 cells (data not shown). The maximal dopamine (10 µM)-mediated stimulation of [³⁵S]GTPγS binding as observed with the D_{2S} receptor fusion proteins was about twice decreased as compared to the wild-type D_{2S} receptor, but its potency was either not altered for the D_{2S}:EYFP fusion protein or slightly decreased for the D_{2S}:ECFP fusion protein. (–)-NPA behaved as an efficacious agonist as compared to dopamine with a potency in the nanomolar range, slightly decreased for both D_{2S} receptor fusion proteins as compared to the wild-type D_{2S} receptor (Table 1). A weak (49–59% stimulation) [³⁵S]GTPγS binding response was obtained for the wild-type D_{2L} receptor; this response was decreased (52–65%) at the D_{2L}:ECFP and D_{2L}:EYFP fusion proteins, therefore agonist dose–response curves were not performed.

3.2. Oligomerization of D₂ receptors

Basal fluorescence emission (445–495 nm) upon laser excitation at 457 nm yielded 1586 ± 112 AFU (*n* = 8) for Cos-7 cells expressing a D_{2S}:ECFP fusion protein; this value was similar for Cos-7 cells transfected with either empty plasmid or plasmid containing a wild-type D_{2S} or D_{2L} receptor or a D_{2L}:ECFP fusion protein and corresponded to background

Table 1

 $[^3\text{H}]$ Nemonapride and $[^3\text{S}]\text{GTP}\gamma\text{S}$ binding responses to either wild-type or dopamine $\text{D}_{2\text{S}}$ and $\text{D}_{2\text{L}}$ receptor ECFP and EYFP fusion proteins

Cell type G_α protein	$[^3\text{H}]$ Nemonapride					$[^3\text{S}]\text{GTP}\gamma\text{S}$			
	Cos-7					CHO-K1			
	none					recombinant $\text{G}_{\alpha\text{O}}\text{Cys}^{351}\text{Ile}$ protein			
Receptor/Ligand	Expression (pmol/mg protein)	IC_{50} (nM)				E_{max} (%)			
		(-)-NPA	Bromocriptine	(+)-UH-232	Nemonapride	Dopamine	Dopamine	(-)-NPA	(-)-NPA
Wild-type $\text{D}_{2\text{S}}$	8.51 \pm 0.53	75 \pm 13	28 \pm 8	120 \pm 25	0.72 \pm 0.04	141 \pm 14	11.8 \pm 3.1	132 \pm 12	2.7 \pm 0.7
$\text{D}_{2\text{S}}:\text{ECFP}$	9.40 \pm 1.25	107 \pm 29	40 \pm 10	170 \pm 6	0.83 \pm 0.09	58 \pm 10	21.0 \pm 1.0	60 \pm 9	8.6 \pm 0.4
$\text{D}_{2\text{S}}:\text{EYFP}$	15.6 \pm 2.16	96 \pm 15	31 \pm 6	147 \pm 27	0.75 \pm 0.07	67 \pm 4	14.8 \pm 4.6	66 \pm 2	5.1 \pm 0.8
Wild-type $\text{D}_{2\text{L}}$	7.89 \pm 1.55	73 \pm 18	27 \pm 4	123 \pm 16	0.67 \pm 0.06	49–55	nd	54–59	nd
$\text{D}_{2\text{L}}:\text{ECFP}$	12.9 \pm 2.39	76 \pm 24	25 \pm 8	132 \pm 14	0.90 \pm 0.10	19–25	nd	22–28	nd
$\text{D}_{2\text{L}}:\text{EYFP}$	14.9 \pm 2.99	90 \pm 18	30 \pm 4	113 \pm 7	0.84 \pm 0.08	17–21	nd	19–23	nd

The wild-type and D_2 receptor fluorescent fusion proteins were expressed in Cos-7 or CHO-K1 cells in the co-presence of a recombinant $\text{G}_{\alpha\text{O}}\text{Cys}^{351}\text{Ile}$ protein to measure, respectively, $[^3\text{H}]$ nemonapride (0.10 nM) and $[^3\text{S}]\text{GTP}\gamma\text{S}$ (0.5 nM) binding as described in Section 2. Maximal $[^3\text{S}]\text{GTP}\gamma\text{S}$ binding responses (E_{max}) were expressed as a percentage above the basal $[^3\text{S}]\text{GTP}\gamma\text{S}$ binding. Data correspond to the mean or mean \pm S.E.M. of two or four to five independent transfection experiments, each experimental point being performed in duplicate. nd: not determined.

fluorescence. This value was similar with the 500–540 nm filter set, indicating that this filter set is unable to detect a fluorescent ECFP emission. When cells expressed a $\text{D}_{2\text{S}}:\text{EYFP}$ fusion protein, the emitted fluorescence in the 500–540 nm window increased to 2125 ± 190 AFU ($n=8$; Fig. 1), suggesting a weak excitation of the EYFP fluorochrome in the fusion protein at 457 nm as expected by the EYFP excitation spectrum [29]. Co-expression in equivalent plasmid amount of $\text{D}_{2\text{S}}:\text{ECFP}$ and $\text{D}_{2\text{S}}:\text{EYFP}$ yielded a significant $26 \pm 5\%$ ($n=15$) increase in the emitted fluorescence, suggesting a fluo-

rescence transfer from the laser-excited $\text{D}_{2\text{S}}:\text{ECFP}$ to a $\text{D}_{2\text{S}}:\text{EYFP}$ in close proximity (less than 100 nm [30]). A similar experiment with an equimolar amount of $\text{D}_{2\text{L}}:\text{ECFP}$ and $\text{D}_{2\text{L}}:\text{EYFP}$ did not result in energy transfer. A 27-fold excess in $\text{D}_{2\text{L}}:\text{ECFP}$ versus $\text{D}_{2\text{L}}:\text{EYFP}$ plasmid yielded a significant $16 \pm 3\%$ ($n=6$) increase in the FRET signal, indicating spatial proximity between the two $\text{D}_{2\text{L}}$ receptor fusion proteins. This observed emission was independent of agonist stimulation. In addition, (-)-NPA dose-dependently increased the energy transfer to $23 \pm 4\%$ ($n=5$) above the ligand-independent $\text{D}_{2\text{S}}$ -mediated fluorescence level with a potency of 0.23 ± 0.06 nM (Fig. 2). Dopamine (10 μM) also increased the FRET signal to 21.5% (20–23%, $n=2$). The (-)-NPA (10 nM)-mediated $\text{D}_{2\text{S}}$ receptor oligomerization could be inhibited by co-treatment with the D_2 receptor antagonist haloperidol (1 μM , Fig. 2). Agonist stimulation of the $\text{D}_{2\text{L}}$

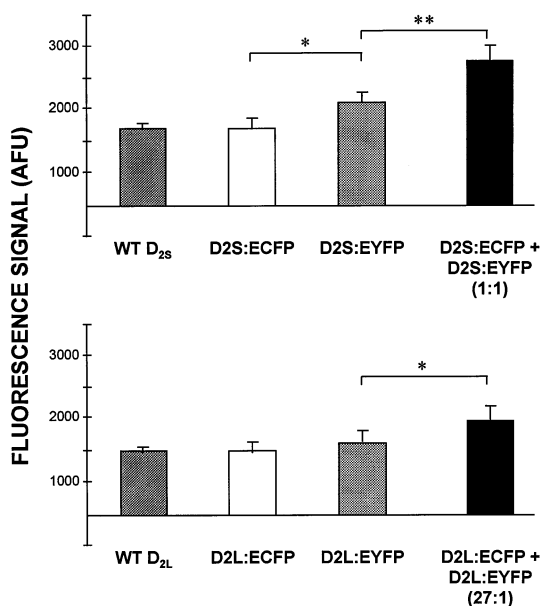


Fig. 1. Constitutive dopamine D_2 receptor oligomerization. Cos-7 cells were transfected with both D_2 receptor isoforms (short, S or long, L) either in their wild-type (WT) form or fused to ECFP or EYFP fluorescent protein alone or co-expressed, as described in Section 2. Molecular ratio were 1:1 for $\text{D}_{2\text{S}}:\text{ECFP}+\text{D}_{2\text{S}}:\text{EYFP}$ plasmids and 27:1 for $\text{D}_{2\text{L}}:\text{ECFP}+\text{D}_{2\text{L}}:\text{EYFP}$ plasmids. Fluorescent readings were performed 0.8 s after laser excitation at 457 nm. Data correspond to mean \pm S.E.M. of six to eight independent transfection experiments, each experimental point corresponding to the mean of at least six wells on a culture plate. Statistical analysis was performed on the AFU using Student's t -test. * $P < 0.05$ vs. the fluorescence emitted with $\text{D}_{2\text{S}}:\text{EYFP}$ or $\text{D}_{2\text{L}}:\text{EYFP}$ alone.

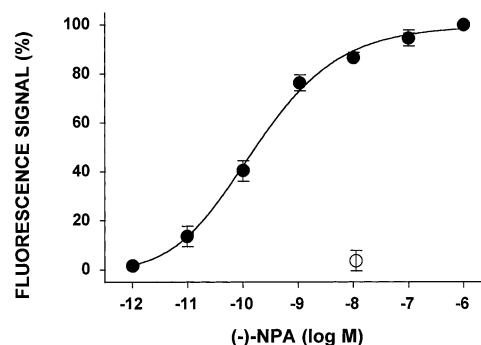


Fig. 2. Ligand-dependent dopamine $\text{D}_{2\text{S}}$ receptor oligomerization. Cos-7 cells were co-transfected with both $\text{D}_{2\text{S}}:\text{ECFP}$ and $\text{D}_{2\text{S}}:\text{EYFP}$ fusion proteins as described in Section 2. Fluorescent readings were performed as described in Fig. 1. Constitutive energy transfer fluorescence resulting from $\text{D}_{2\text{S}}$ receptor oligomerization was 2680 ± 112 AFU. Maximal stimulation as obtained with (-)-NPA was 3298 ± 136 AFU. Dose-response curve for (-)-NPA (●) and co-treatment of (-)-NPA (10 nM)+haloperidol (1 μM) (○) are shown. Data are expressed as a percentage of the maximal stimulation of specific FRET signal (difference in fluorescence between the co-expression of $\text{D}_{2\text{S}}:\text{ECFP}+\text{D}_{2\text{S}}:\text{EYFP}$ and of $\text{D}_{2\text{S}}:\text{EYFP}$ alone) obtained with 1 μM (-)-NPA and correspond to mean \pm S.E.M. of three to four independent transfection experiments, each experimental point corresponding to the mean of at least six wells on a culture plate.

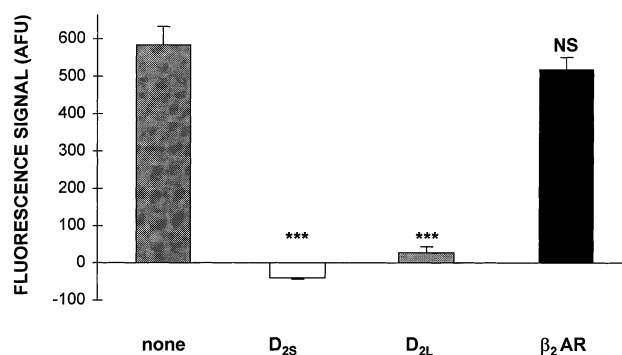


Fig. 3. Inhibition of constitutive D_{2S}:ECFP and D_{2S}:EYFP oligomerization. Cos-7 cells were co-transfected with both D_{2S}:ECFP and D_{2S}:EYFP fusion proteins in either the absence (none) or presence of plasmid containing wild-type, non-fluorescent D_{2S} or D_{2L} receptor or β_2 -adrenoceptor (β_2 AR), as described in Section 2. Fluorescent readings were performed as described in Fig. 1. Data were expressed as the ligand-independent specific FRET signal, corresponding to the difference in fluorescence between the co-expression of D_{2S}:ECFP+D_{2S}:EYFP and of D_{2S}:EYFP alone. Values correspond to the mean \pm S.E.M. of three independent transfection experiments, each experimental point corresponding to the mean of at least six wells on a culture plate. Statistical analysis was performed on the AFU using Student's *t*-test. ****P* < 0.001; NS: *P* > 0.05 vs. the fluorescence emitted with both D_{2S}:ECFP+D_{2S}:EYFP.

receptor fluorescent fusion proteins did not yield an enhancement of the fluorescent signal. Mixture of Cos-7 cells independently expressing D_{2S}:ECFP or D_{2S}:EYFP following transfection did not generate a fluorescent signal (not shown). The specificity of the interaction between D_{2S}:ECFP and D_{2S}:EYFP was confirmed by co-expression of both fluorescent fusion proteins with a wild-type, non-fluorescent D_{2S} receptor. This condition fully abolished the energy transfer (Fig. 3), in agreement with the formation of D_{2S}:ECFP plus wild-type D_{2S} and D_{2S}:EYFP plus wild-type D_{2S} oligomers unable to generate a FRET signal. A similar result was obtained by co-expression with a wild-type D_{2L} receptor. This suggests indirectly the existence of oligomers involving both short and long isoforms of the D₂ receptor (Fig. 3). Co-expression of D_{2S}:ECFP and D_{2S}:EYFP with a distinct β_2 -adrenoceptor did not modify the fluorescent signal in a significant manner (Fig. 3).

4. Discussion

The present report demonstrates that oligomeric forms of both dopamine D_{2S} and D_{2L} receptors can be obtained by the association of fluorescently labelled receptors, fused to either ECFP or EYFP in Cos-7 cells. Such C-terminal fluorescent fusion proteins yielded a radioligand binding profile similar to the wild-type, non-fluorescent D₂ receptor, indicating that the fusion process did not modify the global receptor conformation. Because the intracellular loops of GPCR have been implicated in the interaction with G proteins [31], the fusion of the 26 kDa ECFP or EYFP portion at the C-terminal end of the D₂ receptor could alter the receptor-dependent G protein activation. This has been tested upon co-expression of a recombinant, pertussis toxin-resistant G_{ao}Cys³⁵¹Ile protein and monitoring of its activation by the binding of [³⁵S]GTPγS. A slightly decreased potency and a twice decreased maximal stimulation for the agonists (–)-NPA and dopamine were

observed for the fluorescent fusion proteins as compared to the wild-type D_{2S} receptor, suggesting a less efficient G_{ao} protein coupling. These data indicate that D_{2S}:ECFP, D_{2S}:EYFP, D_{2L}:ECFP and D_{2L}:EYFP are functional receptor proteins, they are therefore suitable for use in a FRET assay. Fluorescence energy transfer has successfully been applied to demonstrate receptor homo-oligomerization of various GPCRs including opioid receptors and yeast Ste2 receptor [7,9] and hetero-oligomerization between SSTR5 and D₂ receptors [15]. It has been reported that FRET results in donor photobleaching due to fluorescent excitation and autofluorescence [32]. In the present study, photobleaching was not observed during the time course of data recording since emission was measured less than 1 s after excitation, and for the entire 96 well plate. We were unable to detect basal fluorescence from the donor protein (ECFP, D_{2S}:ECFP or D_{2L}:ECFP). This may be due either to the weak fluorescence intensity of the ECFP molecule [29] or to a phenomenon of autoquenching, which may also exist for the EYFP-derived fusion proteins. A low level of autofluorescence of the acceptor EYFP was observed for D_{2S}:EYFP when excitation was performed at 457 nm. Nevertheless, co-expression of both D_{2S}:ECFP+D_{2S}:EYFP and D_{2L}:ECFP+D_{2L}:EYFP resulted in respectively a 26% and 16% increase in the signal emitted in the 500–540 nm filter window as compared to the D₂ receptor EYFP fusion proteins. The detection of a FRET signal under basal conditions (i.e. in the absence of ligand) demonstrated a close proximity between the ECFP-labelled and EYFP-labelled D₂ receptor subtypes as the maximum distance allowing energy transfer between the two fluorescent proteins is approximately 100 nm [30]. The FRET signal resulting from co-expression of wild-type, non-fused ECFP and EYFP could not be evaluated in our experimental system because the fluorescent signal of wild-type EYFP expressed alone saturated the camera (not shown). Nevertheless, independent expression of D_{2S}:ECFP and D_{2S}:EYFP in individual cells and mixing did not result in a FRET signal, thus there is no intercellular energy transfer. Thus, the herein observed results can be best explained by the formation of constitutive D₂ receptor dimers or oligomers since the FRET signal could result from dimeric and/or oligomeric D₂ receptor complexes. The oligomerization seems more efficient for the D_{2S} receptor subtype than for the D_{2L} receptor isoform (Fig. 1).

D_{2S} oligomer formation was also enhanced by agonist activation, the magnitude of the agonist effect was as important as the constitutive D_{2S} oligomerization (about 20%). This enhancement occurred in a dose-dependent manner, but the potency of (–)-NPA for receptor oligomerization was 10-fold increased as compared to its potency at wild-type D_{2S} receptors to stimulate [³⁵S]GTPγS binding via a recombinant G_{ao}Cys³⁵¹Ile protein (see Table 1). Agonist promotion of FRET could indicate either an increase in the amount of D_{2S} receptor oligomers and/or a ligand-induced conformational change in pre-existing D_{2S} receptor oligomers which results in a closer contact or more favorable orientation of the ECFP and EYFP portion, thereby increasing the energy transfer. Ligand-dependent promotion of D_{2L} receptor oligomerization could not be observed in our experimental system and may be linked to the weaker ligand-independent FRET signal which is observed. Recently, activation of the metabotropic glutamate mGluR1 receptor by glutamate has been

shown to stabilize pre-existing receptor dimers; movements of the receptor portions involved in dimerization affected the separation of transmembrane domains and intracellular portions [33]. For other GPCRs, dimerization only occurred in the presence of agonists like for SSTR5 [15] and gonadotropin-releasing hormone receptor [10].

Competition of fluorescent D_{2S} receptor oligomerization by wild-type, non-fluorescent receptors was performed to confirm the specificity of the receptor interaction. Co-expression of D2S:ECFP and D2S:EYFP with wild-type D_{2S} receptors fully abolished the energy transfer, suggesting the formation of D2S:ECFP+D_{2S} and D2S:EYFP+D_{2S} receptor oligomers. No competition was obtained with a distinct β_2 -adrenoceptor, indicating a specific interaction between D_{2S} receptors. Co-expression of D2S:ECFP and D2S:EYFP with the D_{2L} receptor subtype also totally abolished the FRET signal. These data indirectly suggest oligomerization between the short and long isoforms of the D₂ receptor. Nevertheless, these two receptor subtypes seem to possess a differential compartmentalization. Immunohistochemical studies showed that the D_{2S} receptor predominates in the cell bodies and axons of dopaminergic neurons of the mesencephalon and hypothalamus. On the other hand, the D_{2L} receptor isoform is more strongly expressed in the striatum and nucleus accumbens [34]. Oligomerization between D_{2S} and D_{2L} receptor isoforms is nevertheless not surprising because they only differ by a 29 amino acid portion in their third intracellular loop [16]. Moreover, GPCR oligomerization is likely to involve a portion encompassing the sixth transmembrane domain as shown for the β_2 -adrenoceptor [35] and dopamine D₁ receptor [36]. Involvement of the C-terminal intracellular portion, which is directly linked to the fluorescent proteins in the fusion proteins used herein, cannot be excluded.

In conclusion, we applied the energy transfer assay between two fluorescent proteins to demonstrate dimerization/oligomerization of dopamine D₂ receptors in both the absence and presence of agonist. A plausible association between the long and short isoforms of the D₂ receptor cannot be excluded. The occurrence of GPCR oligomers may be a general phenomenon which constitutes a new challenge in the pharmacology of GPCRs.

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