ACCELERATED COMMUNICATION

Ligand-Dependent Oligomerization of Dopamine D2 and Adenosine A2A Receptors in Living Neuronal Cells

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ABSTRACT

Adenosine A2A and dopamine D2 receptors (A2A and D2) associate in homo- and heteromeric complexes in the striatum, providing a structural basis for their mutual antagonism. At the cellular level, the portion of receptors engaging in homo- and heteromers, as well as the effect of persistent receptor activation or antagonism on the cell oligomer repertoire, are largely unknown. We have used bimolecular fluorescence complementation (BiFC) to visualize A2A and D2 oligomerization in the Cath.a differentiated neuronal cell model. Receptor fusions to BiFC fluorescent protein fragments retained their function when expressed alone or in A2A/A2A, D2/D2, and A2A/D2 BiFC pairs. Robust fluorescence complementation reflecting A2A/D2 heteromers was detected at the cell membrane as well as in endosomes. In contrast, weaker BiFC signals, largely confined to intracellular domains, were detected with A2A/dopamine D1 BiFC pairs. Multicolor BiFC was used to simultaneously visualize A2A and D2 homo- and heteromers in living cells and to examine drug-induced changes in receptor oligomers. Prolonged D2 stimulation with quinpirole lead to the internalization of D2/D2 and A2A/D2 oligomers and resulted in decreased A2A/D2 relative to A2A/A2A oligomer formation. Opposing effects were observed in cells treated with D2 antagonists or with the A2A agonist 5’-N-[11032]-N-methylcarboxamidoadenosine (MECA). Subsequent radioreceptor binding analysis indicated that the drug-induced changes in oligomer formation were not readily explained by alterations in receptor density. These observations support the hypothesis that long-term drug exposure differentially alters A2A/D2 receptor oligomerization and provide the first demonstration for the use of BiFC to monitor drug-modulated GPCR oligomerization.

A growing number of G protein-coupled receptors (GPCRs) have been shown to exist as oligomers with unique functional properties and physiological relevance (Pin et al., 2007). Evidence suggests that A2A and D2 form receptor heteromers. Both receptors are highly expressed in the striatum, where they colocalize on spiny neurons (Fink et al., 1992). The receptors have opposing actions on adenylyl cyclase activity, through coupling to Gs (A2A) or Gi/o (D2) proteins. Biochemical and behavioral evidence also indicates antagonistic A2A/D2 interactions (Ferre et al., 1991; Agnati et al., 2003; Fuxe et al., 2007). Moreover, persistent D2 activation sensitizes A2A receptor-stimulated cAMP accumulation (Vorthers and Watts, 2004). A2A and D2 have been shown to oligomerize in resonance energy transfer as well as coimmunoprecipitation experiments (Hillion et al., 2002; Canals et al., 2003; Kamiya et al., 2003). Therefore, a direct A2A/D2 interaction may account for the antagonism between the two receptors. In addition to forming heteromers, A2A and D2 also exist as homomers (Lee et al., 2000; Armstrong and Strange, 2001; Gazi et al., 2003; Canals et al., 2004; Guo et al., 2005). The stoichiometry of A2A and D2 in A2A/D2 heteromers is unknown, as is the relative proportion of A2A and D2 recep-
tors engaging in hetero- or in homomers (or existing as monomeric receptors). Although A2A and D2 homo- and heteromerization was shown to be constitutive and was not affected by acute receptor activation (Canals et al., 2003; Gazi et al., 2003; Canals et al., 2004), the effect of persistent receptor activation or antagonism on the relative homo-/heteromer population has not been investigated.

Bimolecular fluorescence complementation (BiFC) is an emerging technique to monitor protein-protein interactions (Hu et al., 2002; Shyu et al., 2006). Whereas most currently available techniques are restricted to the detection of two interacting proteins, multicolor BiFC (i.e., the reconstitution of distinct spectral GFP variants) allows the simultaneous detection of two distinct protein-protein interactions in living cells (Hu and Kerppola, 2003). We have applied multicolor BiFC to simultaneously visualize A2A/D2 heteromers and A2A homomers in the Cath.a differentiated (CAD) neuronal cell model (Qi et al., 1997). The results indicate that A2A/D2 heteromers coexist and colocalize with A2A homomers. Prolonged (18-h) treatment with the selective D2 agonist quinpirole or the D2 antagonist sulpiride had opposing effects on the proportion of A2A/D2 heteromers relative to A2A homomers. These observations have clinical implications in the management of Parkinson's disease and schizophrenia, which rely on long-term treatment with drugs targeting dopaminergic receptors.

Materials and Methods

Materials. D2L, A2A, and D1 cDNAs were obtained from the Missouri S&T cDNA Resource Center. Growth media and reagents (unless otherwise stated) were purchased from Sigma-Aldrich (St. Louis, MO). [3H]CAMP (25 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). [3H]Spiperone (85 Ci/mmol) was from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK).

Cell Culture. CAD cells were maintained as described previously (Vortherms and Watts, 2004).

Expression Vectors. Full-length human D2L, A2A, or D1 cDNAs were amplified by polymerase chain reaction using oligonucleotides incorporating EcoRI and Xbal or XhoI restriction sites and omitting stop codons. Polymerase chain reaction fragments digested with EcoRI/Xbal or EcoRI/XhoI were ligated into the corresponding sites from pBIFC vectors (Shyu et al., 2006). These vectors contain fragments from the yellow Venus [V (Nagai et al., 2002)] or the cyan Cerulean [C (Rizzo et al., 2004)] enhanced fluorescent proteins. N-terminal fragments (VN or CN) include residues 1 to 172, whereas C-terminal fragments (VC or CC) include residues 155 to 238. Cloning into pBIFC vectors incorporates MYC (pBIFC-VN), HA (pBIFC-VC and pBIFC-CC), or FLAG (pBIFC-CN) N-terminal epitope tags to the fusion proteins to ease their detection. Receptor fusions to Venus or Cerulean were obtained by swapping BiFC fragments with Venus or Cerulean coding sequences. Constructs were verified by DNA sequencing.

Imaging and Image Analysis. CAD cells were grown to 70% confluence in four-well Lab-Tek coverslips (Nalge Nunc International, Rochester, NY) and transfected using 1 μl/well Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's recommendations. DNA amounts per well were 500 ng (D2L and D1 constructs), 200 ng (A2A-VN), 100 ng (A2A-CN, A2A-CC), or 20 ng (nCherry-Mem, YFP-Endo, YFP-Golgi, and YFP-ER). Twenty-four hours after transfection, the growth media was replaced with phosphate-buffered saline, and images were captured using a charge-coupled device camera mounted on a TE2000-U inverted fluorescence microscope (Nikon Instruments Inc., Melville, NY) equipped with a 100-W mercury lamp and band-pass filters (Chroma, Rockingham, VT) for Venus (excitation at 500/20 nm; emission at 530/30 nm), Cerulean (excitation at 430/25 nm; emission at 470/30 nm), or mCherry (excitation at 572 nm/23 nm). Fluorescent images were acquired using the MetaMorph software (Molecular Devices, Sunnyvale, CA) and AutoDeblur (MediaCybernetics, Bethesda, MD) was used for three-dimensional deconvolution. Blind selection and analysis of the cells avoided experimental bias. Quantification of BiFC signals was performed as described previously (Hu et al., 2002), using the ImageJ software (http://rsb.info.nih.gov/ij/). Stacks of fluorescent images were analyzed as follows. Background fluorescence intensities were determined by measuring areas devoid of cells and were subtracted from each pixel intensity measurement. After background removal, pixel intensities were scaled by a factor equal to the inverse of the exposure time. Images from the mCherry-Mem membrane marker were used to select cells for analysis and to normalize BiFC signals. As an approximation of plasma membrane signals, maximal pixel intensities along lines traced across plasma membranes were measured. Intracellular signals were measured by tracing regions of interest and determining average pixel intensities. Cells with saturated signals, as well as cells with signals lower than 1.5 times background values, were not considered for analysis. Because Venus/mCherry fluorescence ratios exhibited non-Gaussian distributions, median values were calculated and averaged between different experiments. In multicolor BiFC experiments, median Venus/Cerulean fluorescence ratios were measured. For each condition, approximately 40 cells were analyzed. Median values from at least three independent experiments were averaged and used for statistical analysis.

Fluorescence Measurement in Cell Suspensions. CAD cells were grown in 12-well plates, transfected as above, suspended in phosphate-buffered saline, and transferred into 96-well plates (40 μg protein/well; Naige Nunc International). Cerulean and Venus fluorescence were measured with a Fusion plate reader (Packard, Waltham, MA) using 430/25 nm and 500/20 nm excitation as well as 470/30 nm and 535/30 nm emission filters, respectively. Background from mock-transfected cells was subtracted from fluorescent signals. Bleed-through and cross-talk coefficients for Cerulean and Venus channels were calculated with cells expressing either V or C (or corresponding BiFC pairs). The V/C fluorescence ratio (noted x coefficient) in cells expressing only Venus was 0.00005 ± 0.00002 (n = 7), x in cells expressing VN/CC BiFC fragments was 0.00276 ± 0.00065 (n = 5), and the V/C fluorescence ratio (y coefficient) in cells expressing Cerulean was 0.00256 ± 0.00018 (n = 7). Corrected Venus (Vcor) and Cerulean (Ccor) signals were calculated using the equations Vcor = (V - xV1 - xy) and Ccor = (C - xV1 - xy), with V and C indicating the measured Venus and Cerulean fluorescence intensities.

Protein Analysis. Protein concentration was determined using the BCA method (Pierce, Rockford, IL). BiFC-tagged GPCR expression was quantified by dot blot (Zeder-Lutz et al., 2006). Cell suspensions were lysed with SDS [2% (w/v)], and proteins (5 μg) were spotted onto nitrocellulose membranes using a bio-dot apparatus (Bio-Rad Laboratories, Hercules, CA). Anti-βA (Sigma) or anti-c-MYC (Clontech, Mountain View, CA) mouse antibodies as well as anti-mouse-HRP conjugated antibodies (Bio-Rad Laboratories) were used for immunodetection. Enhanced chemiluminescence signals (ECL+, GE healthcare) were detected and quantified using a Typhoon scanner and the ImageQuant software (Amersham, Chalfont St. Giles, Buckinghamshire, UK).

cAMP Accumulation Assays. Cells were seeded in 48-well plates (approximately 105 cells/well) and transiently transfected with 200 ng of plasmid DNA using the Lipofectamine 2000 reagent (0.4 μl/well; Invitrogen). At 24 h after transfection, cells were stimulated for 15 min on ice with drugs diluted in Earle's balanced salt solution assay buffer (Earle's balanced salt solution containing 2% bovine calf serum, 0.025% ascorbic acid, and 15 mM HEPES). In experiments with cells expressing D2L (or D1L fusion proteins), forskolin (30 μM) was used to stimulate adenyl cyclase. Quinpirole (10 μM) and spiperone (1 μM) were used as D2-like agonist and antagonist.
antagonist, respectively. 5′-N-Methylcarboxamidoadenosine (MECA; 1 μM) and CGS15943 (1 μM) were used as A2A agonist and antagonist, respectively. Dopamine (10 μM) and butaclamol (10 μM) were used as D1-like agonist and antagonist, respectively. Stimulation experiments were performed in the presence of the phosphodiesterase inhibitors 3-isobutyl-1-methylxanthine (500 μM) or Ro 20-1724 (100 μM for MECA stimulations) and terminated by the addition of 3% trichloroacetic acid. A competitive binding assay was used for cAMP quantification (Vothknecht and Watts, 2004).

Radioligand Binding Experiments. Radioreceptor binding experiments were performed as described previously (Watts and Neve, 1996), with minor modifications. A point binding technique was employed to estimate A2A and D2 receptor densities, by saturating concentrations of radioligand. These concentrations were based on full receptor isotherms, which revealed similar binding properties between the fusion proteins and wild-type receptors (data not shown). Twenty-four hours after transfection and 18 h after drug treatment, cells in 12-well plates were washed and lysed in 1 ml of ice-cold lysis buffer (1 mM HEPES and 2 mM EDTA, pH 7.4) and membranes were collected by centrifugation (10 min at 13,000 g). Membrane pellets were resuspended by mechanical homogenation in a final volume of 500 μl of radioligand binding buffer (50 mM Tris and 4 mM MgCl2, pH 7.4). Membranes for A2A receptor experiments were treated with 2 U/ml adenosine deaminase (Roche Applied Science, Indianapolis, IN) at 37°C for 30 min to remove endogenous adenosine. Treated membranes (10–20 μg of protein in 100 μl) were added in duplicate to assay tubes to determine total and nonspecific binding (defined by 50 μM adenosine-5′-N-ethylcarboxamide). All tubes contained [3H]ZM 241-385 (~2.5 nM; American Radioligand Chemicals, St. Louis, MO) and were incubated for 1 h at 25°C. D2 binding experiments were performed in a similar fashion, excluding adenosine deaminase treatment. Total binding at D2 was determined by incubating 10 to 20 μg of protein (in 100 μl) membrane suspensions with [3H]spiperone (~0.5 nM; Amersham) at 37°C for 30 min in a total volume of 500 μl receptor binding buffer. Nonspecific binding was defined by using 5 μM (+)-butaclamol. A2A and D2 binding assays were terminated by filtration onto FB glass fiber plates with ice-cold wash buffer (10 mM Tris and 0.9% NaCl) using a cell harvester (FilterMate; Packard). Radioactivity was determined using a Packard TopCount scintillation counter. Specific binding for each sample was determined as the difference between the average counts for total versus nonspecific binding. The specific binding values were normalized to the amount of protein added per well, as determined by the Pierce BCA Protein Assay. Within an experiment, drug treatments were performed in triplicate, and for each of these, total and nonspecific binding conditions were performed in duplicate.

Statistical Analysis. Statistical analysis was performed using Prism (GraphPad Software Inc., San Diego, CA). Student’s t test or one-way ANOVA followed by post hoc tests are indicated with the corresponding p values in the figure legends. A p value < 0.05 was considered significant.

Results and Discussion

A2A and D2L Oligomerization in the CAD Neuronal Cell Model Detected by BiFC. Interactions between A2A and D2L were studied in CAD neuronal cells. Fluorescence resonance energy transfer studies with cells coexpressing A2A–Cerulean and D2L–Venus suggested heteromerization of A2A and D2L in CAD cells (data not shown), consistent with previous reports (Canals et al., 2003; Kamiya et al., 2003). To establish BiFC as a tool to visualize A2A/D2L heteromerization, we engineered A2A and D2L fusions to VN and VC or CN and CC. Function of the BiFC fusion receptors was addressed by measuring cAMP accumulation in response to agonists in cells expressing -VN or -VC receptor fusions or untagged receptors (Fig. 1). Inhibitory effects on adenylyl cyclase were measured in CAD cells expressing D2L, D2L-VN, or D2L-VC. Treatment with the D2 agonist quinpirole resulted in inhibition of forskolin-stimulated cAMP accumulation, which was

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Fig. 1. Functional characterization of receptor-BiFC fragment fusion proteins. A, inhibition of forskolin (Fsk)-stimulated cAMP accumulation was measured in CAD cells expressing D2L, D2L-VN, or D2L-VC after stimulation with quinpirole (Quin, 10 μM) in the absence or presence of spiperone (Spip, 1 μM). B, cAMP accumulation in HEK-293 cells expressing A2A, A2A-VN, or A2A-VC was measured under basal conditions or in the presence of MECA (1 μM) in the absence or presence of CGS15943 (1 μM) as indicated. C, receptor function in cells expressing D2L-VN/D2L-VC, A2A-VN/A2A-VC, or A2A-VN/A2A-VC BiFC pairs. cAMP accumulation was measured in CAD cells as in A for the left panel and in HEK-293 cells as in B for the right panel. D, cAMP accumulation in CAD cells expressing D1 or D1-VC under basal conditions or in the presence of dopamine (DA; 10 μM) in the absence or presence of butaclamol (10 μM), as indicated. Data are means ± S.E.M. of at least three experiments assayed in duplicate. *, p < 0.001 compared with empty vector transfections (one-way ANOVA followed by Dunnett’s post hoc test).
blocked by the addition of the D₄ antagonist spiperone (Fig. 1A). Because CAD cells endogenously express A₂A (Voth-erms and Watts, 2004), A₂A-, A₂A-VN, or A₂A-VC were expressed in HEK-293 cells to verify their function. Short-term activation of A₂A receptors with the adenosine analog MECA resulted in increased cAMP accumulation in cells expressing each of the constructs. This effect was blocked by the A₂A antagonist CGS15943 (Fig. 1B). Subsequent studies examined agonist responses in cells coexpressing D₂L-VN/D₂L-VC, A₂A-VN/D₂L-VC, or A₂A-VN/A₂A-VC (Fig. 1C). The results of these experiments demonstrated that fluorescence complementation did not disrupt quinpirole- or MECA-stimulated receptor function. Function of the dopamine D₁ receptor (D₁) fusion to VC was also addressed in CAD cells expressing D₁ or D₁-VC. D₁-mediated dopamine stimulation of cAMP accumulation was similar in both transfections and was blocked by the dopamine receptor antagonist butaclamol (Fig. 1D). Together, these results revealed that VN and -VC fusion receptors retained ligand-dependent function in the conditions tested. Because Cerulean and Venus have virtually identical structures, we assume that the functional data described above are relevant for receptor fusions to both Venus and Cerulean fragments.

Subsequent experiments used the novel BiFC fusion receptors to explore the localization and specificity of fluorescence complementation in CAD cells. Cells coexpressing A₂A-VN and D₂L-VC exhibited robust Venus fluorescence detected in whole-cell fluorescence measurements (Fig. 2A) or using microscopy (Fig. 2B). Because biochemical evidence suggests a weak A₂A/D₁ interaction (Hillion et al., 2002), control experiments in which D₁-VC replaced D₂L-VC were used to address the specificity of the fluorescent complementation. Fluorescent signals from cells expressing A₂A-VN and D₂L-VC were significantly higher than signals in A₂A-VN and D₁-VC transfections (Fig. 2, A and B). Analysis of receptor expression levels in dot-blot experiments suggested that this difference was not a result of reduced D₁-VC protein expression levels compared with D₂L-VC (Fig. 2C). Likewise, A₂A-VN levels were not significantly different when cotransfected with D₂L-VC or D₁-VC (data not shown). The localization of the A₂A/D₂L and A₂A/D₁ interactions was then analyzed at the subcellular level. Strong BiFC signals were observed at the plasma membrane in cells expressing A₂A-VN and D₂L-VC (Fig. 2, B and D). Signals were also detected in intracellular compartments, presumably reflecting receptor internalization or localization at the endoplasmic reticulum (ER). In experiments using A₂A-VN and D₁-VC pairs, BiFC signal intensity at the plasma membrane was reduced by more than 60% compared with A₂A/D₂L signals (Fig. 2, B and D). Cells expressing A₂A-VN and D₁-VC seemed to display an increased proportion of intracellular fluorescence (Fig. 2E), suggesting more extensive internalization or less efficient export to the plasma membrane of A₂A/D₁, possibly as a consequence of quality-control mechanisms at the endoplasmic reticulum (Bulenger et al., 2005).

**A₂A and D₂L Homo- and Heteromerization Monitored Simultaneously in Living Cells.** Multicolor BiFC (Hu and Kerppola, 2003) was used to simultaneously visualize and compare A₂A/D₂L heteromers and A₂A homomers. The D₂L-VN construct was cotransfected with A₂A fusions to N- and C-terminal fragments of Cerulean (A₂A-CN and A₂A-CC) in CAD cells. Reconstitution of Venus-like fluorescence was

![A](image1)

![B](image2)

![C](image3)

![D](image4)

![E](image5)
indicated to D2A/D2L heteromer formation, whereas Cerulean fluorescence reflected A2A homomerization (Fig. 3A, Supplemental Fig. 1A). Cells imaged by fluorescence microscopy displayed both Venus and Cerulean signals, indicating coexistence of A2A/D2L hetero- and A2A homomers within a cell. Both fluorescent signals largely colocalized at the plasma membrane as well as in intracellular compartments. Similar observations were made with cells transfected with D2L-VN, A2A-CN, and D2L-CC (Supplemental Fig. 1B). Venus (D2L/D2L) and Cerulean (A2A/A2A) fluorescent signals coexisted and largely colocalized at the plasma membrane and in intracellular vesicular structures.

To our knowledge, this is the first study in which multiple GPCR interactions were simultaneously visualized in living cells. The localization of the A2A/D2L oligomer was further addressed by cotransfecting A2A-CN and D2L-CC with three distinct fluorescent markers (Fig. 3B). There was virtually no overlap of A2A-CN/D2L-CC fluorescence with the transmedial Golgi marker (YFP-Golgi; YFP fusion to residues 1–81 of the β1,4-galactosyltransferase). Consistent with recent studies suggesting biogenesis of serotonin 5-HT2C homomers, β2 adrenergic receptor homomers, and D1/D2 heteromers at the ER (Salahpour et al., 2004; So et al., 2005; Herrick-Davis et al., 2006), A2A-CN/D2L-CC fluorescence showed a moderate level of overlap with the ER marker (YFP-ER; YFP fused to the ER targeting sequence of calreticulin and the KDEL ER retrieval sequence). There was also significant overlap of A2A/D2L and structures labeled with the endosomal marker RhoB (Adamson et al., 1992) fused to YFP (YFP-Endo), suggesting trafficking of A2A/D2L heteromers through early endosomes as reported for a number of GPCRs including D2 (Seachrist and Ferguson, 2003). Collectively, these results indicate proper trafficking of BiFC-tagged fusion receptors.

**Effect of Ligands on Receptor Localization.** Having established multicolor BiFC as a tool to detect receptor homo- and heteromerization in a neuronal model, subsequent microscopic studies were designed to examine the effect of long term D2 agonist and antagonist treatments on D2L and A2A homo- and heteromer localization. In cells expressing D2L-VN, A2A-CN, and A2A-CC, 18-h quinpirole treatment resulted in a decreased ratio of surface to intracellular Venus (A2A/D2L) signals (Fig. 3C and Supplemental Fig. 1A). This effect was blocked by coapplication of the D2 antagonist sulpiride. In reciprocal experiments, cells transfected with D2L-VN, A2A-CN, and D2L-CC revealed a similar reduction of surface to intracellular fluorescence for BiFC signals of Cerulean (A2A/A2A) and Cerulean (A2A/D2L) oligomers (Fig. 3D and Supplemental Fig. 1B). Sulpiride blocked the effect of quinpirole on A2A/D2L fluorescence and seemed to increase intracellular fluorescence consequent to quinpirole treatment. The effect of quinpirole was reversed by the D2 antagonists spiperone or sulpiride. Furthermore, D2 antagonists alone caused a marked increase of Venus over Cerulean fluorescence. This increase was similar to that observed when antagonists were coapplied with quinpirole. No significant effect of the quinpirole treatment was observed in control experiments where the D1 receptor replaced D2L (data not shown). In experiments with cells expressing D2L-VN, A2A-CN, and D2L-CC, prolonged quinpirole treatment led to increased Venus over Cerulean fluorescence (Supplemental Fig. 2), indicative of increased D2L/D2L over A2A/D2L oligomerization.

The effect of persistent A2A stimulation on A2A homo- and A2A/D2L heteromerization was addressed by treating cells expressing D2L-VN, A2A-CN, and A2A-CC with the adenosine receptor agonist MECA (Fig. 3F). Treatment with MECA increased the proportion of A2A/D2L (Venus) over A2A/A2A (Cerulean) oligomers and this effect was blocked by coapplication of the adenosine antagonist CGS15943. When applied alone, CGS15943 had no effect on BiFC fluorescence. Control experiments determined that the D2 and A2A ligands tested did not emit fluorescence when excited with wavelengths corresponding to Venus or Cerulean excitation (data not shown).

The differential effect of ligands on BiFC fluorescence may reflect ligand-dependent alterations of receptor density. Thus, we measured D2 and A2A receptor levels in cells transfected with D2L-VN, A2A-CN, and A2A-CC using single-point radioreceptor binding assays. Both quinpirole and sulpiride treatments led to increased D2 receptor density, whereas MECA and CGS15943 had no effect on D2 expression (Table 1). An up-regulation of D2L-VN protein levels after quinpirole, sulpiride, or spiperone treatments was also revealed in dot-blots experiments (data not shown). These results are consistent with a quinpirole-induced increase of D2L relative to A2A oligomer formation (Fig. 3, D and E). The inclusion of sulpiride prevented this change and reversed the fluorescence ratio. Intracellular fluorescence was similarly influenced by the drug treatments, indicating that changes of fluorescence intensity at the plasma membrane did not solely result from altered receptor trafficking. To validate the microscopic analysis, nonbiased whole-cell fluorescence measurements were taken (Fig. 3F). These studies also revealed a decrease in Venus (A2A/D2L) over Cerulean (A2A/A2A) fluorescence consequent to quinpirole treatment. The effect of quinpirole was reversed by the D2 antagonists spiperone or sulpiride. Furthermore, D2 antagonists alone caused a marked increase of Venus over Cerulean fluorescence.

To our knowledge, this is the first study in which multiple GPCR interactions were simultaneously visualized in living cells. The localization of the A2A/D2L oligomer was further addressed by cotransfecting A2A-CN and D2L-CC with three distinct fluorescent markers (Fig. 3B). There was virtually no overlap of A2A-CN/D2L-CC fluorescence with the transmedial Golgi marker (YFP-Golgi; YFP fusion to residues 1–81 of the β1,4-galactosyltransferase). Consistent with recent studies suggesting biogenesis of serotonin 5-HT2C homomers, β2 adrenergic receptor homomers, and D1/D2 heteromers at the ER (Salahpour et al., 2004; So et al., 2005; Herrick-Davis et al., 2006), A2A-CN/D2L-CC fluorescence showed a moderate level of overlap with the ER marker (YFP-ER; YFP fused to the ER targeting sequence of calreticulin and the KDEL ER retrieval sequence). There was also significant overlap of A2A/D2L and structures labeled with the endosomal marker RhoB (Adamson et al., 1992) fused to YFP (YFP-Endo), suggesting trafficking of A2A/D2L heteromers through early endosomes as reported for a number of GPCRs including D2 (Seachrist and Ferguson, 2003). Collectively, these results indicate proper trafficking of BiFC-tagged fusion receptors.
Fig. 3. Effect of ligands on A2A and D2L homo- and heteromer formation and trafficking. A, complemented Venus and Cerulean fluorescence in cells expressing D2L-VN, A2A-CN, and A2A-CC was monitored by fluorescence microscopy. B, localization of intracellular A2A/D2L heteromers. Microscopic images from cells cotransfected with A2A-CN, D2L-CC (in cyan), and either YFP-Golgi, YFP-ER, or YFP-Endo (in yellow). Merged images are shown with YFP signals in red and CN/CC signals in green. Overlapping signals are in yellow. C, internalization of A2A and D2L homo- and heteromers after prolonged (18 h) D2L activation with quinpirole (Quin, 10 μM) in the absence or presence of sulpiride (Sulp, 10 μM). Surface over intracellular fluorescence was measured in cells transfected with D2L-VN, A2A-CN, and A2A-CC (solid bars) or with D2L-VN, A2A-CN, and D2L-CC (striped bars). D and E, relative oligomer formation after prolonged exposure to quinpirole or sulpiride in cells expressing D2L-VN, A2A-CN, and A2A-CC. D, ratiometric images (Ratio) represent Venus over Cerulean signals and are displayed in pseudocolors. The corresponding intensity scale is shown. E, fluorescent signals at the plasma membrane or in intracellular compartments were measured and expressed as Venus/Cerulean fluorescence ratio. Note: portions of data used for C (D2L-VN, A2A-CN, and A2A-CC transfected cells) were also used in E. F, whole-cell fluorescence after treatment with quinpirole (10 μM), spiperone (Spip, 1 μM), sulpiride (10 μM), MECA (10 μM), or CGS15943 (CGS, 10 μM) was measured by fluorometry. Scale bars: 5 μm. *, p < 0.05; **, p < 0.01; ***, p < 0.001 (compared with vehicle, one-sample t test, n = 3–11).
consistent with previous reports (Filtz et al., 1994; Zhang et al., 1994; Starr et al., 1995) and likely reflect a pharmacological chaperone effect (Bernier et al., 2004; Conn et al., 2007) on D2 by its ligands, as previously reported for α opioid (Petajá-Repo et al., 2002) and D4 dopamine (Van Craenenbroeck et al., 2005) receptors. Although previous reports failed to observe an effect of prolonged (14 h) A2A stimulation on A2A expression (Chern et al., 1993), MECA treatment increased A2A receptor density (Table 1). Unexpectedly, both quinpirole and sulpiride also caused a significant increase in A2A density in D2VN, A2A-CN, and A2A-CC transfected cells (Table 1). The mechanisms underlying the modest up-regulation of A2A by MECA, quinpirole, and sulpiride are not clear and probably involve multiple pathways. For example, prolonged stimulation or antagonism of A2A or D2 receptors may lead to changes in intracellular CaMP concentrations and as a result modify extracellular adenosine levels altering A2A density (Do et al., 2007). In addition, pharmacological chaperone effects of D2 ligands may help A2A/D2 heteromers pass quality control at the ER (Bulenger et al., 2005) and therefore promote both D2 and A2A expression. These possibilities will be explored in future experiments.

The drug-induced up-regulation of receptor levels may (at least partially) account for the change in receptor oligomerization monitored with BiFC. The increased A2A/D2L relative to A2A/A2A BiFC signals after prolonged D2 antagonism may be consistent with greater D2 versus A2A up-regulation by sulpiride (1.72- and 1.24-fold over vehicle, respectively; Table 1). In contrast, increased A2A/D2L relative to A2A/A2A oligomerization resulting from persistent A2A stimulation was accompanied with increased A2A in the absence of D2 level changes; inconsistent with BiFC signals simply reflecting receptor densities. Furthermore, both sulpiride and quinpirole increased D2 and A2A levels but had opposing effects on receptor oligomerization. These observations suggest that a quinpirole-induced up-regulation of D2 and A2A was not responsible for the observed reduction of A2A/D2L relative to A2A/A2A oligomers. Rather, we propose that ligand-mediated changes in receptor conformation and/or microenvironmental localization may influence the formation of receptor oligomers. For example, the activation of D2 may result in a stronger propensity to form homomers and/or decrease D2 affinity for A2A receptors by modifying the interaction interface. Consistent with this hypothesis is the observation that prolonged quinpirole treatment lead to increased D2L/D2L relative to A2A/D2L (Supplemental Fig. 2) and decreased A2A/D2L relative to A2A/A2A oligomer formation (Fig. 3).

We used a novel approach to study GPCR interactions and observed ligand-mediated effects on oligomer formation (Pfeifer and Eidne, 2005). GPCR oligomerization has been proposed to be altered in pathogenic situations or by long-term drug administration such as in Parkinson’s disease therapies (Javitch, 2004; Fuxe et al., 2007). Therapies for Parkinson’s disease largely rely on long-term dopamine receptor stimulation with l-DOPA to compensate for the loss of striatal dopaminergic neurons and are often accompanied with dyskinesias. A2A antagonists have recently been applied with reduced l-DOPA doses in clinical studies and were shown to prevent and alleviate l-DOPA-induced dyskinesias (Schwarzchild et al., 2006; Morelli et al., 2007). Although a precise understanding of the molecular mechanisms underlying this adjunctive therapy are lacking, it has been proposed that long-term l-DOPA treatment may alter A2A and D2 homo- and heteromerization on striatal neurons (Antonelli et al., 2006). The present studies provide support for that model. In particular, the D2 agonist-induced decrease in A2A/D2 heteromers relative to A2A homomers may alleviate the constitutive D2 antagonism of A2A signaling. Such an increase in A2A signaling may play a role in the sensitization of A2A-mediated cAMP accumulation after activation of D2 receptors (Vorherrs and Watts, 2004). Moreover, a D2 agonist-induced enhancement of A2A signaling also provides a molecular explanation for the beneficial effects of A2A antagonists in l-DOPA-induced dyskinesias (Morelli et al., 2007). These observations and the results in the present study highlight the applicability of multicolor BiFC as a novel approach to examine physiologically relevant GPCR interactions. Moreover, it may offer a novel technique for screening drugs that target GPCR oligomers.

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