Dopamine Receptor Oligomerization Visualized in Living Cells*

Received for publication, April 26, 2005, and in revised form, July 8, 2005. Published, JBC Papers in Press, August 22, 2005, DOI 10.1074/jbc.M504562200

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G protein-coupled receptors occur as dimers within arrays of oligomers. We visualized ensembles of dopamine receptor oligomers in living cells and evaluated the contributions of receptor conformation to the dynamics of oligomer association and dissociation, using a strategy of trafficking a receptor to another cellular compartment. We incorporated a nuclear localization sequence into the D1 dopamine receptor, which translocated from the cell surface to the nucleus. Receptor inverse agonists blocked this translocation, retaining the modified receptor, D1-nuclear localization signal (NLS), at the cell surface. D1 co-translocated with D1-NLS to the nucleus, indicating formation of homooligomers. (+)-Butaclamol retained both receptors at the cell surface, and removal of the drug allowed translocation of both receptors to the nucleus. Agonist-nonbinding D1(S198A/S199A)-NLS, containing two substituted serine residues in transmembrane 5 also oligomerized with D1, and both were retained on the cell surface by (+)-butaclamol. Drug removal disrupted these oligomerized receptors so that D1 remained at the cell surface while D1(S198A/S199A)-NLS trafficked to the nucleus. Thus, receptor conformational differences permitted oligomer disruption and showed that ligand-binding pocket occupancy by the inverse agonist induced a conformational change. We demonstrated robust heterooligomerization between the D2 dopamine receptor and the D1 receptor. The heterooligomers could not be disrupted by inverse agonists targeting either one of the receptor constituents. However, D2 did not heterooligomerize with the structurally modified D1(S198A/S199A), indicating an impaired interface for their interaction. Thus, we describe a novel method showing that a homogeneous receptor conformation maintains the structural integrity of oligomers, whereas conformational heterogeneity disrupts it.

G protein-coupled receptors (GPCRs) form dimers and higher order oligomers, as inferred from a large body of evidence garnered from a variety of methodological approaches (1–3), and their static configuration has been visualized by atomic force microscopy in the case of rhodopsin to reveal dimers arranged in clustered rows, with each row accommodating 10–20 dimers (4, 5). Rhodopsin-related GPCRs function as arrays of oligomers (5, 6) and form complexes with identical or other GPCRs, generating homo- and hetero-oligomers (7–10). The structural details involved in the formation of receptor dimers or oligomers have not been elucidated, with little experimental evidence for fundamental questions, such as their behavior at the cell surface, whether the oligomers remain intact or separate, and if homooligomers and heterooligomers behave differently. That the oligomerized GPCR structures modulate the properties and conformations of the individual constituent receptors involved has been shown for µ- and δ-opioid receptor heterooligomers (8, 11, 12) and D1 and D2 dopamine receptor heterooligomers (13). In these structures, the ligand binding properties and/or the coupling properties were altered, depending on whether a receptor was within a homooligomer or heterooligomer.

The biophysical techniques utilized to investigate GPCR oligomers, such as bioluminescence resonance energy transfer (14) or fluorescence resonance energy transfer (9, 15), permitted the analysis of receptor-receptor or receptor-protein interactions in situ, within living cells. However, these techniques still have only limited ability to investigate the many aspects of oligomer structure or function that still remain unknown. There is a need to provide insight into the questions regarding receptor oligomeric complexes, such as (i) the precise sites of interactions maintaining monomers in a dimer formation; (ii) whether true heterodimers exist or only homodimers within a heterooligomeric complex; (iii) whether the homodimer interactions differ from the heterodimer; (iv) the numbers of dimers in an oligomer; and (v) whether oligomers form larger complexes and can be functionally regulated. Thus, elucidation of the mechanism underlying the formation of oligomeric structures, analysis of their functional properties, or analysis of their behavior in cells requires new experimental paradigms.

We wished to explore some of the above aspects of oligomerization to understand the dynamics governing the formation and trafficking of oligomers, to ultimately understand the functional relevance of oligomers in cellular processes. We devised a strategy that engineered the trafficking of a GPCR to another cellular compartment and hypothesized that if it took with it its oligomeric partner, this would provide definitive proof of oligomerization and provide a tool to study its dynamics in the cell. We and others determined that homodimerization in the rhodopsin-like GPCRs utilizes a transmembrane domain dimer interface (6, 16, 17, 18), and we predicted that this interaction would remain intact during the engineered receptor trafficking. Moreover, this process may permit us to visualize homo- and heterooligomer formation and, using conformationally altered receptor variants, to probe the contribution of the receptor structure to the stability of oligomers. To achieve our goals, a nuclear translocation pathway was exploited for GPCRs. Translocation of proteins to the nucleus involves nuclear transport proteins that recognize nuclear localization signal sequences (NLSs) (19), which recruit importin carrier proteins, that mediate pro-

* This work was supported by a Proof of Principle Grant from the Canadian Institutes for Health Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: GPCR, G protein-coupled receptor; NLS, nuclear localization signal; BTC, butaclamol; (+)-BTC and (-)-BTC, (+)- and (-)-butaclamol, respectively; GFP, green fluorescent protein; RFP, red fluorescent protein; mRFP, monomerized red fluorescent protein; IC1, -2, and -3, intracellular loop 1, 2, and 3; HA, hemagglutinin; FITC, fluorescein isothiocyanate.
tein translocation to the nucleus. Only a few GPCRs contain endogenous NLSs (20–22). One example, the angiotensin AT₁ receptor, contains an endogenous NLS in helix 8, which serves to direct the receptor into the nucleus in certain cells (10, 20).

The NLS strategy was evaluated using dopamine receptors. Dopamine D₁ and D₂ receptors each have been shown to form homooligomers and together to form heterooligomers (13, 23, 24). We were the first to demonstrate that D₂ receptors exist as homodimers in human and rat brain (25), and our demonstration of D₁ and D₂ receptor complexes by co-immunoprecipitation from rat brain and heterologous cells demonstrated that these receptors heterooligomerize (13). The functional synergism between D₁ and D₂ dopamine receptors was evidenced by the generation of a novel calcium signal by receptor co-activation (13). By fluorescence resonance energy transfer analysis, we revealed that the D₁ and D₂ receptors exist in close proximity on the cell surface, presumably within a heterooligomeric complex. Furthermore, the D₁ and D₂ receptor heterooligomers displayed novel agonist-induced internalization and trafficking patterns, distinct from that of D₁ and D₂ receptor homooligomers (26).

In this report, we show that incorporating an NLS into several of the dopamine receptors mediated receptor translocation to the nucleus. We used this translocation strategy as outlined (Fig. 1a) to understand the ability of these GPCRs to co-trafficking with their oligomerization partners as a test of the robustness of the interaction between them and to probe the structural conformation of homo- and heterooligomers after ligand occupancy and after introduction of structural variation by point mutagenesis. The method enabled the identification of both homo- and heterooligomers for the dopamine receptors and demonstrated that both types of interactions were robust enough to result in co-trafficking of oligomeric partners to the nucleus. Conformational homogeneity of the receptors was necessary to maintain the integrity of a homooligomer, and the interaction between similar receptors within a homomeric structure could not be disrupted. Furthermore, the introduction of any structural dissimilarity of the receptors within the homooligomer, whether induced by drug occupancy or point mutation, resulted in the ability to disrupt these oligomeric structures in living cells. However, within the D₁-D₂ heterooligomer, conformational alteration by antagonist occupancy of one receptor was unable to affect the conformation of the other, and we defined a structural alteration that prevented oligomerization of D₁ and D₂ receptors, indicating that the arrangement of the receptors within the heterooligomer may be substantially different from that within a homooligomer.

Importantly, the strategy described provides a means of testing the robustness of the interaction between receptors, which will be useful to determine points of contact forming the oligomers.

**Visualize of Dopamine Receptor Oligomers**

**MATERIALS AND METHODS**

**Fluorescent Proteins**—cDNA sequences encoding GFP (27), pDsRed2 (28), and pDsRed2-nuc were obtained from CLONTECH (Palo Alto, CA).

**Cell Culture**—HEK cells grown on 60-mm plates in minimum essential medium were transfected with 0.5–2 μg of cDNA using Lipofectamine (Invitrogen). Dopamine antagonists (+)-butaclamol or SCH 23390, when used, was added to cells at 6, 22, 30, and 42 h, and cells were visualized by confocal microscopy at 48 h post-transfection.

**Microscopy**—Live cells expressing GFP and pDsRed2 fusion proteins were visualized with an LSM510 Zeiss confocal laser microscope. In each experiment, 5–8 fields, containing 50–80 cells/field were evaluated, and the entire experiment was repeated 2–4 times (n = 3–5).

**Fluorocytometry**—50,000 cells were added to each well (96-well plate) and transfected with 0.5 μg of cDNA. Minimum essential medium containing antagonists at varying concentrations were added to wells in pentuplicate. The drugs were prepared as 1 mM concentration stock and diluted in growth medium to achieve a concentration between 10 nM and 10 μM. After 48 h, cells were fixed with 4% paraformaldehyde and incubated with the primary antibody (rat anti-HA antibody, 1:200 dilution; Roche Applied Science) and secondary antibody conjugated to FITC (goat anti-rat antibody, 1:32 dilution; Sigma). Cell surface fluorescence was detected using a Cytofluor 4000 (PerSeptive Biosystems). Each experiment was repeated three or four times. Background fluorescence from media, cells, plastic, etc. was evaluated in each experiment and subtracted from the readings.

**DNA Constructs**—All of the DNA encoding the GPCRs were from humans. Sequences encoding GPCRs were cloned into plasmid pEGFP, pDsRed2-N1, or pcDNA3.

**Receptor Constructs**—The D₁(S198A/S199A) and D₁(S199A/S202A) receptors were prepared using the Quikchange mutagenesis kit (Stratagene) according to the manufacturer’s instructions using the following sets of primers: D₁(S198A/S199A), forward (5’-GGACATATGCTATGTACCGGCAGCGCCTCAATTTACATCCC-3’) and reverse (5’-GGGATTAAGTTACGATTTACGCCTGAGATGCATATGCTCC-3’); D₁(S199A/S202A), forward (5’-GCAGAGCATATGCGCTTCATCCCCGCGTAATAAGCTTTTACATCC-3’) and reverse (5’-CCACAGGAGATGTATAGCAGCAGTATGTCATATGCCTGG-3’).

**D₁-NLS-GFP**—Receptor DNA was subjected to PCR as previously reported (29). The reaction mixture consisted of H₂O (32 μl), 10× Pfu buffer (Stratagene) (5 μl), dNTP (10 mM, 5 μl), MgSO₄ (5 μl), oligonucleotide primers (100 ng, 1 μl each), DNA template (100 ng), and Pfu enzyme (5 units). Total volume was 50 μl. PCR conditions were as follows: one cycle at 94°C for 2 min and 30–35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min per cycle and then one cycle at 72°C for 5 min.

**Primer Set for Amplification of the DNA Encoding the D₁ Receptor**—Primers were as follows: HD₁-P1, 5’-GGAGACTCCTGTACGAACCCGAAATTCGGCAGCATGGAGGAGCTGGCTGGTG-3’; HD₁-P2, 5’-GTGTGGCAGATTCTATGTCAGGTGCTACGCCTGT-3’.

The restriction site EcoRI was incorporated in the primer HD₁-P1, and KpnI was incorporated into HD₁-P2. The PCR product, containing no stop codon was subcloned into vector pEGFP at EcoRI and KpnI and in frame with the start codon of GFP. The NLS sequence, KKKKR, was inserted into DNA encoding the base of TM7 (helix 8) of the D₁ dopamine receptor by PCR, replacing the sequence 335DFRKA.

**Primer Set for the Construction of DNA Encoding D₁-NLS**—Primers were as follows: HD₁-NLS, 5’-CTTACAAGAGGTGAAATCTTTTAAATTTGACCATTTAAGGCTATTATG-3’; HD₁-NLSR, 5’-GGCTTATAGCTAAAAATTTAAAGGTATTCTTTAACCCTTTAGGATG-3’.

Using the DNA encoding D₁-GFP as template, PCR with the primers HD₁-P1 and HD₁-NLS resulted in a product of 1000 bp (PCR1). Using DNA encoding D₁-GFP, PCR with primers HD₁-P2 and HD₁-NLS resulted in a product of 300 bp (PCR2). A subsequent PCR carried out with HD₁-P1 and HD1-P2 primers resulted in a product of 1300 bp using the product from PCR1 and the product from PCR2 as templates. The resulting DNA encoding D₁-NLS was subcloned into vector pEGFP at EcoRI and KpnI restriction sites.

All of the additional constructs described below were made using the same PCR method and experimental conditions as described above for
the D1 dopamine receptor, but with the specific primers as described below.

**Primer Set for the Construction of D1-IC1-NLS**—Primers were as follows: D1-NLSF-IC1, 5’-GTGCTCGCTTAAAAAGAGGTCAGAGCATCCGCTGAG-3’; D1-NLSR-IC1, 5’-GCGGTGCGGCTGAGATCCGTTAAC-3’. KKKF was inserted into the IC1 segment of the D1 receptor, replacing 133MKTP.

**Primer Set for the Construction of the D1-IC2-NLS**—Primers were as follows: D1-NLSF-IC2, 5’-CCGGTATTGAAGAATTAAAGCCGAAATGTC-3’; D1-NLSR-IC2, 5’-GGGCGCGGCTGAGATCCGTTAAC-3’. KKKF was inserted into the IC2 segment of the D1 receptor, replacing 203MSFR.

**Primer Set for D2-NLS-GFP**—Primers were as follows: HDNLSF, 5’-CACCACCTTCAACAAAAATTCTCAGAGCTCTTCTC-3’; HD2-NLSR, 5’-GGATCTTCAAGAGGCGCTTCTTTGAAAATTTTTTGCACGTTGC-3’. The sequence KKKF was inserted in the D2 receptor, replacing 816IEFRK.

**Primer Set for the Construction of SPGFP-D2**—The D2 cDNA was isolated by the PCR method using the following set of primers: D2sp-BsrGI, 5’-TCCGATTCTTTTTCAGAGATTCATTT-3’; D2sp-NotI, 5’-GAGTCGCGCTGAGATCCGTTAAC-3’. This PCR product was then subcloned into the SPGFP vector at restriction sites BsrGI and NotI.

**Primer Set for the Construction of SPGFP-D5**—Using the SPGFP-D2 as template, the NLS KKKF was introduced into helix 8 of the D2 by the PCR method using the following set of primers: HD2-NLSF, 5’-GTGCTCGCTTAAAAAGAGGTCAGAGCATCCGCTGAG-3’; HD5-Kpn (5’-CCGGTATTGAAGAATTAAAGCCGAAATGTC-3’) and HD5-PstI (5’-CGCCAATGGACCGTGC-3’). This PCR product was isolated by PCR with the following set of primers: D5-BsrGI, 5’-AACGCGTTCGTAGAGATTCATTT-3’; D5-NotI, 5’-GAGTCGCGCTGAGATCCGTTAAC-3’. The sequence KKKF was inserted in the D5 receptor, replacing 483IEFRK.

**Primer Set for the Construction of DS-GFP**—The human D5 was isolated by PCR with primers HD5-EcoRI (5’-CTGGATACTTCGAGATGCAGCGCAGCCGACG-3’) and HD5-Kpn (5’-CGCCAATGGACCGTGC-3’) and HD5-PstI (5’-CGCCAATGGACCGTGC-3’). The sequence KKKF was inserted into the D5 receptor before the NLS, replacing 503IEFRK.

**Primer Set for the Construction of DS-5**—The human D5 was isolated by PCR with the following set of primers: D5-BsrGI, 5’-CCAGCGTTCGTAGAGATTCATTT-3’; D5-NotI, 5’-GAGTCGCGCTGAGATCCGTTAAC-3’. This PCR product was then subcloned into the SPGFP vector at restriction sites BsrGI and NotI.

**The Construction of D1-mRFP**—mRFPI in the pReSTb vector was a gift from Dr. Irene Prastio (Howard Hughes Medical Institute, University of California, San Diego). Using this vector as template, the mRFPI was isolated by PCR using the following two primers: mRFPI-BAMHI, 5’-GATAAGGA-TCCGATGGCTCTTCTCGAGG-3’; mRFPI-NotI, 5’-GCGGCGGCTGAGATCCGTTAAC-3’. The sequence KKKF was inserted into the D1-mRFP vector at restriction sites EcoRI and KpnI and subcloned into the mRFPI vector at the same restriction sites EcoRI and KpnI and in frame with the mRFPI.

**Primer Set for CysLT2-NLS-GFP**—Primers used were LT2-NLSF, 5’-GCTGGAAAATTTAAGAGACTAAATGTCGCTCA-3’ and LT2-NLSR (5’-GCTTTTTTTTTAATTCTTTCCAGGAAAGAGTAGAGC-3’). The sequence KKKF was inserted into the cysteinyl leukotriene receptor 2, replacing 320ENFKD.

**RESULTS**

The initial objective was to generate a GPCR that would traffic to the nucleus under basal conditions and permit ligand-occupied conformational changes to modulate this process.

**Optimization of the Position of the NLS within the D1 Dopamine Receptor in a Conformation-dependent Site**—To determine the optimal site for NLS incorporation that would provide the most efficient receptor translocation to the nucleus, the D1 dopamine receptor was modified. Since the proteins that carry NLS-containing proteins to the nucleus, such as the importins, are cytoplasmic, the optimal placement of the NLS was investigated by its introduction into various positions within the three intracellular loops, helix 8, and the carboxyl tail of D1; each of these locations is illustrated in Fig. 1b.

When expressed in cells, the unmodified D1 receptor tagged with GFP was localized on the cell surface in the majority of the cells (>90%) at 48 h post-transfection as visualized by confocal microscopy (Fig. 2a, upper left panel). Cells transfected with the D1 receptor containing the NLS in helix 8 (D1-NLS) revealed a basal localization of receptor in the nucleus at 48 h post-transfection (Fig. 2a, upper right panel) observed in over 90% of cells. The localization of the receptor in the nucleus was confirmed using a nuclear dye (Hoechst 33342) (Fig. 2a, lower panels). Thus, incorporation of a NLS into the D1 receptor sequence in helix 8 resulted in efficient trafficking of the receptor under basal conditions to the nucleus. A series of tomographic images (Z-stacks) obtained by confocal microscopy confirmed the nuclear localization of the D1-NLS.

Investigation of the effect of the NLS inserted in various other intracellular positions of the receptor revealed that D1 with an NLS inserted in the first intracellular cytoplasmic loop (D1-IC1-NLS) was expressed and detected in the nucleus in 85% of cells (Fig. 2b, left). D1 with the NLS inserted in the second intracellular cytoplasmic loop (D1-IC2-NLS) was expressed and detected in the nucleus in 51% of cells. In this case, over 40% of cells still had receptor detectable on the cell surface, indicating that incorporation of the NLS in this position was not as efficient in translocating the receptor from the cell surface. D1 with the NLS inserted in the third intracellular cytoplasmic loop (D1-IC3-NLS) was expressed and detected in the nucleus of 85% of cells (Fig. 2b, middle). D1 with an NLS inserted in a distal position in the carboxyl tail (D1-CT-NLS) was expressed and was detected at the cell surface and also in the nucleus (Fig. 2b, right). The distribution of D1-GFP-NLS where the NLS was attached distal to the GFP fused to D1, showed expression in the cytoplasm and cell surface but little expression in the nucleus (data not shown).

To determine the effect of occupancy of the binding pocket on the nucleus within various positions of the receptor on trafficking to the nucleus, we used several antagonists with inverse agonist properties to induce a conformational change in the receptor. Although receptor agonists would also induce conformational changes in the receptor, we wished to preclude agonist-induced internalization mechanisms, which would confound the evaluation of NLS-mediated trafficking. Cells expressing D1-NLS were treated with the dopamine receptor antagonist (+)-butaclamol ((+)-BTC) or SCH 23390 6 h post-transfection.
With (+)BTC (1 μM) treatment for 48 h, there was a very efficient retention of the D1-NLS receptor on the cell surface (85% of cells) with little translocation to the nucleus (Fig. 2c, left). This effect was also found with the D1 receptor-selective antagonist SCH 23390. Stereoselectivity of this effect was demonstrated by the lack of effect of (−)BTC (1 μM) treatment of the cells. In addition, the specificity of the D1 antagonist on receptor translocation was tested by treating D1-NLS-expressing cells with the D2 receptor-selective antagonist raclopride (1 μM), which was unable to retain the receptor at the cell surface.

D1-NLS was expressed and treated with (+)BTC (500 nM) for 48 h, and at this time, the receptor was located at the cell surface (100% of cells). The (+)BTC was removed, and the receptor distribution was examined at 3, 6, 13, 16, 19, and 24 h. Between 13 and 16 h, the receptor had left the cell surface and was distributed to the nucleus in 80% of cells. Cells expressing D1-IC1-NLS treated with either (+)BTC (1 μM) or SCH 23390 (1 μM) also revealed retention of receptor at the cell surface in 82% of cells (Fig. 2c, middle) and 77% of the cells, respectively, compared with 76% of cells with receptor expression in the nucleus with no treatment. In contrast, cells expressing D1-IC3-NLS treated with (+)BTC (1 μM) was localized largely on the cell surface and cytoplasm and was absent from the nucleus. D1-IC3-NLS treated with (+)BTC (1 μM) was localized in the nucleus, indicating no effect of drug. Nuclei were identified by co-expression with DsRed2-nuc. c, effect of exposure to antagonist. D1-NLS-expressing cells were treated with the D1 antagonist (+)BTC, 1 μM (left), and were located at the cell surface. D1-IC1-NLS was treated with (+)BTC (1 μM) and was localized largely on the cell surface and cytoplasm and was absent from the nucleus. D1-IC3-NLS treated with (+)BTC (1 μM) was localized in the nucleus, indicating no effect of drug. Nuclei were identified by co-expression with DsRed2-nuc. d, demonstration of cell surface expression of D1-NLS 9 and 24 h following transfection. Cells expressing D1-NLS were treated with concanavalin A for 24 h.

**Visualization of Dopamine Receptor Oligomers**

**FIGURE 2.** a, effect of incorporation of an NLS into helix 8 of the D1 dopamine receptor (D1-NLS). The D1 dopamine receptor-GFP (D1) fusion protein was expressed in HEK cells and visualized by confocal microscopy. D1 was localized on the cell surface and was absent from the nucleus (upper left panel). D1 dopamine receptor-GFP with an NLS in helix 8 (D1-NLS) expression was located in the nucleus of the cells and not at the cell surface (upper right panel). Nuclei were identified by co-expression with DsRed2-nuc or staining with Hoechst 33342 (lower panel). b, effect of incorporation of an NLS into other positions of the D1 receptor; IC1 (D1-IC1-NLS), IC3 (D1-IC3-NLS), and the carboxyl tail (D1-CT-NLS) were expressed in HEK cells and visualized. D1-IC1-NLS and D1-IC3-NLS were localized in the nucleus, and D1-CT-NLS was localized on the cell surface and inside the cell. In some cases, nuclei were identified by co-expression with DsRed2-nuc. c, effect of exposure to antagonist. D1-NLS-expressing cells were treated with the D1 antagonist (+)BTC, 1 μM (left), and were located at the cell surface. D1-IC1-NLS was treated with (+)BTC (1 μM) and was localized largely on the cell surface and cytoplasm and was absent from the nucleus. D1-IC3-NLS treated with (+)BTC (1 μM) was localized in the nucleus, indicating no effect of drug. Nuclei were identified by co-expression with DsRed2-nuc. d, demonstration of cell surface expression of D1-NLS 9 and 24 h following transfection. Cells expressing D1-NLS were treated with concanavalin A for 24 h.
Visualization of Dopamine Receptor Oligomers

transfection and treated with concanavalin A to retard internalization of the receptor from the cell surface. D1-NLS was visualized and detected at the cell surface between 7 and 10 h post-transfection, at a time when no receptor was visualized in the nucleus (Fig. 2d, left), subsequently showing a progressive translocation to the nucleus (Fig. 2d, middle). D1-NLS was retained on the cell surface with no receptor located in the nucleus following treatment with concanavalin A for 24 h after transfection (Fig. 2d, right). These results indicated that the receptor trafficked first to the cell surface after biosynthesis and then was translocated to the nucleus. Cells expressing D1-IC3-NLS were treated with concanavalin A for 24 h after transfection and showed undiminished robust nuclear expression with no cell surface expression, indicating that the receptor may have traveled directly to the nucleus.

The incorporation of the NLS into the helix 8 of the D1 receptor did not alter the binding pocket of the receptor, with preserved agonist-detected high affinity and low affinity states, indicative of intact receptor-G protein coupling and ligand affinities. The D1-NLS receptor had a $K_{d_{\text{high}}}$ value of $4.17 \times 10^{-7}$ M and $K_{d_{\text{low}}}$ of $1.19 \times 10^{-7}$ M detected by agonist SKF 81297 not different from unmodified D1 receptor.

Co-translocation of NLS-modified D1 and Wild Type D1 Receptors—The helix 8 NLS-containing D1 receptor (D1-NLS) had efficient ability to translo-
FIGURE 5. Disruption of D1 receptor oligomers by conformational alteration. a, HEK cells expressing D1(S198A/S199A) (A198/A199) showed that the receptors were located at the cell surface, b, D1(S198A/S199A)-NLS (GFP) (green) and D1 (RFP) (red), showing D1(S198A/S199A)-NLS trafficked with D1 to the nucleus. Cells expressing only D1 show exclusive cell surface localization (arrow). c, cells expressing D1(S198A/S199A)-NLS and D1 when treated with (+)BTC revealed robust co-localization at the cell surface. d, co-expression of D1(S198A/S199A)-NLS with D1, following removal of the (+)BTC. The red and green colors are distinct with no overlap, indicating that the oligomerized receptors have separated.

FIGURE 6. Lack of D1 oligomer disruption by a minor receptor modification. a, D1(S199A/S202A)-NLS (A199/A202) expressed in HEK cells treated with vehicle and in the presence of (+)BTC revealed robust effects to retain the receptor. b, D1-S199A/S202A-NLS (GFP) (green) and D1 (RFP) (red) co-expressed and treated with (+)BTC, which retained both receptors at the cell surface. c, cells co-expressed D1-S199A/S202A-NLS and D1 and visualized following removal of the (+)BTC. The majority of both receptors co-migrated together from the cell surface to the nucleus.

A D1 receptor generated with two of these serines, Ser^{198} and Ser^{199}, substituted with alanine (D1(S198A/S199A)) did not bind dopamine or SCH 23390, but we determined that it did bind (+)BTC. The D1(S198A/S199A) receptor was efficiently expressed on the cell surface (100% of cells) (Fig. 5a). This receptor formed homooligomers as detected by Western blot analysis. Cells expressing D1(S198A/S199A)-NLS, when treated with (+)BTC (1 μM) but not SCH 23390, resulted in retention of receptor at the cell surface (100% of cells).

To investigate the cellular route by which this mutated receptor reached the nucleus, cells expressing D1(S198A/S199A)-NLS were treated with concanavalin A. The receptor was retained on the cell surface with no receptor located in the nucleus following treatment for 24 h after transfection. This indicated that the receptor trafficked to the cell surface prior to translocation to the nucleus (data not shown).

In order to explore whether structural variations in the D1 receptor could affect oligomerization, D1 was expressed with D1(S198A/S199A)-NLS, and these receptors were visualized to translocate together to the nucleus, indicating efficient oligomer formation (90% of cells) (Fig. 5b). Cells in the microscopic field expressing only D1 showed cell surface expression exclusively (indicated by an arrow). Cells expressing both D1 and D1(S198A/S199A)-NLS were treated with (+)BTC (1 μM), and both receptors were seen to be located on the cell surface (100% of cells) (Fig. 5c). (+)BTC was removed, and the cellular distribution of the receptors 24 h later was examined. At this time point, D1 had remained at the cell surface, and D1(S198A/S199A)-NLS translocated alone to the nucleus (Fig. 5d). D1(S198A/S199A)-NLS was in the nucleus in 40% of cells only, and 60% of the cells had receptor in the nucleus and cell surface. These data indicated that treatment with (+)BTC had probably induced a conformational change within the two receptors, which were now different from each other, resulting in their separation. By this method, we could detect differences in the conformation of the D1 and D1(S198A/S199A)-NLS receptors. Thus, D1(S198A/S199A) and D1 appear to oligomerize under basal conditions, but unlike the structurally homogenous D1 and D1-NLS oligomers, these oligomers were more easily disrupted, probably due to conformational differences between the receptors.

To further test this hypothesis, D1(S198A/S199A) was expressed with D1(S198A/S199A)-NLS, and these receptors translocated together to the nucleus (97% of cells). In the presence of (+)BTC, both receptors were located at the cell surface (100% of cells). Following the removal of (+)BTC, both D1(S198A/S199A) and D1(S198A/S199A)-NLS moved from the cell surface and translocated to the nucleus (93% of cells). These data confirmed, therefore, that there were differences in the conformations of D1 and D1(S198A/S199A) receptors, not just within the binding pocket, as predicted by the mutations, but in the overall structure. Under basal conditions, the difference was such that it permitted efficient oligomer formation, but it was more exaggerated by antagonist binding; hence, the oligomer was disrupted. Thus, as shown by the pairs D1 and D1-NLS, and D1(S198A/S199A) and D1(S198A/S199A)-NLS, stable oligomers were formed only between conformationally identical receptors.
When D1 was co-expressed with D1(S199A/S202A)-NLS, both receptors co-trafficked to the nucleus (100% of cells) (Fig. 6c), suggesting an incomplete separation of the two receptors. These data indicated that considerable conformational similarity existed between these receptors capable of forming stable oligomers.

**Evaluating the Ability of D1-NLS Receptor to Remove D1 from the Cell Surface Using Fluorometric Analysis**—Analysis of the density of cell surface receptors was used to monitor the ability of D1-NLS to translocate D1 off the cell surface. Using fluorescent labeling of epitope-tagged D1 receptors, cell surface receptor detection was achieved, and the signal was detected by a plate reader fluorometer. Cells expressing HA-tagged D1-NLS receptors were treated with varying doses of (+)BTC for 24 h, some D1 remained at the cell surface, but some translocated with D1(S199A/S202A)-NLS to the nucleus (100% of cells) (Fig. 6c), showing retention of the receptor on the cell surface, indicating that the antagonist reduced D1-NLS trafficking from the cell surface. Stereoselectivity of this effect was demonstrated by the lack of effect of (−)BTC (1 μM). HA-tagged D1 receptors were co-expressed with increasing amounts of a FLAG-tagged D1-NLS. There was a concentration-dependent effect of FLAG-D1-NLS to remove the HA-D1 from the cell surface (Fig. 7b).

**Visualization of Dopamine Receptor Oligomers**—The D2-NLS, with the NLS in helix 8, did not translocate as efficiently as D1-NLS from the cell surface to the nucleus, presumed to be due to the absence of an extended carboxyl tail on the D2 receptor, placing the attachment of the GFP in too close proximity to the helix 8 NLS and impeding importin access. A D2 receptor with GFP located in the amino terminus of the receptor (amino-D2-NLS) was therefore prepared. Following the removal of (+)BTC for 24 h, some D1 remained at the cell surface, but some translocated with D1(S199A/S202A)-NLS to the nucleus (100% of cells) (Fig. 6c), suggesting an incomplete separation of the two receptors. These data indicated that considerable conformational similarity existed between these receptors capable of forming stable oligomers.

**Heterooligomerization of D1 and D2 Dopamine Receptors**—The D2-NLS, with the NLS in helix 8, did not translocate as efficiently as D1-NLS from the cell surface to the nucleus, presumably due to the absence of an extended carboxyl tail on the D2 receptor, placing the attachment of the GFP in too close proximity to the helix 8 NLS and impeding importin access. A D2 receptor with GFP located in the amino terminus of the receptor (amino-D2-NLS) was therefore prepared.
D1 expression was not equivalent and markedly lesser amounts of the NLS-containing receptor were present.

In order to investigate the effect of conformational change in one or the other or both protomers within the heterooligomer, we examined the effect of selectively or jointly manipulating the receptor conformations. Cells expressing amino-D2-NLS and D1 were treated with the selective D1 receptor antagonist SCH 23390 (500 nM) for 24 h, and cells were observed 24 h after drug wash off. In the presence of SCH 23390, both amino-D2-NLS and D1 receptors trafficked together robustly to the nucleus (90% of cells) (Fig. 8b). The receptor distributions remained unaltered after drug removal. Treatment with the D2-selective antagonist raclopride (500 nM) in a similar experimental design revealed, in the presence of drug, that both receptors were localized together on the cell surface (100% of cells) (Fig. 8c). After raclopride removal, both receptors moved off the cell surface, but after drug wash off, the receptors were again seen to separate, with amino-D2-NLS translocating to the nucleus (100% of cells) (Fig. 8d) and moved off together to the nucleus when the drug was removed (35% of cells in nucleus only, 65% of cells in nucleus and cell surface). Thus, the conformational change occurring in one constituent of the heterooligomer could not be imparted to the other constituent, as indicated by the inability of the SCH 23390 occupying the D1 receptor to affect the conformation of the D2-NLS.

We also located the NLS in intracellular loop one of the D2 receptor (D2-IC1-NLS), distant from the carboxyl tail, and this receptor also translocated efficiently to the nucleus (100% of cells). The heterooligomeric association between D1 and D2 receptors was confirmed with this construct as well, since the D1 receptor translocated with the D2-IC1-NLS to the nucleus (100% of cells).

To probe the effect of a structural conformational difference in the stability of the heterooligomer, we co-expressed amino-D2-NLS and D1(S198A/S199A). These cells showed that amino-D2-NLS had trafficked to the nucleus (100% of cells), whereas D1(S198A/S199A) remained on the cell surface (56% of cells in the nucleus only and 44% of cells nucleus and cell surface). The effect of co-manipulation of both receptor conformations simultaneously was tested by treating cells expressing amino-D2-NLS and D1 receptors with (+)-BTC (500 nm). When (+)-BTC was present, both receptors were localized on the cell surface (100% of cells) (Fig. 8d) and moved off together to the nucleus when the drug was removed (35% of cells in nucleus only, 65% of cells in nucleus and cell surface). Thus, the conformational change occurring in one constituent of the heterooligomer could not be imparted to the other constituent, as indicated by the inability of the SCH 23390 occupying the D1 receptor to affect the conformation of the D2-NLS.

Visualization of Dopamine Receptor Oligomers
and D1(S198A/S199A) remaining on the cell surface (100% of cells) (Fig. 9b). We also expressed amino-D2-NLS with D1(S199A/S202A), and both trafficked together to the nucleus under basal conditions (100% of cells) (Fig. 9c). When treated with (+)BTC (500 nM), both receptors were co-localized on the cell surface (100% of cells) (Fig. 9d). After drug wash off, both receptors trafficked together to the nucleus (97% of cells) (Fig. 9e), indicating that this heterooligomer remained intact.

The incorporation of the NLS into the D2 receptor did not alter the binding pocket of the receptor, with preserved agonist-detected high affinity and low affinity states, indicative of intact receptor-G protein coupling. The D2 receptor had a $K_{\text{high}}$ value of $1.51 \times 10^{-9} \text{M}$ and $K_{\text{low}}$ of $6.67 \times 10^{-6} \text{M}$ for quinpirole. Similarly, D2-NLS had a $K_{\text{high}}$ value of $3.22 \times 10^{-9} \text{M}$ and $K_{\text{low}}$ of $4.16 \times 10^{-6} \text{M}$ for quinpirole, not different from the D2 receptor.

**Formation of D5 and D1 Dopamine Receptor Heterooligomers**—The D5 dopamine receptor structure shares extensive homology with the D1 receptor; however, there are pronounced sequence differences in the carboxyl tail regions of these receptors. When expressed in cells, the D5 was located primarily at the cell surface and also in the cytoplasm. The D5 was modified with the NLS incorporated in helix 8 (D5-NLS). However, D5-NLS did not translocate as efficiently as D1-NLS to the nucleus. Additionally, the D5 receptor with an NLS incorporated into intracellular loop one (D5-IC1-NLS) also did not translocate as efficiently to the nucleus, compared with D1-IC1-NLS. These experiments indicated significant differences in the structural conformation of the D5 carboxyl tail compared with D1. In support of this, D5-IC1-NLS with a carboxyl tail truncated at Asp$^{399}$ was very efficiently translocated to the nucleus (95% of cells). From these results, we suspected that the GFP moiety attached to the carboxyl tail of the D5 dopamine receptor in the above experiments may be positioned by the carboxyl tail conformation to sterically hinder access by the importin protein to the NLS. A construct with the GFP moiety placed in the amino terminus of the receptor (amino-D5) when expressed with D1-NLS showed that these receptors translocated together to the nucleus (85% of cells), confirming that the D1 and D5 receptors formed heterooligomers (Fig. 10a).

**Removal of D5 Receptor from the Cell Surface by D1-NLS**—Using fluorescence cell surface labeling, HA-D5 cell surface detection was measured. Cells co-expressing HA-D5 receptors with increasing amounts of FLAG-D1-NLS revealed a robust effect of FLAG-D1-NLS to remove the HA-D1 from the cell surface (Fig. 10b).

**DISCUSSION**

We developed a novel strategy that enabled elucidation of some structural features and architecture of dopamine receptor homooligomers and heterooligomers in living cells. The method, unlike static in-cell fluorescent methodologies currently available, was able to probe structural variations within dopamine receptor oligomers and test the robustness of the receptor-receptor interactions, including demonstration of disruption of the oligomers from the cell surface in living cells. These are aspects that cannot be evaluated by any current methodology. The occupancy of the ligand-binding pocket by an antagonist/inverse agonist was able to shift the conformation of the D1 receptor sufficiently to disrupt the oligomer and result in separation of the components when there was a receptor with a structural variation expressed with the wild type receptor. These data provide, for the first time, evidence of the ability to regulate GPCR oligomers at the cell surface, showing that the relative activation state or the conformation of oligomers contributes to whether oligomers remain together or separate from one another. These capabilities inherent within this method provide a unique alternative for the study of properties of GPCR oligomers not possible otherwise.

We visualized that the D1 receptor formed homooligomers with D1-NLS and readily trafficked with it to the nuclear compartment. When the co-expressed D1 and D1-NLS receptors were treated with (+)-butaclamol or SCH 23390, both forms remained on the cell surface, and then, when the drug was removed, both trafficked together off the cell surface to the nucleus. These data indicated that these receptors interacted to form an oligomer, robustly enough to survive co-trafficking to another cellular compartment. Since any conformational change in the receptors induced by the drug would occur in all the receptors within the D1/D1-NLS oligomer and would therefore be identical, the oligomer was seen to remain intact through antagonist occupancy and after drug wash off. To investigate the characteristics of the oligomers formed between D1 and D1-NLS, two amino acid mutations were introduced into transmembrane domain V of the receptor at Ser$^{198}$ and Ser$^{199}$. The D1(S198A/S199A) receptor did not bind to agonist or the antagonist/inverse agonist SCH 23390 but was shown to bind the antagonist/inverse agonist (+)-butaclamol. When expressed individually with its NLS-containing counterpart, these receptors were co-trafficked and oligomerized. The oligomers between D1(S198A/S199A)-NLS and D1(S198A/S199A) were not separated and remained intact, whereas the oligomers formed between D1 and D1(S198A/S199A) receptors, could be disrupted and separated. This suggested that these two receptors, the D1 and D1(S198A/S199A), with the small structural difference in transmembrane V, were structurally similar in the basal state to oligomerize but sufficiently distinct conformationally when the wild type receptor was bound by (+)-butaclamol for the oligomer to separate. An overall interpretation of these data is that stable oligomers only form between receptors that have identical conformations and that introduction of a conformational difference between them may be a mechanism by which

**FIGURE 10. Oligomerization of D1 and D5 dopamine receptors.** a, D1-NLS (GFP) (green) and D5 (RFP) (red) co-traffick to the nucleus. HA-D5 expressed in HEK cells. Cells expressing HA-D5 were co-expressed with FLAG-D1-NLS. The HA-D5 cell surface receptors were quantified by the fluorescent signal of an FITC-conjugated second antibody directed at the HA antibody. Co-expression with FLAG-D1-NLS resulted in a decrease in cell surface expression of HA-D5, indicating that FLAG-D1-NLS formed oligomers with HA-D5.
Visualization of Dopamine Receptor Oligomers

oligomer size is delimited and regulated. It is intriguing to speculate that this may provide a physiological mechanism for oligomer disruption, whereby the ligand-occupied receptor, which would be in an activated conformation, could separate from neighboring receptors that are not activated and therefore not in an identical conformation. However, since the building block of GPCR oligomers appears to be a dimer, it remains to be determined whether this process could separate the receptors into monomers or rather break up an oligomer into dimeric components. Indeed, it has been suggested that the weakest interaction may be between dimer rows within oligomeric arrays (5).

D1(S199A/S202A) bound both antagonist/inverse agonist ligands similarly to the D1 receptor, implying that this receptor was less altered conformationally, compared with wild-type D1, than D1(S198A/S199A). This was confirmed by the robustness of its oligomerization to D1 and the inability to separate these proteins by ligand occupancy.

The specific sites of interaction maintaining the monomeric receptor components in both dimer and oligomer formation have yet to be completely elucidated. We and others have reported that transmembrane 4 is involved in receptor dimer formation (5, 16, 17), and other transmembranes have been reported to participate in oligomer formation (18, 31). Thus, alterations in the receptor hydrophobic core, particularly residues containing the ligand-binding site, predictably may affect the receptor conformation, such as in D1(S198A/S199A).

We also showed the ability of dopamine D1 and D2 receptors to oligomerize and traffic together. In this case, the use of selective antagonists to attempt to introduce a conformational difference showed that manipulation of one receptor could not influence the conformation of the other. An explanation for this may be that the D1 and D2 receptors do not form heterodimers, although they form heterooligomers. It is possible that heterooligomers are formed between arrays of homodimers but heterodimers are not formed. The separation induced by the antagonist with heterooligomers may suggest that they were never heterodimers. The inability of the D1(S198A/S199A) to oligomerize with the D2 receptor indicated that the conformational alteration induced in the D1 receptor impaired an interface necessary for D1 and D2 heterooligomerization. However, since the D1(S198A/S199A) was able to oligomerize with the wild type D1 receptor, it indicated a lesser conformational difference between these two receptors. Whether this indicates that different interfaces are responsible for the assembly of homooligomers versus heterooligomers remains to be shown. We have shown that the basal intrinsic activity of D1(S198A/S199A) on adenyl cyclase activation was comparable with that of the wild type D1 receptor. This, together with the demonstrated ability of this receptor to bind and respond to (+)-butaclamol, indicated that the conformational change resulting from the mutation of the two serine residues in transmembrane domain V has not led to a major disruption of receptor structure but has been relatively small.

The data also reveal differences in the nuclear translocation of the highly homologous D1 and D5 receptors when modified with the insertion of a NLS, which indicated the novel conclusion of significant conformational differences between the intracellular domains of these receptors, despite the extensive similarities in the primary structures between D1 and D5 receptors (75% overall). The D1 dopamine receptor, fused with GFP with the NLS incorporated in IC1, IC3, or helix 8 each resulted in a robust translocation to the nucleus, whereas each of these equivalent modifications made with the D5 (also fused with GFP) showed less robust nuclear localization. However, as determined by fluorometric analysis, the HA-tagged D5-NLS was efficiently translocated from the cell surface. This indicated that the positioning of the GFP impeded importin access to NLS binding. In confirmation of this, D5-IC1-NLS with a truncated carboxyl tail or D5 with the GFP positioned at the amino terminus translocated efficiently to the nucleus.

Our strategy to examine the properties of the dopamine receptor oligomers capitalized on the physiological mechanism whereby sequences specifying an NLS, when incorporated into cytoplasmic proteins, enabled the proteins to be relocated to the nucleus by the importin translocation pathway. This is a well-characterized process, the mechanism of which is well understood (10, 19, 32). Therefore, the incorporation of an NLS into a GPCR and its translocation from the cell surface toward and into the nucleus formed the basis for the investigation of dopamine receptor oligomerization.

In summary, we have developed an engineered translocation method that enables the study of GPCR oligomer complexes directly in living cells. This method provides insight into the dynamics of these complexes in situ following changes in configuration of the component receptors, introduced by selective mutagenesis or that occur with antagonist occupancy of the binding pocket. This strategy provides an entirely novel approach to examine homo- and heterooligomerization in Family A GPCRs. We have shown that an oligomer composed of homogenous structural units behaves as a single complex, its integrity due to receptor-receptor interactions maintained even when trafficking to a distant cellular compartment. However, an oligomer composed of heterogeneous units, even if generated from the same GPCR, was prone to easier disruption by altering the conformation of the components. This may provide a means of physiological regulation of the size and kinetics of an oligomer. In addition, the clear segregation of the differentially tagged component receptors upon oligomer disruption suggested that they were heterooligomeric and probably not heterodimeric in nature. Furthermore, we were also able to demonstrate that small conformational changes could prevent heterooligomerization, suggesting that alterations in the structure may affect the interaction interfaces. We have shown, therefore, that the multisubunit GPCR oligomeric complexes can be regulated at the cell surface, and these data provide insight into the dynamic nature of the structure and the stability of these protein complexes within cells.

REFERENCES

1. George, S. R., O’Dowd, B. F., and Lee, S. P. (2002) Nat. Rev. Drug Discovery 1, 808 – 820
2. Milligan, G. (2004) Mol. Pharmacol. 66, 1–7
3. Bouvier, M. (2001) Nat. Rev. Neurosci. 2, 274 – 286
4. Fotiadis, D., Liang, Y., Filipek, S., Saperstein, D. A., Engel, A., and Palczewski, K. (2003) Nature 421, 127 – 128
5. Liang, Y., Fotiadis, D., Filipak, S., Saperstein, D. A., Palczewski, K., and Engel, A. (2003) J. Biol. Chem. 278, 21655 – 21662
6. Lee, S. P., O’Dowd, B. F., Ng, G. Y., Varghese, G., Akil, H., Mansour, A., Nguyen, T., and George, S. R. (2000) Mol. Pharmacol. 58, 120 – 128
7. Jordan, B. A., and Devi, L. A. (1999) Nature 399, 697 – 700
8. George, S. R., Fan, T., Xie, Z., Tse, R., Tam, V., Varghese, G., and O’Dowd, B. F. (2000) J. Biol. Chem. 275, 26128 – 26135
9. McVey, M., Ransmay, D., Ketlet, E., Rees, S., Wilson, S., Pope, A. J., and Milligan, G. (2001) J. Biol. Chem. 276, 14092 – 14099
10. Lee, D. K., Lanca, A. J., Cheng, R., Nguyen, T., Ji, X. D., Gobeil, F., Ir., Chemtob, S., George, S. R., and O’Dowd, B. F. (2004) J. Biol. Chem. 279, 7901 – 7908
11. Gomes, I., Jordan, B. A., Gupta, A., Trapaidze, N., Nagy, V., and Devi, L. A. (2000) J. Neurosci. 20, Rapid Communication 110, 1–5
12. Lavec, B. A., O’Dowd, B. F., and George, S. R. (2002) Curr. Opin. Pharmacol. 2, 76 – 81
13. Lee, S. P., So, C. H., Rashid, A. J., Varghese, G., Cheng, R., Lanca A. J., O’Dowd, B. F., and George, S. R. (2000) J. Biol. Chem. 275, 35671 – 35678
14. Gales, C., Rebois, R. V., Hogue, M., Trieu, P., Breit, A., Hebert, T. E., and Bouvier, M. (2005) Nat. Methods 2, 177 – 184
15. Rocheville, M., Lange, D. C., Kumar, U., Patel, S. C., Patel, R. C., and Patel, Y. C. (2000) Science 288, 154 – 157

4. M. C. Kong, G. Varghese, T. Fan, S. P. Lee, B. F. O’Dowd, and S. R. George, submitted for publication.
Visualization of Dopamine Receptor Oligomers

Lee, S. P., O’Dowd, B. F., Rajaram, R. D., Nguyen, T., and George, S. R. (2003) Biochemistry 42, 11023–11031
17. Guo, W., Shi, L., and Javitch, J. A. (2003) J. Biol. Chem. 278, 4385–4388
18. Carrillo, J. J., Lopez-Gimenez, J. F., and Milligan, G. (2004) Mol. Pharmacol. 66, 1123–1137
19. Jans, D. A., Xiao, G.-Y., and Lam, M. H. C. (2000) BioEssays 22, 532–544
20. Lu, D., Yang, H., Shaw, G., and Raizada, M. K. (1998) Endocrinology 139, 365–375
21. Chen, R., Mukhin, Y. V., Garnovskaya, M. N., Thielen, T. E., Iijima, Y., Huang, C., Raymond, J. R., Ullian, M. E., and Paul, R. V. (2000) Am. J. Physiol. 279, F440–F448
22. Watson, P. H., Fraher, L. J., Natale, B. V., Kisiel, M., Hendy, G. N., and Hodson, A. B. (2000) Bone 26, 221–225
23. Ng, G. Y. K., Trogadis, J., Stevens, J., Bouvier, M., O’Dowd, B. F., and George, S. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10157–10161
24. Ng, G. Y., O’Dowd, B. F., Lee, S. P., Chung, H. T., Brann, M. R., Seeman, P., George, S. R. (1996) Biochem. Biophys. Res. Commun. 227, 200–204
25. Zawarynski, P., Tallerico, T., Seeman, P., Lee, S. P. O’Dowd, B. F., and George, S. R. (1998) FEBS Lett. 441, 383–386
26. So, C. H., Varghese, G., Curley, K. J., Kong, M. M., Alijaniaram, M., Ji, X., Nguyen, T., O’Dowd, B. F., and George, S. R. (2005) Mol. Pharmacol. 68, 568–578
27. Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G., and Cormier, M. J. (1992) Gene (Amst.) 111, 229–233
28. Matz, M. V., Fradkov, A. F., Labas, Y. A., Savitsky, A. P., Zaraisky, A. G., Markelov, M. L., and Lukyanov, S. A. (1999) Nat. Biotechnol. 17, 969–973
29. Marchese, A., George, S. R., and O’Dowd, B. F. (1998) in Identification and Expression of G Protein Coupled Receptors (Lynch, K., ed) pp. 1–26, Wiley-Liss, New York
30. Tomic, M., Seeman, P., George, S. R., and O’Dowd, B. F. (1993) Biochem. Biophys. Res. Commun. 191, 1020–1027
31. Overton, M. C., and Blumer, K. J. (2002) J. Biol. Chem. 277, 41463–41472
32. Jong, Y. J., Kumar, V., Kingston, A. E., Romano, C., and O’Malley, K. L. (2005) J. Biol. Chem. 280, 30469–30480