

MOL/2005/012229

**D1 and D2 Dopamine Receptors Form Heterooligomers and Co-internalize Following  
Selective Activation of Either Receptor  $\phi$**

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MOL/2005/012229

**Running Title:** Novel trafficking of D1-D2 dopamine receptor heterooligomers

**Number of text pages:** 29

**Number of tables:** 0

**Number of figures:** 8

**Number of References:** 29

Number of words (abstract): 177

Number of words (introduction): 587

Number of words (discussion): 1443

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**Abbreviations:** CHO, chinese hamster ovary; GPCR, G-protein coupled receptor; HEK 293T, human embryonic kidney 293T; MEM, minimum essential media; GFP, green fluorescent protein; mRFP, monomeric red fluorescent protein; HA, hemagglutinin; trFRET, time- resolved fluorescence resonance energy transfer; APC, Allophycocyanin; PBS, phosphate buffered saline; FITC, Fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; SDS, Sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; SEM, standard error of the mean; AFU, absolute fluorescence unit; GRK2, G- protein receptor kinase 2; PKA, protein kinase A.

## ABSTRACT

We provided evidence for the formation of a novel phospholipase C mediated calcium signal arising from co-activation of D1 and D2 dopamine receptors. In the present study, robust fluorescence resonance energy transfer showed these receptors exist in close proximity indicative of D1-D2 receptor heterooligomerization. The close proximity of these receptors within the heterooligomer allowed for cross-phosphorylation of the D2 receptor by selective activation of the D1 receptor. D1-D2 receptor heterooligomers were internalized when the receptors were co-activated by dopamine or either receptor was singly activated by the D1-selective agonist SKF 81297 or D2-selective agonist quinpirole. The D2 receptor expressed alone did not internalize following activation by quinpirole except when co-expressed with the D1 receptor. D1-D2 receptor heterooligomerization resulted in an altered level of steady-state cell surface expression compared to D1 and D2 homooligomers, with increased D2 and decreased D1 receptor cell surface density. Collectively, these results demonstrated that D1 and D2 receptors formed heterooligomeric units with unique cell surface localization, internalization and transactivation properties that are distinct from that of D1 and D2 receptor homooligomers.

The dopamine receptor family is subdivided into 2 distinct subclasses, based on structural similarities, pharmacological profiles and signal transduction mechanisms, into the D1-like and D2-like receptors. Although D1 and D2 receptor subclasses are biochemically distinct in that D1 receptors couple positively, whereas the D2 receptors couple negatively to adenylyl cyclase, many physiological functions are known to be mediated by the co-activation of both receptors. For example, the augmentative effect of cocaine on locomotion and intracranial self-stimulation is mediated by the activation of both D1 and D2 receptors (Kita et al., 1999). Dopamine receptor synergism could occur either at the level of neuronal networks through D1-like and D2-like receptors expressed in separate neuronal populations, or alternatively, within the same neurons through convergent post-receptor mechanisms. The latter mechanism of D1-D2 receptor synergism is possible since dopamine receptor subclasses are co-localized in rat brain, with co-localization of D1-like and D2-like receptors in virtually every neuron in the neonatal striatum (Aizman et al., 2000). Furthermore, our own studies have demonstrated robust co-localization of D1 and D2 receptors in a subset of neurons in human caudate nucleus, rat striatum and cortex (Lee et al., 2004). These data suggest, therefore, that functional synergism could occur within individual neurons. In fact, the co-activation of both D1 and D2 receptors has been shown to result in a significant increase in action potential frequency in neurons of the substantia nigra pars reticulata (Waszczak et al., 2002) and a potentiation of arachidonic acid release in CHO cells co-expressing both receptors (Piomelli et al., 1991). Our recent discovery of a common functional output generated by the concurrent co-activation of D1 and D2 receptors within the same cells resulting in the activation of a novel phospholipase C-dependent calcium signaling pathway (Lee et al., 2004), provides the long awaited putative biochemical mechanism for dopamine receptor synergism (Pollack, 2004). These data, together with the demonstration that

the receptors could be co-immunoprecipitated from cells and from brain (Lee et al., 2004), indicate that D1 and D2 receptor synergism, rather than resulting from a convergence in the signaling pathways distal to the receptors, may occur directly at the receptor level.

G-protein coupled receptors (GPCRs) have been demonstrated to exist as both homooligomers, forming complexes with identical receptors, and heterooligomers, forming complexes with other types of receptors (George et al., 2002). Receptor heterooligomerization is thought to account for the observed synergism between adenosine and dopamine receptors (Franco et al., 2000), as well as the mu and delta opioid receptors (George et al., 2000), among others (George et al., 2002). Since dopamine D1 and D2 receptors have been shown to form homooligomers (George et al., 1998; Lee et al., 2000), it is possible that some of the functional synergism observed within the dopamine receptor subclasses may be mediated by a physical interaction between receptors expressed in the same neuron, forming heterooligomers. Our observations of D1 and D2 receptors within the same signaling complexes by co-immunoprecipitation from rat brain and heterologous cells co-expressing both receptors (Lee et al., 2004) suggested the receptors may heterooligomerize. In this report, we demonstrate definitively that D1 and D2 receptors existed as heterooligomers at the cell surface. D1 and D2 receptor conformations within the heterooligomer permitted cross-phosphorylation of the D2 receptor by D1 receptor activation. Furthermore, D1 and D2 receptor heterooligomerization resulted in D1 and D2 receptor co-internalization by selective activation of either the D1 or D2 receptor within the heterooligomeric complex, and altered steady-state cellular distribution of both receptors, with overall enhanced D2 and decreased D1 receptor cell surface expression.

## MATERIALS AND METHODS

**Cell culture.** All cell culture reagents, media and antibiotics, mammalian expression vectors and transfection reagents were obtained from Invitrogen (Carlsbad, CA). COS7 and Human Embryonic Kidney (HEK) 293T cells (American Tissue Culture Collection, Manassas, VA) were maintained as monolayer cultures at 37° C. COS7 cells were maintained in  $\alpha$ - minimum essential medium (MEM) supplemented with 10% fetal bovine serum. HEK 293T cells were maintained in advanced MEM supplemented with anti-biotic/antimycotic (Invitrogen) and 6% fetal bovine serum.

**Dopamine receptor constructs.** For transient transfections, DNA encoding the human D1 receptor, the long and short isoforms of the human D2 receptor and the human D5 receptor were each inserted into the mammalian expression vector pcDNA3.1 (Invitrogen). For confocal microscopy, D1 and D2 receptors were cloned in-frame into the pEGFP-N1 vector (BD Biosciences Clontech, Palo Alto, CA) or the mRFP-1 vector. The cDNA encoding monomeric red fluorescent protein mRFP1 was a gift from Dr. Roger Tsien (University of California, San Diego).

For generation of D1, D2 and D1-D2 receptor stable cell lines, hemagglutinin- (HA) epitope-tagged D1 receptor cDNA and FLAG epitope-tagged D2 receptor cDNA were introduced alone or together into the pBUDCE4.1 expression vector (Invitrogen). All experiments were performed with the long isoform of the D2 receptor except where indicated.

**Transient Transfections.** Cells were transiently transfected using Lipofectamine reagent (Invitrogen) and membranes were prepared 48 hours post-transfection. To obtain equivalent cell

surface expression levels of D1 and D2 receptors, the amount of D1 receptor cDNA was transfected in a 1:2 ratio in relation to the amount of D2 receptor cDNA. pcDNA3 vector was used to keep the total amount of cDNA in each transient transfection constant.

**Stable Transfections.** HEK 293T cells were transfected as described above and selection was performed in the presence of 200  $\mu$ g zeocin from Invitrogen. Between 10-20 clones expressing varying receptor densities were screened to select those with comparable expression levels.

**Time Resolved Fluorescence Resonance Energy Transfer (trFRET).** This protocol was similar to that of McVey and colleagues with minor modifications (McVey et al., 2001). Briefly, the donor and acceptor fluorophores were Europium chelate and Allophycocyanin (APC), respectively (Perkin Elmer, Fremont, CA). Europium chelate was conjugated to the anti-FLAG antibody. APC was conjugated to the anti-HA antibody. Each antibody was diluted in a solution of 50% phosphate buffered saline (PBS): 50% fetal bovine serum (FBS). Cells expressing HA-D1 and FLAG-D2 receptors together or separately and mixed were incubated with these antibodies for two hours at 37° C on a rotating wheel. After incubation, samples were pelleted at 5000 rpm and washed twice with PBS and then resuspended in a final volume of 300 $\mu$ l PBS. 100  $\mu$ l of each sample was then aliquoted to a 96-well plate in triplicate. Fluorescence analysis was performed on a Victor<sup>2</sup> multilabel plate reader (Perkin-Elmer) with excitation at 340 nm and emission measured after a 400  $\mu$ s delay at 615 nm (Eu<sup>3+</sup> signal) and 665 nm (trFRET signal). Energy transfer (E) was calculated as follows:

$$E = (E_{AD665}/E_{AD615}) - (E_{D665}/E_{D615})$$

Where  $E_{AD665}$  and  $E_{AD615}$  is the emission at 665 nm and 615 nm respectively, in the presence of both the donor and acceptor fluorophores and  $E_{D665}$  and  $E_{D615}$  represent the emission at 665 nm and 615 nm from samples containing the donor only.

**Confocal Microscopy.** HEK-293T cells were transfected with DNA encoding D1-GFP with or without D2-mRFP for 48 hours. Live cell confocal microscopy was performed with a Zeiss LSM 510 microscope. Fifteen to twenty optimal sections along the z axis were acquired in increments of 0.1  $\mu\text{m}$ . Figures showed the central image or, where indicated, z sections from the adherent surface through the nucleus. Images were acquired and processed with Zeiss LSM Image Browser software. For immunocytochemistry, HEK 293T stable cell lines expressing D1-HA, D2-FLAG or co-expressing both receptors were fixed by 4% paraformaldehyde, permeabilized with 0.1% triton and incubated with 3F10 anti-HA antibody (Roche Diagnostics Canada, Laval, Quebec, Canada) and anti-FLAG antibody (Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada). The antibody-tagged receptor antibody-labeled receptors were visualized by incubating permeabilized specimens in the presence of FITC-conjugated anti-rat IgG or TRITC anti-rabbit IgG (Sigma).

**Membrane Preparations.** Cells transiently or stably expressing dopamine receptors were washed with PBS, resuspended in hypotonic lysis buffer with protease inhibitors (5 mM Tris-HCl, 2 mM EDTA, 5  $\mu\text{g}/\text{mL}$  leupeptin, 10  $\mu\text{g}/\text{mL}$  benzamide, and 5  $\mu\text{g}/\text{mL}$  soybean trypsin inhibitor (pH 7.4)) and homogenized with a Brinkman Polytron apparatus. The homogenate was centrifuged to pellet unbroken cells and nuclei, and the supernatant was collected. The

supernatant was centrifuged at 40000g for 30 minutes to isolate a membrane fraction enriched in plasma membrane and the resulting pellet (P2 membranes) was washed and resuspended in lysis buffer.

For subcellular fractionation studies, S1 supernatant was layered on top of a sucrose density column, which then was subjected to centrifugation at 150,000 g for 90 minutes at 4°C. Precipitates were collected at the 15%:30% interface, consisting of the light vesicular membrane fractions, and at the 30%:60% interface, consisting of mostly surface membrane (Toews, 2000). The receptor density in each fraction was quantified by radioligand binding of 1 nM [<sup>3</sup>H]SCH 23390 for the D1 and 1 nM [<sup>3</sup>H]raclopride for the D2 receptor and expressed as a percentage of the total number of receptors in the entire cell, which was determined by adding the radioligand binding in light and heavy membrane fractions of each individual experiment.

Protein content was determined by the Bradford method (Bio-rad, Hercules, CA) with bovine serum albumin as the standard.

**SDS PAGE and immunoblotting.** The procedures used for protein gel electrophoresis and immunoblotting were identical to those described previously (Lee et al., 2000).

**Radioligand Binding.** Saturation binding experiments were performed with 15-30 µg of membrane protein with increasing concentrations of [<sup>3</sup>H] raclopride (250 pM to 10,000 pM, final concentration) for estimation of the D2 receptor density or [<sup>3</sup>H] SCH 23390 (100-4000 pM, final concentration) for estimation of the D1 receptor density. Non-specific binding was determined by 10 µM (+)butaclamol.

Competition experiments were done in duplicate with increasing concentrations

( $10^{-11}$ - $10^{-3}$ M) of unlabelled agonists. The concentration of [ $^3$ H]SCH 23390 used in the competition assays for the D1 receptor was approximately equivalent to its K<sub>d</sub> (1 nM). The final concentration of [ $^3$ H]raclopride used in the competition assays for the D2 receptors was 2 nM. Bound ligand was isolated by rapid filtration through a 48-well cell harvester (Brandel, Montreal, Canada) using Whatman GF/C filters. Data was analyzed by nonlinear least-squares regression using GraphPad Prism. Data from multiple experiments were averaged and expressed as the means  $\pm$  SEM. The results were considered significantly different when the probability of randomly obtaining a mean difference was  $p < 0.05$  using the paired Student's *t* test.

**Cell surface Immunofluorometry.** HEK 293T and COS7 cells were transfected and maintained for 48 hours post-transfection. Cells were plated in 96-well clear bottom plates (Corning, NY) at a confluency of 50,000 cells per well. Cells were then fixed by 4% paraformaldehyde, blocked with 4% bovine serum albumin in PBS, incubated with 1:200 dilution of anti-HA (Roche) or 1:1000 dilution of anti-FLAG (Invitrogen) antibodies for 1 hour, washed with PBS, and then labeled with a secondary antibody conjugated with FITC (Invitrogen). Fluorescence was detected with a spectrophotometer (Cytofluor 4400 <sup>TM</sup>, Perseptive Biosystems, St. Louis, MO). Internalization studies on HEK 293T cells were used for internalization studies. Cells were treated 30 minutes with agonist prior to fixation and processed as described above.

**Agonist-induced Phosphorylation.** Phosphorylation assays were performed identical to those described previously (Lamey et al., 2002).

## RESULTS

In order to gain some insight into the cell surface arrangement of D1 and D2 receptors within the calcium signaling complexes, trFRET was performed on whole cells which co-expressed HA-D1 and FLAG-D2 receptors. Robust energy transfer between the antibodies bound to the N-termini of D1 and D2 receptors was observed in cells co-transfected with both receptors (Figure 1A, bar 1). Cells expressing D1 and D2 receptors singly and mixed together, showed no trFRET (Figure 1A, bar 2). Since only cell surface receptors were detected by this method, this suggested that the D1 and D2 receptors were within proximity of 50-100 angstroms on the cell surface and most likely existed within the same oligomeric complex. To determine if D1 and D2 receptor heterooligomers were preformed within intracellular compartments, trFRET was performed on light and heavy sucrose gradient fractions enriched in, respectively, endoplasmic reticulum and plasma membranes. Equivalent robust energy transfer was detected in both the heavy and light membrane fractions (Figure 1B), suggesting these receptors were assembled as heterooligomers in intracellular compartments before being presented on the cell surface. To verify the purity of the fractions, subcellular markers were used. Although the ER marker calnexin and the recycling endosomal marker Rab 11 were observed within both fractions, the plasma membrane marker Na<sup>+</sup>/K<sup>+</sup> ATPase was exclusively localized in the heavy fraction (Figure 1C).

In order to determine if the formation of D1 and D2 receptor heterooligomers resulted in a change in the conformation of the binding pocket of either receptor, ligand-binding studies were performed using the ligands that were previously used to demonstrate the novel calcium signal- dopamine, the D1-specific agonist SKF 81297 and the D2-specific agonist quinpirole (Lee et al., 2004). Competition of the D1-specific antagonist [<sup>3</sup>H]SCH 23390 binding with dopamine (D1R alone,  $K_{high} = 116 \pm 14$  nM, fraction of receptors in high affinity state =  $0.15 \pm 0.04$ ,  $K_{low} = 4251 \pm 691$  nM; D1R co-expressed with D2R,  $K_{high} = 118.50 \pm 5.50$  nM, fraction of receptors in high affinity state =  $0.17 \pm 0.06$ ,  $K_{low} = 3950 \pm 848$  nM) or SKF 81297 (D1R alone,  $K_{high} = 31.78 \pm 7.0$  nM, fraction of receptors in high affinity state =  $0.89 \pm 0.06$ ,  $K_{low} = 2156 \pm 910$  nM; D1R co-expressed with D2R,  $K_{high} = 35.30 \pm 8.4$  nM, fraction of receptors in high affinity state =  $0.90 \pm 0.04$ ,  $K_{low} = 2097 \pm 926$  nM) to membranes of COS7 cells expressing the D1 receptor alone or co-expressing D1 and D2 receptors, demonstrated no significant change in the agonist affinities or the proportion of receptors detected in the agonist-detected high and low affinity states (Figures 2A and 2B,  $p > 0.05$ ). Saturation binding isotherm with [<sup>3</sup>H] SCH 23390 indicated no significant change when D1 and D2 receptors were co-expressed (D1R alone,  $k_d = 0.64 \pm 0.12$  nM; D1R co-expressed with D2R,  $k_d = 0.79 \pm 0.15$  nM,  $p > 0.05$ ). Similarly, competition of the binding of the D2-specific antagonist [<sup>3</sup>H]raclopride with dopamine (D2R alone,  $K_{high} = 10.30 \pm 5.50$  nM, fraction of receptors in high affinity state =  $0.10 \pm 0.03$ ,  $K_{low} = 2478 \pm 639$  nM; D2R co-expressed with D1R,  $K_{high} = 12.53 \pm 6.13$  nM, the fraction of receptors in high affinity state =  $0.11 \pm 0.02$ ,  $K_{low} = 2112 \pm 818$  nM) or quinpirole (D2R alone,  $K_{high} = 149 \pm 45$  nM, fraction of receptors in high affinity state =  $0.14 \pm 0.02$ ,  $K_{low} = 3450 \pm 766$  nM; D2R co-expressed with D1R,  $K_{high} = 129 \pm 63$  nM, fraction of receptors in high affinity state =  $0.19 \pm 0.03$ ,  $K_{low} = 3691 \pm 740$  nM) to membranes from COS7 cells transiently expressing the D2 receptor alone or co-expressing D1

and D2 receptors also indicated no significant change in agonist affinities or the proportion of receptors detected in the high affinity state (Figure 2C and 2D,  $p > 0.05$ ). Saturation binding isotherm with [ $^3\text{H}$ ]raclopride indicated no significant change when D1 and D2 receptors were co-expressed (D2R alone,  $k_d = 0.87 \pm 0.06$  nM, D2R co-expressed with D1R,  $k_d = 0.88 \pm 0.06$  nM,  $p > 0.05$ ).

Since D1 and D2 receptors form heterooligomers, we queried if the configuration of the receptors within the complex would allow for cross-modulation of receptor function, such that selective activation of the D1 receptor could result in the phosphorylation of the D2 receptor in the heterooligomeric complex. We have shown previously that the mutation of a glutamic acid residue at position 359 to alanine in a putative G-protein coupled receptor 2 kinase (GRK2) recognition site on the carboxyl tail of the D1 dopamine receptor (D1-E359A) completely attenuated the rapid agonist-induced phosphorylation of the D1 receptor and the ability of the receptor to undergo homologous desensitization, without effect on adenylyl cyclase activation (Lamey et al., 2002) (Figure 3A, lane 1 and 2). When the D2 receptor was expressed alone and treated with 1 nM SKF 81297, a concentration of the D1 selective agonist which did not activate the D2 receptor and has been shown previously to not activate the robust calcium signal (Lee et al., 2004), no significant agonist-induced phosphorylation of the receptor was observed (Figure 3A, lane 4). When the D2 receptor was co-expressed with D1-E359A and treated with 1 nM SKF 81297, phosphorylation of the D2 receptor was observed (Figure 3A, lane 6). Figure 3B demonstrates that the amount of receptor protein in each lane was approximately equivalent, demonstrating that the increase in phosphate accumulation did not result from changes in D2 receptor expression. Phosphorylation of the D2 receptor by D1 receptor activation was also observed in the presence of the PKA inhibitor H89, suggesting that the mechanism of D2

phosphorylation was not dependent upon the activation of protein kinase A mediated by the occupancy of the D1 receptor (data not shown).

Since D1 and D2 receptors formed heterooligomers at the cell surface, we investigated if the close interaction was maintained during agonist-mediated receptor internalization. To determine if the D1-D2 receptor complex was internalized upon co-activation of both receptors, HEK 293T cells transiently expressing HA-D1, FLAG-D2 or both receptors were subjected to cell surface immunofluorescence assays (Figure 4). Following 10  $\mu$ M dopamine treatment for 30 minutes, internalization of the D1 receptor was observed when expressed alone or co-expressed (16.7 $\pm$ 8.7%, n=4 for D1R; 17.4 $\pm$ 4.0%, n=4 for D1R with D2R, p>0.05,) (Figure 4a). The D2 receptor did not internalize on exposure to dopamine when expressed alone but did internalize when co-expressed with the D1 receptor (1.1 $\pm$ 2.4%, n=4 for D2R alone; 15.1 $\pm$ 1.8%, n=4 for D2R with D1R, p<0.05) (Figure 4b). To confirm our results obtained by transient transfection, HEK 293T cells stably expressing HA-D1 (1559  $\pm$  446 fmol/mg), FLAG-D2 (748  $\pm$  199 fmol/mg) and co-expressing HA-D1 and FLAG-D2 (D1-HA =1762  $\pm$  361 fmol/mg, D2-FLAG=1877  $\pm$  434 fmol/mg) receptors were treated with 10  $\mu$ M dopamine for 30 minutes following which the number of the receptor binding sites in P2 membrane preparations were quantified by radioligand binding and the results observed was similar to that observed in cells transiently expressing the receptors (17  $\pm$  7.5%, n=4 for D1R alone; 16  $\pm$  4.0%, n=4 for D1R with D2R, p>0.05; for the D2R, -2.2  $\pm$  1.7%, n=4 for D2R alone; 21  $\pm$  4.0%, n=4 for D2R with D1R, p<0.05).

Since D1 receptor activation within the heterooligomer allowed for cross-modulation of D2 receptor function, the effect of selective agonists on the internalization of the heterooligomer was tested. To quantify this effect, HEK 293T cells expressing either one or both HA-D1 and

FLAG-D2 receptors were treated for 30 minutes with varying concentrations of SKF 81297 or quinpirole and cell surface expression of each receptor was quantified by cell surface immunofluorescence assays. Dose-dependent internalization of the D1 receptor was detected after 30-minute treatment with increasing concentrations of SKF 81297 when the D1 receptor was expressed alone or co-expressed with the D2 receptor ( $19 \pm 5.8\%$ ,  $n=4$  for the D1R alone;  $23 \pm 8.1\%$ ,  $n=4$  for the D1R with D2R,  $p>0.05$ ) with a significant decrease in the potency of the D1 agonist to result in the internalization of the D1-D2 receptor heterooligomer ( $EC_{50}=13 \pm 7.7$  nM,  $n=4$  for the D1R alone;  $EC_{50}=45 \pm 4.0$  nM,  $n=4$  for D1R with D2R,  $p<0.05$ ) (Figure 5A). To observe if the D2 receptor co-internalized with the D1 receptor upon selective D1 receptor activation, the concentration-dependent response to the D1 selective agonist SKF 81297 was analyzed. Whereas no internalization of the D2 receptor was observed in response to SKF 81297 when expressed alone, internalization of the D2 receptor was observed when co-expressed with the D1 receptor (Figure 5B) ( $13 \pm 3.9\%$  internalized;  $EC_{50}=12 \pm 6.0$  nM,  $n=3$  for D2R with D1R).

Dose-dependent internalization of the D2 receptor was not detected after 30-minute treatment with increasing concentrations of quinpirole when D2 receptor was expressed alone. However, on co-expression with the D1 receptor, internalization of the D2 receptor was observed in response to quinpirole (Figure 5C) ( $14 \pm 4.8\%$  internalized;  $EC_{50}=24.5 \pm 5.7$  nM,  $n=4$  for D2R with D1R). Since D1 and D2 receptors physically interact, it may be possible that the D1 receptor would co-internalize with the D2 receptor, similar to what was observed for the D2 receptor when co-expressed with the agonist activated D1 receptor. Concentration-dependent internalization of the D1 receptor in response to the D2 selective agonist quinpirole was observed upon co-expression with the D2 receptor ( $31 \pm 7.7\%$  internalized;  $EC_{50}=13.2 \pm 5.4$  nM,  $n=4$  for

D1R with D2R) (Figure 5D). No internalization of the D1 receptor was observed in response to quinpirole when expressed alone (Figure 5D).

Since the D1-D2 receptor heterooligomers function as a unit at the cell surface and internalize as such, the steady state cellular distribution of the receptors was investigated. The subcellular localization of the transiently transfected D1-GFP and D2-mRFP receptors in cells expressing one or both receptors was examined by confocal microscopy, with receptor distribution displayed by serial z-sections through HEK 293T cells (Figure 6). When expressed alone, D1 receptors were found predominantly on the cell surface (Figure 6A) whereas D2 receptors were localized at the cell surface with a significant proportion present intracellularly (Figure 6B). When co-expressed, D1 and D2 receptors were observed to have a similar distribution on the plasma membrane as well as in intracellular compartments (Figure 6C) suggesting a common localization of D1 and D2 receptors, likely stemming from the formation of D1-D2 heterooligomers. This distribution was also observed when receptors were co-expressed in COS7 cells and in the neuroblastoma cell line N1E 115 (data not shown). Similar localization of D1 and D2 receptors was also observed in stable cell lines expressing the receptors alone or co-expressing both receptors (Figure 6D-F), indicating these observations were due to an alteration in the steady state distribution of the receptors upon co-expression and not due to transient transfection.

To further assess the cellular redistribution of the receptors upon co-expression, radioligand binding was performed on heavy and light subcellular membrane fractions obtained using a discontinuous sucrose gradient, which contained distinct cellular markers (Figure 1C). Expressed alone, the D1 receptor was detected predominantly in the heavy membrane fraction. Upon co-expression with the D2 receptor, an increased proportion of the D1 receptor was

detected in the light membrane fraction (Figure 7A) ( $40 \pm 4\%$  for D1R alone;  $64 \pm 5.9\%$  for D1R with D2R,  $p < 0.05$ ) with a decrease observed in the heavy membrane fraction ( $68 \pm 4.6\%$  for D1R alone;  $34 \pm 5.9\%$  for D1R with D2R,  $p < 0.05$ ). When the D2 receptor was expressed alone, the receptor was detected to be approximately equally distributed in both the heavy and light membrane fractions. Upon co-expression with the D1 receptor, an increase in the proportion of the D2 receptors in the heavy membrane fraction was observed ( $47.8 \pm 3.2\%$  for D2R alone;  $66 \pm 5.3\%$  for D2R with D1R,  $p < 0.05$ ) with a decrease observed in the light fraction ( $51.4 \pm 3.4\%$  for D2R alone;  $32.6 \pm 5.4\%$  for D2R with D1R,  $p < 0.05$ ) (Figure 7B). These results supported the observations from confocal microscopy indicating changes in receptor localization upon co-expression. Interestingly, the redistribution of the receptors in co-transfected cells changed the overall receptor density of both receptors. When total receptor expression was quantified, both by radioligand binding on whole cell lysates and from the addition of the number of binding sites in heavy and light sucrose gradient fractions, the amount of D2 receptor binding sites was increased when co-expressed with the D1 receptor and the amount of D1 receptor binding sites was decreased when co-expressed with D2 receptor (data not shown).

To quantify changes at the cell surface, COS7 cells were co-transfected with D1 and D2 receptors and the surface expression of the receptors was estimated by cell surface immunofluorescence assays. A 51% increase in cell surface expression of the D2 receptor was documented upon co-expression with the D1 receptor ( $11416 \pm 525$  AFU for D2R,  $22009 \pm 3245$  AFU for D2R with D1R,  $p < 0.05$ ). (Figure 8A). Binding of [ $^3$ H]raclopride in the plasma membrane enriched P2 fraction also showed a significant 50% ( $p < 0.05$ ) increase in the number of D2 receptor binding sites when co-expressed with the D1 receptor compared to when the D2 receptor was expressed alone ( $B_{max} = 876 \pm 112$  fmol/mg,  $n=5$  for D2R alone;  $B_{max} = 1600 \pm$

164 fmol/mg, n=5 for D2R with D1R,  $p<0.05$ ) (Figure 8B). This increase in the number of D2 receptor-binding sites also correlated with the increase in receptor protein species as observed by western blot (Figure 8B). To determine if the enhancement upon co-expression with the D1 receptor was also observed for the short isoform of the D2 receptor, which lacks 26 amino acids in the third intracellular loop, the D2(short) receptor was co-expressed with the D1 receptor in COS7 cells. A significant 50% ( $P<0.05$ ) increase in the number of D2(short) receptor binding sites was observed when co-expressed with the D1 receptor compared to when it was expressed alone ( $B_{max}=1412 \pm 237$  fmol/mg, n=5 for D2R(short) alone;  $B_{max}=2578 \pm 208$  fmol/mg, n=3 for D2R(short) with D1R,  $p<0.05$ ) with no change in the affinity of the receptor for [<sup>3</sup>H]raclopride ( $K_d= 1.2 \pm 0.25$  nM, n=5 for D2R(short) alone;  $K_d= 1 \pm 0.12$  nM, n=3 for D2R(short) with D1R,  $p>0.05$ ) (Figure 8C). This increase in D2 receptor-binding sites also correlated with the increase in receptor protein species visualized by western blot (Figure 8C). No enhancement of the D2 receptor was observed when co-expressed with the D5 receptor (data not shown).

To examine if cell surface expression of the D1 receptor was altered in parallel with the expression of the D2 receptor when co-expressed, the number of D1 receptors was quantified by cell surface immunofluorescence assays. Decreased cell surface expression of the D1 receptor when co-expressed with the D2 receptor was quantified ( $29014 \pm 4995$  AFU for D1R alone;  $14653 \pm 2464$  AFU for D1R with D2R,  $p<0.05$ ). By radioligand binding studies, the number of D1 receptor binding sites within the plasma enriched P2 membrane fractions was decreased by 55% ( $p<0.05$ ) when co-expressed with the D2 receptor ( $B_{max}= 3156 \pm 415$  fmol/mg, n=10 for D1R alone;  $B_{max}=1447 \pm 375$  fmol/mg, n=10 for D1R with D2R,  $p<0.05$ ) (Figure 8E). This

decrease in D1 receptor binding sites corresponded to a decrease in the amount of receptor in the P2 fraction as verified by western blot (Figure 8E).

## DISCUSSION

Our discovery of the phospholipase C mediated calcium signal generated by the co-activation of D1 and D2 dopamine receptors (Lee et al., 2004) has provided a novel insight into the unique ability of these co-activated receptors to access a completely new signaling pathway. In this report, we definitively demonstrate that D1 and D2 receptors heterooligomerize within these signaling complexes. FRET analysis demonstrated that within these complexes, D1 and D2 receptors exist as heterooligomers on the cell surface and within intracellular compartments. As a result of heterooligomerization, these receptors displayed altered steady state cellular distribution and unique internalization kinetics compared to D1 and D2 receptor homooligomers. A unique characteristic of the heterooligomer was that it enabled robust internalization of both receptors in response to a D2 specific agonist, whereas the D2 receptor expressed alone did not internalize in response to the same agonist. The close proximity of the receptors within the heterooligomer enabled cross-phosphorylation of the D2 receptor by selective activation of the D1 receptor.

FRET analysis demonstrated that the D1 and D2 receptors were in proximity of 50-100 angstroms to each other at the cell surface and within intracellular compartments. This observation indicated that the D1 and D2 receptors existed as heterooligomers, which may have been formed within the ER before being trafficked to the cell surface. This has been suggested previously by other studies on GPCR homo- and heterooligomerization (Canals et al., 2003; Terrillon et al., 2003). However, based on the presence of the recycling endosomal marker Rab 11 in the light membrane fraction, it may be possible that heterooligomers in the light fraction

may also include those within recycling endosomes. The formation of heterooligomers, however, did not change the affinity of the D1 and D2 receptors to the selected prototypical agonists and antagonists used previously to generate the novel calcium signal, indicating that the ligand binding pocket for these drugs was unchanged by heterooligomer formation.

Despite no observable changes in the affinity of selected agonists and antagonists to the ligand binding pocket, heterooligomer formation enabled cross-modulation of receptor activity by phosphorylation. The activation of the D1 receptor by SKF 81297 resulted in cross-phosphorylation of the D2 receptor. The phosphorylation of the D2 receptor likely occurred because in the heterooligomeric conformation, as suggested by the generation of the novel calcium signal upon co-activation of both receptors and the novel pattern of internalization of the D2 receptor, putative phosphorylation sites located in the third intracellular loop of the D2 receptor may have been located in close enough proximity to the D1 receptor to allow for phosphorylation by kinases such as GRKs. The PKA inhibitor H89 had no effect on the D1-receptor mediated D2 receptor phosphorylation, thus suggesting that the phosphorylation of the D2 receptor was not dependent upon PKA activation.

The conformation of D1-D2 receptor heterooligomers may also have allowed for novel internalization characteristics in response to agonist treatment. The internalization studies revealed that the D1-D2 receptor heterooligomer was internalized by co-activation of both receptors by dopamine or by the single activation of one of the receptors within the heterooligomer. D1-D2 receptor heterooligomers internalized when either the D1 receptor was activated by SKF 81297 or the D2 receptor was activated by quinpirole. These results suggested that the interactions between the receptors within the heterooligomer were maintained upon

agonist exposure, unlike other examples where one receptor within the heterooligomer internalizes separately upon agonist activation (Xu et al., 2003).

The D2 selective agonist quinpirole, which failed to internalize the D2 receptor when expressed alone, internalized the D1-D2 receptor heterooligomer. This is a remarkable finding considering that the characteristics of D2 receptor internalization depends on the cell type and may be related to the variable expression of endogenous GRKs and  $\beta$ -arrestins (Ito et al., 1999; Kim et al., 2004; Zhang et al., 1994), and has also been reported to upregulate on the cell surface after long-term agonist exposure (Ng et al., 1997; Zhang et al., 1994). *In vivo* animal experiments, however, demonstrated that the D2 receptor readily internalized in brain in response to endogenous dopamine (Chugani et al., 1988; Sun et al., 2003). Since D1 and D2 receptors are co-localized in many neurons in human and rat brain (Aizman et al., 2000; Lee et al., 2004), it is possible that the internalized D2 receptor detected was co-localized with the D1 receptor. Oligomerization with the D1 receptor may either put the D2 receptor into a conformation that enabled the D2 receptor to recruit endocytic machinery on its own or enabled the D2 receptor to access the endocytic processes linked to the D1 receptor. The latter is a possibility since activation of D1 receptors resulted in the phosphorylation of the D2 receptor. Another possibility is that the novel entity of the D1 and D2 receptor heterooligomer, due to the new receptor conformations achieved, now has different internalization characteristics compared to that of the D1 and D2 receptor homooligomers. This possibility is indicated by the reduced potency of SKF 81297 -mediated heterooligomer internalization and enhanced potency of quinpirole to induce internalization of the D1 and D2 receptor heteromer.

Heterooligomerization resulted in altered steady-state cellular distribution of D1 and D2 receptors within cells that was distinct from that of D1 and D2 receptor homooligomers. This

situation involving D1 and D2 receptors is unique in that the cell surface expression of two receptors, which independently traffic to the cell surface as homooligomers, were both altered by heterooligomer formation. D1 and D2 receptor homooligomers differed in their cellular distribution in that D1 receptor homooligomers were predominantly expressed on the cell surface with little intracellular localization, whereas D2 receptor homooligomers displayed both prominent cell surface and intracellular localization. Intracellular localization of the D2 receptor was not a product of transient transfection since a similar result was observed in stable cell lines expressing the D2 receptor alone as we have shown. The intracellular localization of the D2 receptor has been suggested previously to stem from a relatively inefficient processing of the D2 receptor protein (Fishburn et al., 1995; Prou et al., 2001) or because the receptor participated in an agonist-independent constitutive internalization process (Vickery and von Zastrow, 1999). Changes in the configuration of the intracellular domains of both D1 and D2 receptors within the heterooligomer may have resulted in changes in receptor processing or cell surface turnover and thus altered the number of receptors available for radioligand binding. These changes may have resulted in the exposure or masking of consensus sequences such as ER retention motifs (Zerangue et al., 1999) or ER export motifs (Ma et al., 2001), and may allow for novel interactions with adaptor proteins involved in receptor trafficking.

In summary, the results presented in this study provides cogent evidence that heterooligomerization occurs between D1 and D2 receptors, and together with our report of the robust D1-D2 receptor mediated calcium signal (Lee et al., 2004), provides a molecular mechanism for how D1-D2 receptor synergism may occur in cells where these receptors co-exist. The extent of receptor heterooligomerization must be significant because several changes in

receptor function were observed such as the generation of a novel calcium signal and the novel pattern of agonist-induced D2 receptor internalization. Furthermore, as reported within our previous paper (Lee et al., 2004), the efficiency of co-immunoprecipitation of D1 and D2 receptors in cell lines and rat brains by selective antibodies was high indicating that a significant proportion of receptors existed as hetero-oligomers. The receptors within the heterooligomeric complex have attained novel properties distinct from the individual D1 and D2 homooligomers, possibly stemming from novel receptor conformations within these heterooligomers. The many lines of evidence we have generated all point to the creation of a novel D1-D2 signaling unit, with ability to internalize following selective activation of either D1 or D2 receptors, but requiring concurrent activation of both receptors for generation of the calcium signal. The enhanced ability of the D2 agonist to internalize the D1-D2 heteromeric complex also signifies important ramifications for the understanding of the action of these clinically relevant drugs in brain. It has been proposed that abnormal calcium signaling may constitute the central dysfunction in schizophrenia (Lidow, 2003). At the same time, the leading mainstay of schizophrenia therapeutics is based on the transmitter dopamine. Since neither D1 nor D2 dopamine receptors individually had been shown consistently in different cellular models to directly alter calcium signaling, it was difficult to reconcile these two streams of evidence. Our findings related to the D1 and D2 receptor heterooligomers may provide a novel insight into how these receptors function synergistically and eventually lead to mechanisms of potential dysfunction. The D1-D2 heterooligomers, therefore, may represent an important and compelling drug target for diseases related to the dopaminergic system.

MOLPHARM/2005/012229

## ACKNOWLEDGEMENTS

We would like to thank Theresa Fan for her excellent technical assistance.

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## FOOTNOTES

∅This work was supported by grants from the National Institute on Drug Abuse and from the Canadian Institutes of Health Research.

Dr. Susan George is the holder of a Canada Research Chair in Molecular Neuroscience.

## FIGURE LEGENDS

Fig. 1. A close proximity of co-expressed D1 and D2 receptors on the cell surface was detected using time resolved FRET using Europium chelate as the donor and APC as the acceptor (n=3 independent co-transfections). (A) Energy transfer on the cell surface was detected in cells co-transfected with the amino terminus tagged HA-D1 receptors (D1R) and amino terminus tagged FLAG-D2 receptors (D2R) (bar 1) but not when these receptors were transfected separately and mixed (bar 2) (\* denotes  $p < 0.05$ ). (B) Energy transfer was detected in both the light and heavy membrane fractions obtained from cells co-transfected with HA-D1 and FLAG-D2 receptors. (C) The plasma membrane marker  $\text{Na}^+/\text{K}^+$  ATPase was detected in the heavy membrane fraction (lane 2) but not in the light membrane fraction (lane 1), whereas the endoplasmic reticulum marker calnexin and the recycling endosomal marker Rab11 was found in both fractions (lanes 1 and 2).

Fig. 2. Co-expression of D1 and D2 receptors does not alter agonist binding affinities and agonist detected high and low affinity states (n=4-5). (A) Competition of [<sup>3</sup>H]SCH 23390 binding by dopamine in cell membranes prepared from cells expressing D1R (filled square) or co-expressing D1R and D2R (clear inverted triangle). (B) Competition of [<sup>3</sup>H]SCH 23390 binding by the D1-specific agonist SKF 81297 in cell membranes prepared from cells expressing D1R (filled square) or co-expressing D1R and D2R (clear inverted triangle). (C) Competition of [<sup>3</sup>H]raclopride binding by dopamine in cell membranes prepared from cells expressing D2R (filled circle) or co-expressing D1R and D2R (clear triangle). (D) Competition of [<sup>3</sup>H]raclopride binding by the D2 selective agonist quinpirole in cell membranes prepared from cells expressing D2R (filled circle) or co-expressing D1R and D2R (clear triangle). Black arrows indicate high and low affinity site for receptor expressed alone; gray arrows indicate the high and low affinity site for receptors co-expressed.

Fig. 3. Phosphorylation of the D2 receptor by selective activation of the D1 receptor (representative of n=3). (A) Cells expressing either HA-E359A-D1 (lanes 1 and 2) or c-myc-D2 receptor alone (lanes 3 and 4) or co-expressing both receptors (lanes 5 and 6) were treated with 1 nM SKF 81297. <sup>32</sup>P incorporation was detected following immunoprecipitation by anti-HA (lanes 1 and 2) or anti-c-myc (lanes 3-6) antibodies. Arrows indicate phosphorylated receptor monomers and oligomers. (B) Immunoblot detection of E359A-HA-D1 (lanes 1 and 2) and c-myc-D2 (lanes 3-6) receptors by anti-HA or anti-c-myc antibodies. The immunoprecipitated samples were subjected to immunoblotting to demonstrate that the amount of receptor in each lane was approximately equivalent.

Fig. 4. Co-internalization of D1 and D2 receptors by 10  $\mu$ M dopamine in HEK 293T cells (n=4). (A) Internalization of D1 receptor in cells expressing the D1 receptor alone or co-expressed with the D2 receptor. (B) Internalization of the D2 receptor in cells expressing the D2 receptor alone or co-expressed with the D1 receptor (\* denotes  $p < 0.05$ ). Percent internalization represented the loss of fluorescence from the cell surface of the whole cell after agonist treatment compared to the vehicle-treated control.

Fig. 5. Co-internalization of D1 and D2 receptors by activation of either receptor was detected cell surface immunofluorometry in HEK 293T cells (n=3-4). (A) Internalization of the D1 receptor by SKF 81297 in cells expressing the HA- D1 receptor alone or co-expressed with the FLAG- D2 receptor. (B) Internalization of the D2 receptor by SKF 81297 in cells expressing the FLAG- D2 receptor alone or co-expressed with the HA- D1 receptor. (C) Internalization of the D2 receptor by quinpirole in cells expressing FLAG- D2 receptor alone or co-expressed with HA- D1 receptor. (D) Internalization of D1 receptor by quinpirole in cells expressing HA- D1 receptor or co-expressed with the FLAG- D2 receptor. Cells were treated for 30 minutes with variable concentrations of agonists. Arrows indicate  $EC_{50}$ . Percent internalization represented the loss of fluorescence from the cell surface of the whole cell after agonist treatment compared to the vehicle-treated control.

Fig. 6. Confocal Microscopy of cells expressing D1 and D2 receptors or co-expressing both receptors. Shown are confocal images of HEK 293T cells transfected with D1-GFP alone (A) D2-mRFP (B) or co-expressing both receptors (C). Serial z-sections from the cell surface through the nucleus are shown for the region indicated by the dashed white square. In (C), D1-

GFP corresponds to upper panels and D2-mRFP corresponds to the lower panels. (D-F) Confocal image of HEK 293T stable cell lines expressing D1-HA alone (D), D2-FLAG alone (E) or co-expressing both receptors (F) in HEK 293T stable cell lines.

Fig. 7. The distribution of the D1 and D2 receptors in heavy (plasma membrane) and light (vesicular intracellular) membrane fractions was changed upon co-expression in COS7 cells. (A) Distribution of D1 receptor in heavy and light fractions in cells expressing D1 receptor alone or co-expressed with D2 receptor determined by specific binding of 1 nM [<sup>3</sup>H]SCH 23390 (n=5-7). (B) Distribution of D2 receptor in heavy and light membrane fractions in cells expressing D2 receptor alone or co-expressed with the D1 receptor determined by specific binding of 1 nM [<sup>3</sup>H]raclopride (n=4). Significant differences between the D1 and D2 receptor distribution in singly and doubly transfected cells was denoted by \* for the light vesicular membrane fractions and the # for heavy membrane fractions (p<0.05). The data was represented as the average % expression of the total radioligand binding ± SEM.

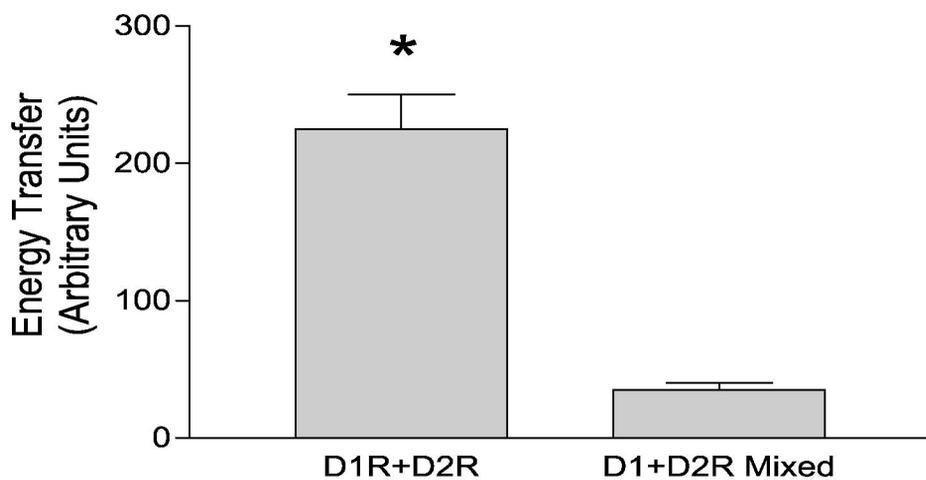
Fig. 8. Membrane expression of D2 receptor was enhanced by co-expression with the D1 receptor in COS7 cells (n=3-5). (A) Cell surface expression of the D2 receptor expressed alone or co-expressed with the D1 receptor as quantified by cell surface immunofluorescence (\* denotes p<0.05). (B) Saturation binding of [<sup>3</sup>H]raclopride to D2 receptor expressed alone or co-expressed with D1 receptor. Immunoblot detecting FLAG- D2 receptor in P2 membranes from cells expressing the D2 receptor alone or co-expressed. (C) Saturation binding of [<sup>3</sup>H]raclopride to D2 receptor (short) expressed alone or co-expressed with D1 receptor. Immunoblot detecting FLAG- D2 receptor (short) expressed in P2 membranes in cells expressing D2 receptor (short)

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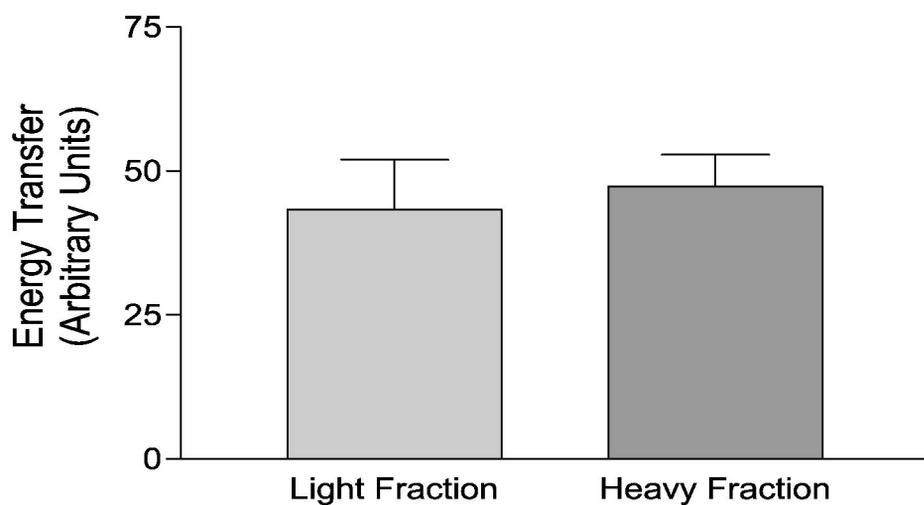
alone or co-expressed. (D) Cell surface expression of the D1 receptor expressed alone or co-expressed with the D2 receptor as quantified by cell surface immunofluorescence (\* denotes  $p < 0.05$ ). (E) Saturation binding of [ $^3\text{H}$ ]SCH 23390 to D1 receptor expressed alone or co-expressed with D2 receptor. Immunoblot detection of the HA- D1 receptor expressed on P2 membranes in cells expressing HA- D1 receptor alone or co-expressed. For immunoblots, arrows indicate monomer, dimer and higher order of oligomeric species.

# Figure 1

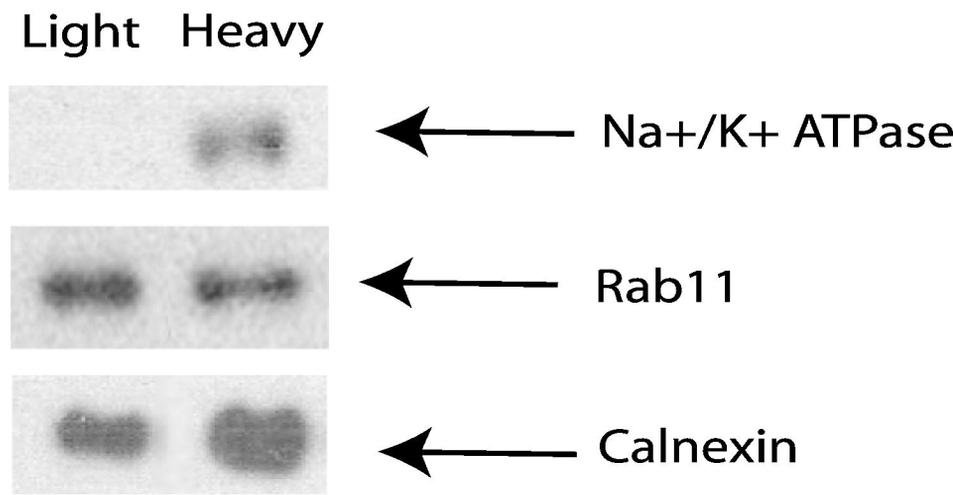
A



B

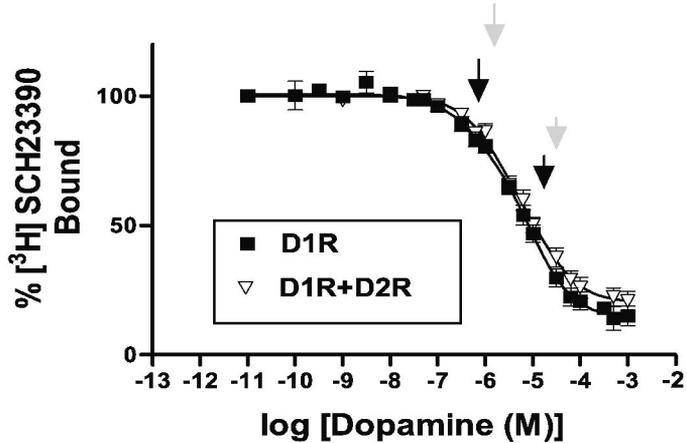


C

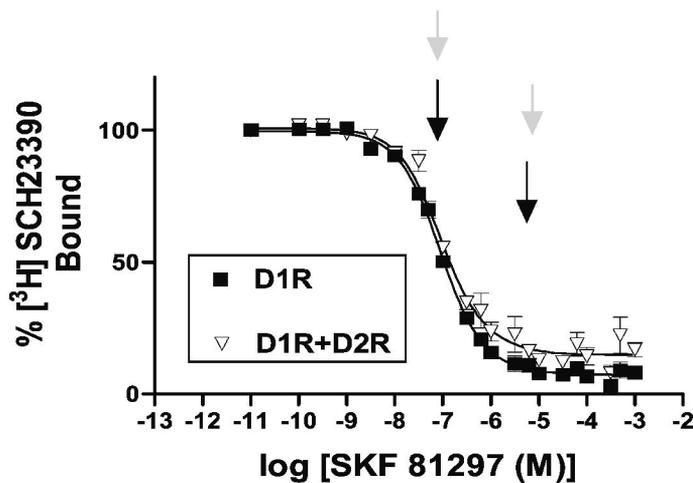


# Figure 2

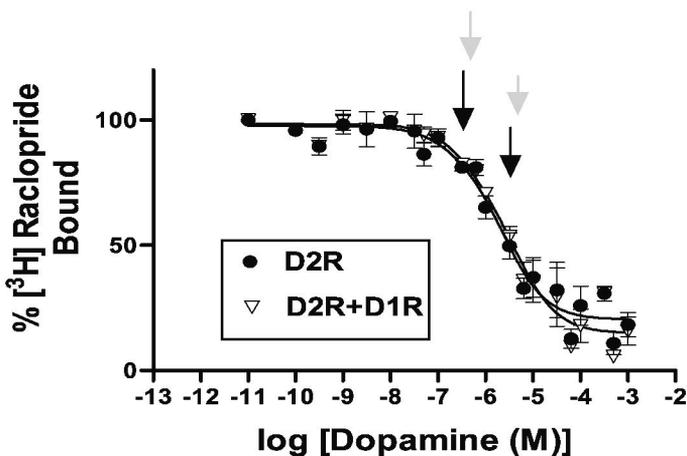
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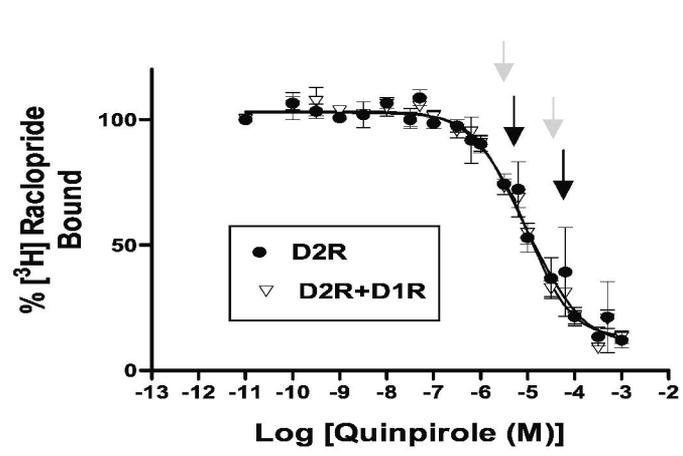
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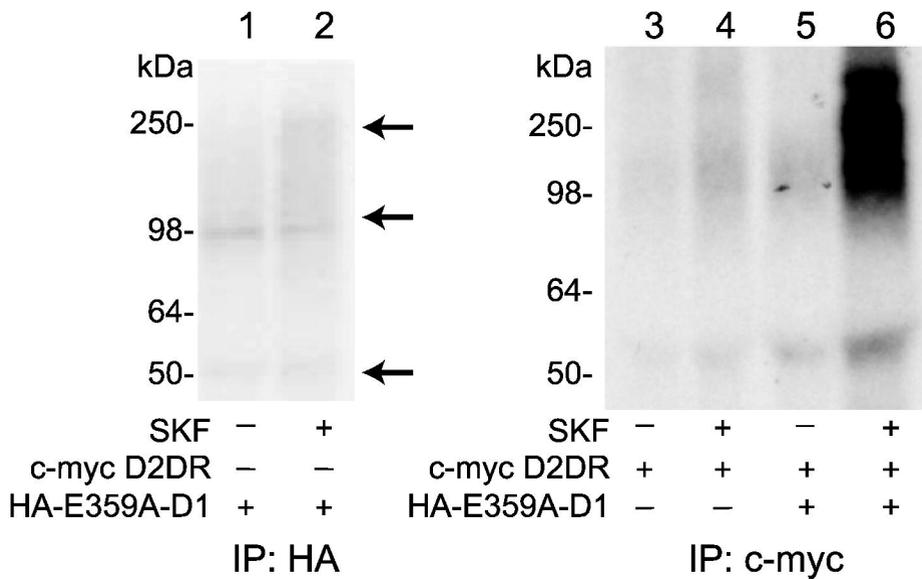


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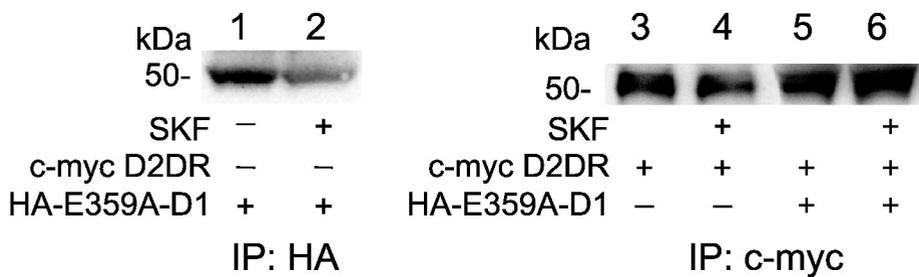


# Figure 3

**A**

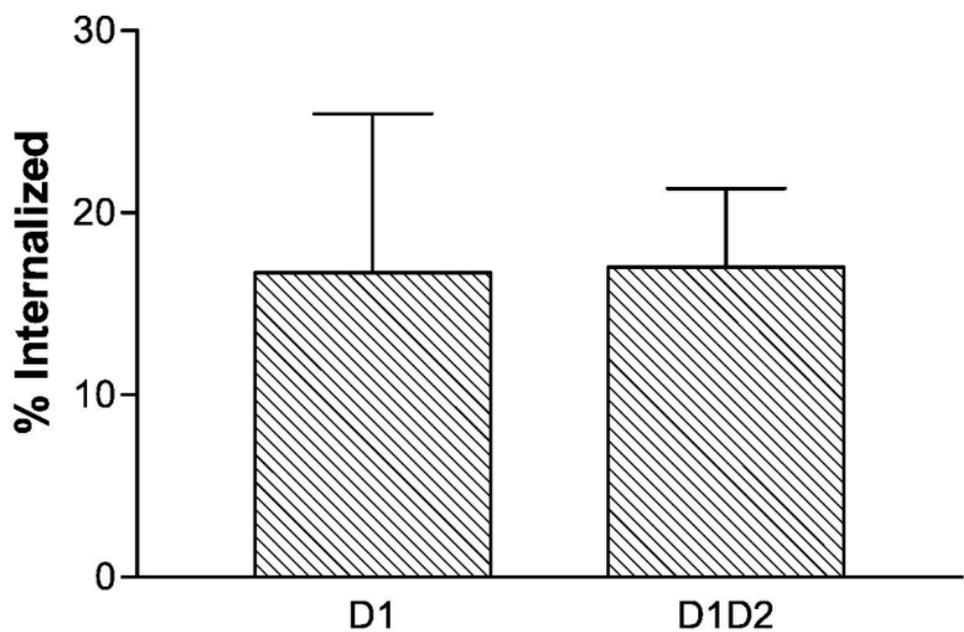


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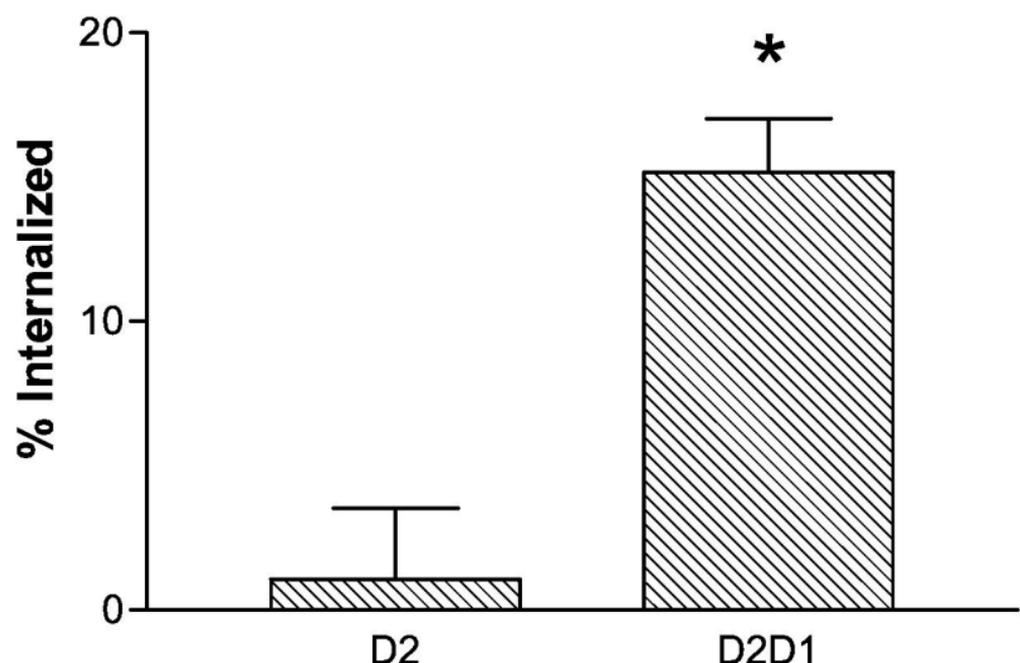


# Figure 4

A

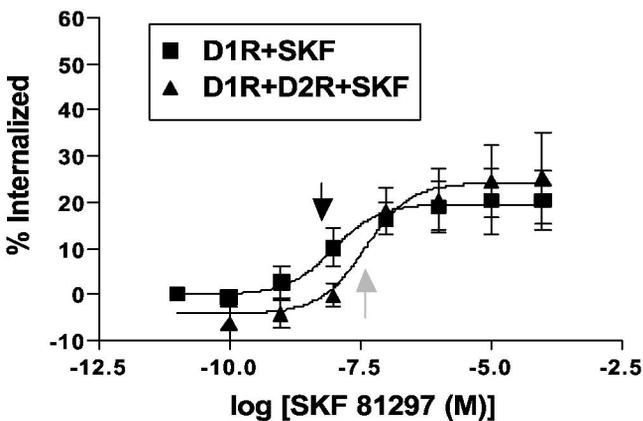


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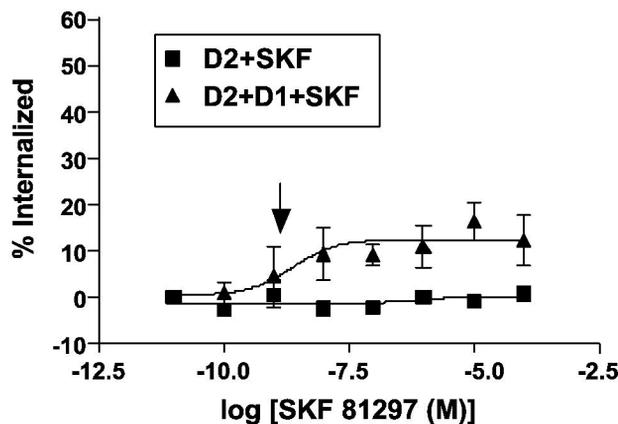


# Figure 5

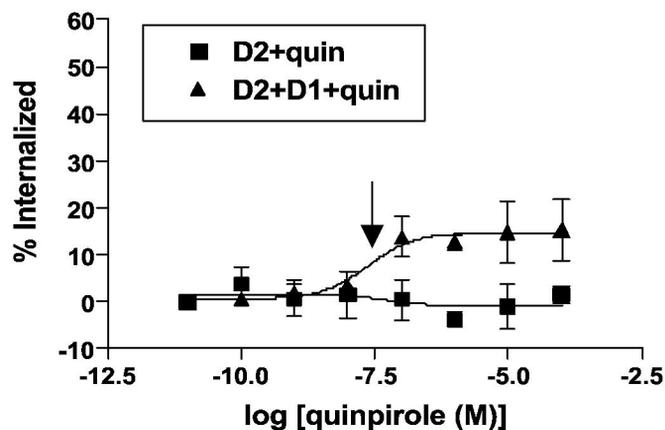
## A



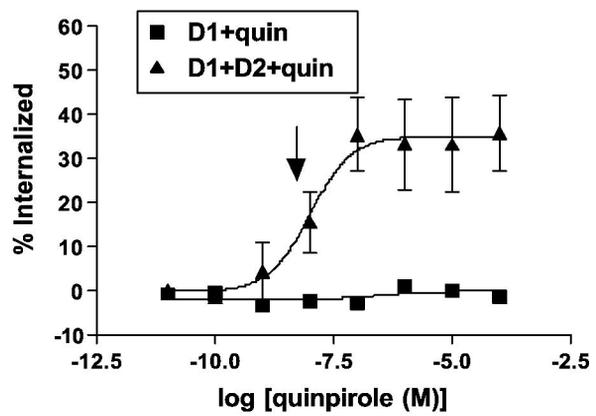
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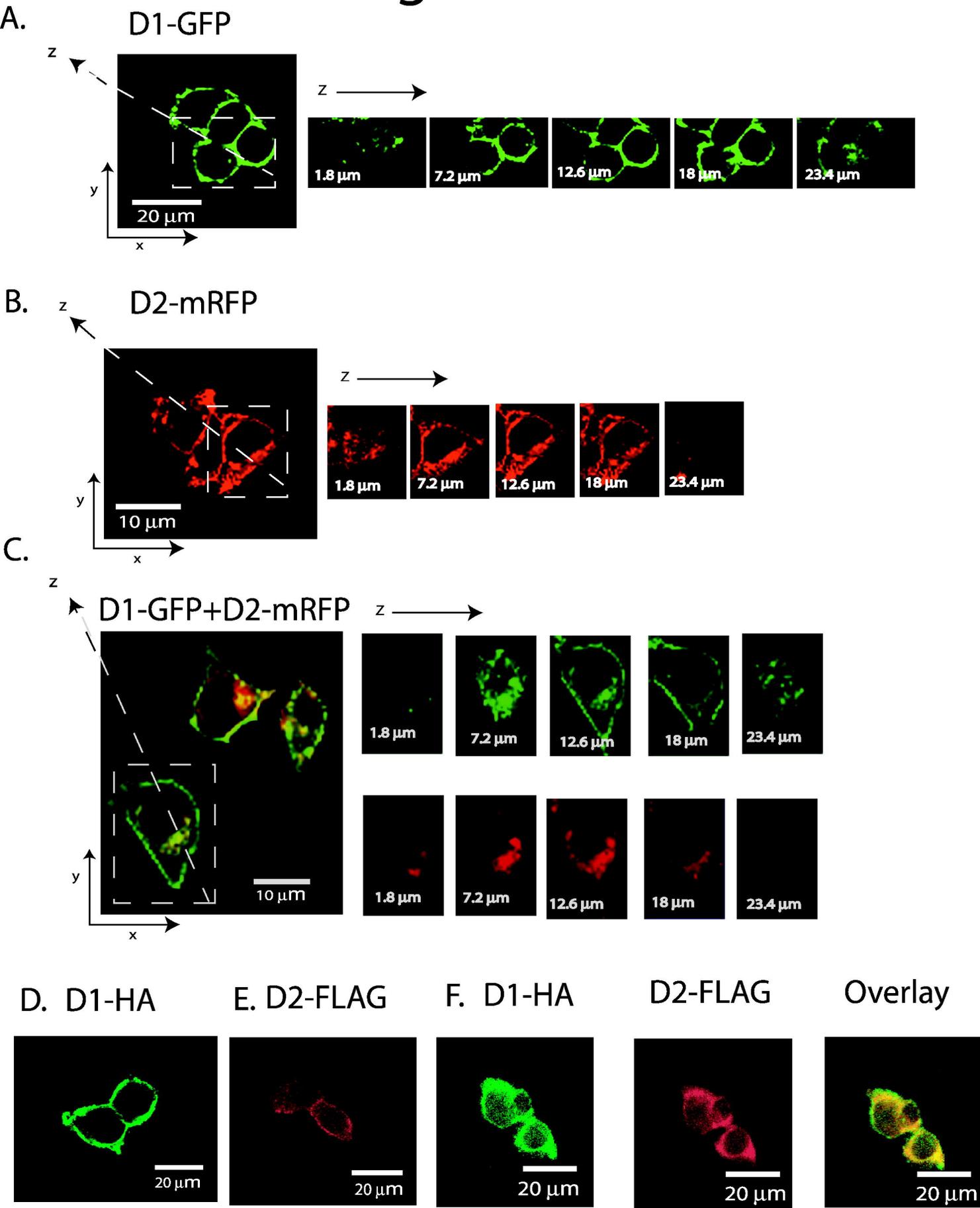
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## D

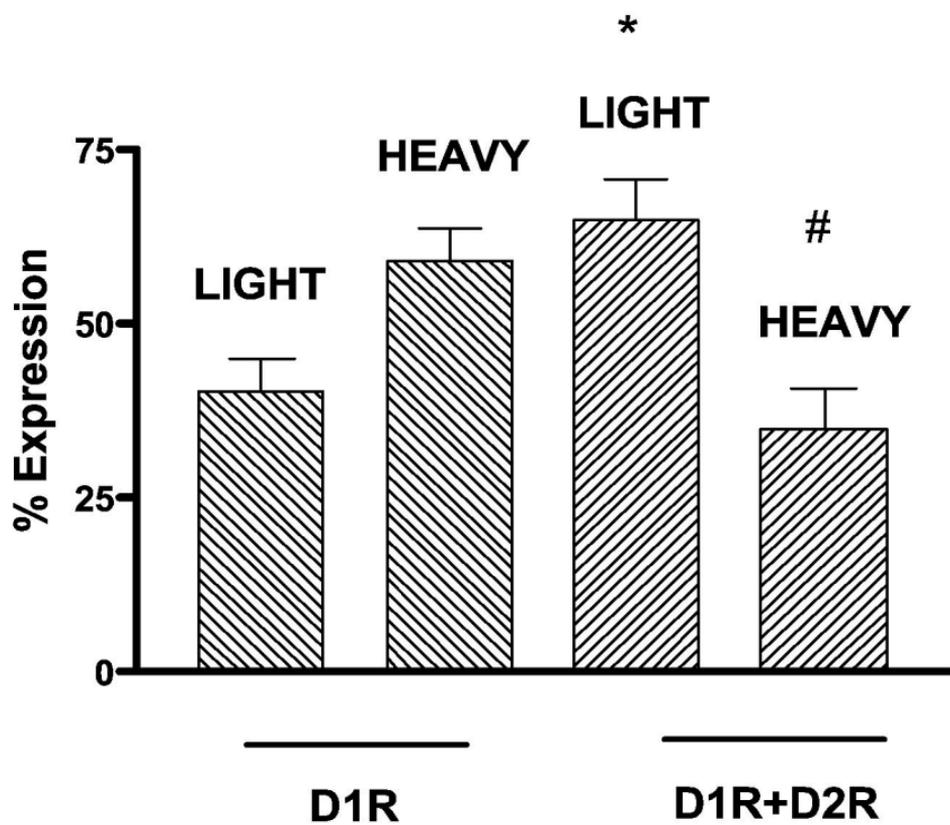


# Figure 6

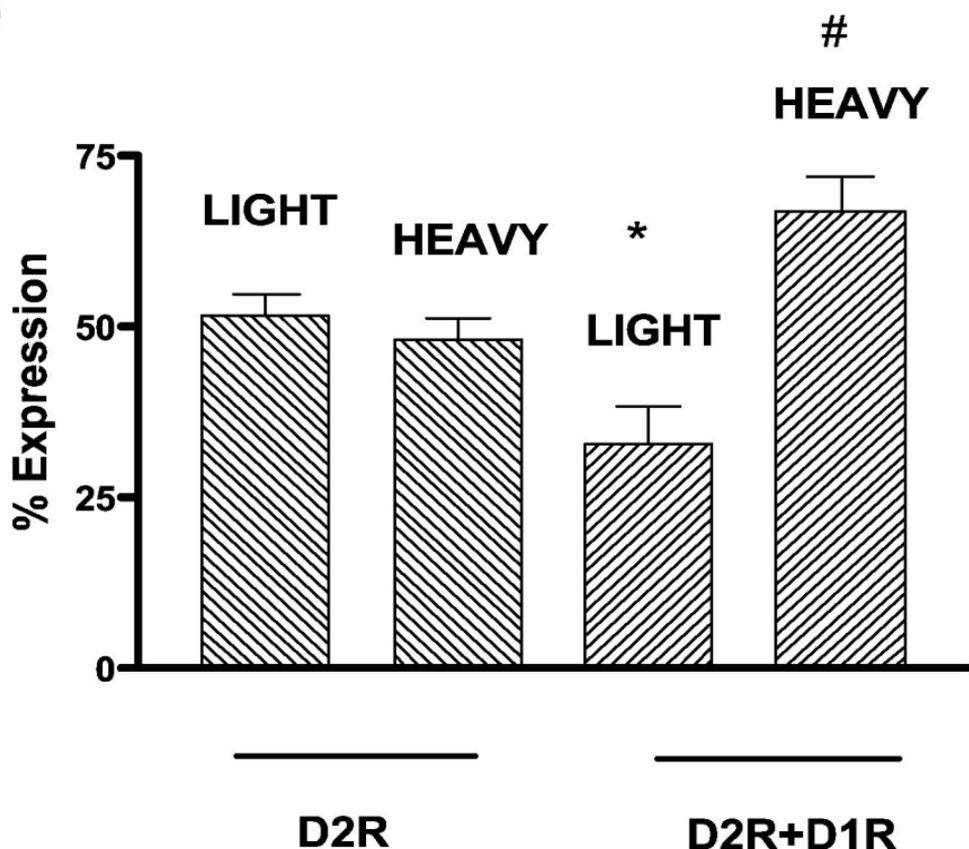


# Figure 7

A

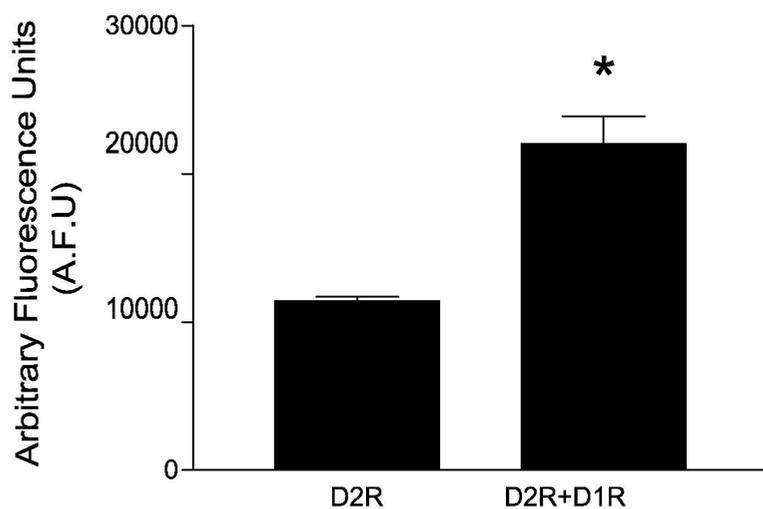


B

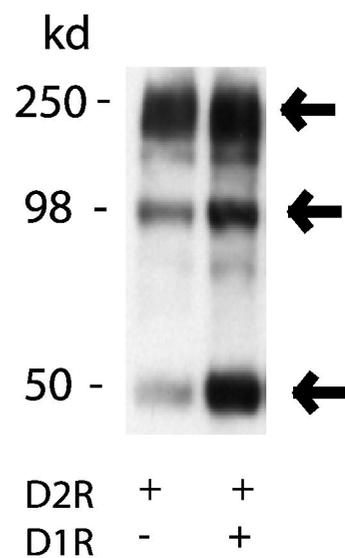
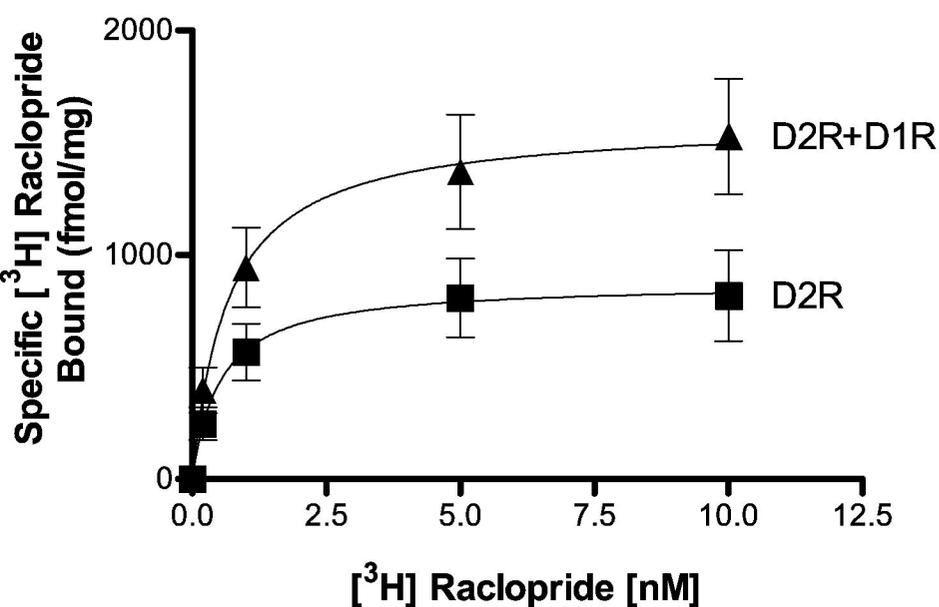


# Figure 8

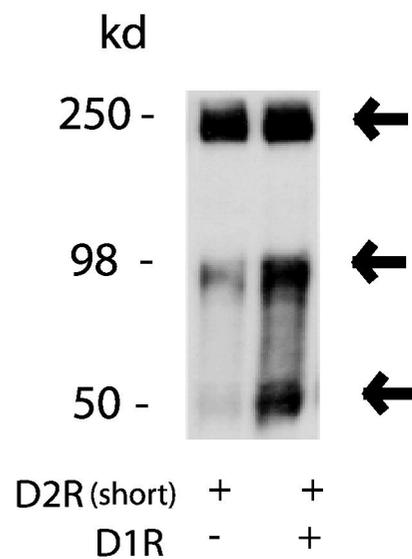
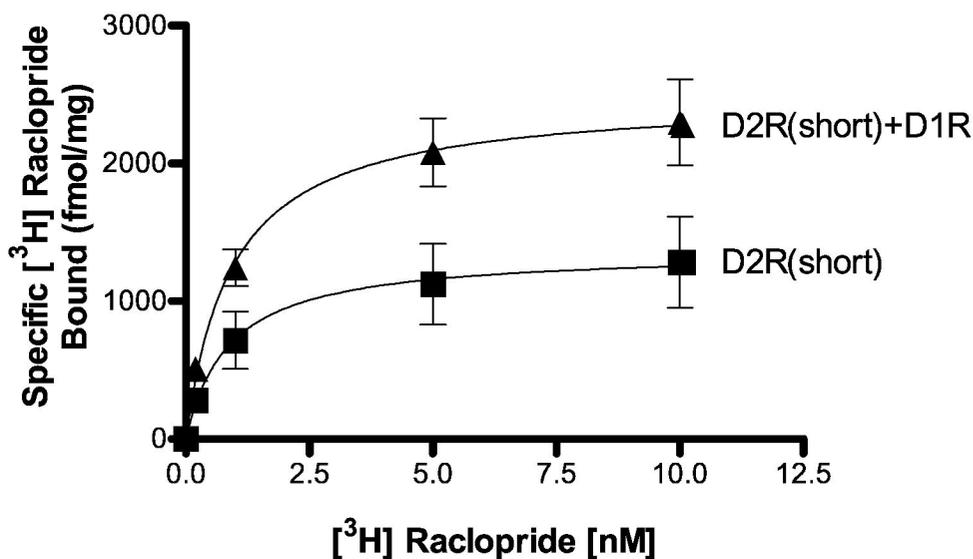
**A**

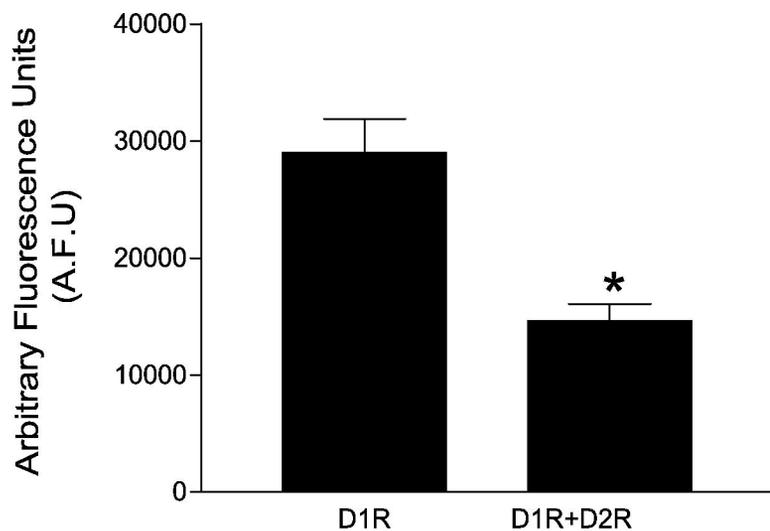


**B**



**C**



**D****E**