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D1-D2 Dopamine Receptor Synergy Promotes Calcium Signaling via Multiple Mechanisms

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D$_{1}$-D$_{2}$ Receptor Synergy and Ca$^{2+}$ Signaling

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**Abbreviations:**

Co-IP: Co-immunoprecipitation  
D$_{1}$R: D$_{1}$ dopamine receptor subtype  
D$_{2}$R: D$_{2}$ dopamine receptor subtype  
D$_{2}$LR: D$_{2}$ long splice variant  
D$_{2}$SR: D$_{2}$ short splice variant  
DA: Dopamine  
DAG: Diacylglycerol  
DAR: Dopamine receptor  
EC$_{50}$: 50% excitatory concentration  
GRK2: G protein receptor kinase 2  
GPCR: G protein-coupled receptor  
IC$_{50}$: 50% inhibitory concentration  
PKC: Protein kinase C  
PLC: Phospholipase C
Abstract

The D₁ dopamine receptor (D₁R) has been proposed to form a hetero-oligomer with the D₂ dopamine receptor (D₂R), which in turn results in a complex that couples to PLC-mediated intracellular calcium release. We have sought to elucidate the pharmacology and mechanism of action of this putative signaling pathway. Dopamine dose response curves assaying intracellular calcium mobilization in cells heterologously expressing the D₁ and D₂ subtypes either alone or in combination, and employing subtype selective ligands, revealed that concurrent stimulation is required for coupling. Surprisingly, characterization of a putative D₁-D₂ heteromer-selective ligand, SKF83959, found no stimulation of calcium release, but a broad range of cross-reactivity with other GPCRs. In contrast, SKF83959 appeared to be an antagonist of calcium mobilization. Over-expression of Gα₁ with the D₁ and D₂ DARs enhanced the dopamine-stimulated calcium response. However, this was also observed in cells expressing Gα₁ with only the D₁R. Inactivation of Gᵢ or Gₛ with pertussis or cholera toxin, respectively, was found to largely, but not entirely reduce the calcium response in D₁R and D₂R co-transfected cells. Moreover, we found that sequestration of Gᵦβγ subunits through over-expression of GRK2 mutants either completely or largely eliminated dopamine-stimulated calcium mobilization. Our data suggests that the mechanism of D₁R-D₂R-mediated calcium signaling involves more than receptor-mediated Gᵢ protein activation, may largely involve downstream signaling pathways, and may not be completely heteromer-specific. In addition SKF83959 may not exhibit selective activation of D₁-D₂ heteromers, and its significant cross-reactivity to other receptors warrants careful interpretation of its use in vivo.
Introduction

Dopamine is a neurotransmitter that functions in the central nervous system to regulate neural processes that include motor control, cognition, and memory. Dysregulation of the DA system is associated with neurological disorders such as Parkinson's disease, schizophrenia, addiction, and ADHD. Five DA receptor (DAR) genes exist in mammals, each of which encodes a DAR subtype (D_{1R} - D_{5R}), which are grouped by structure and function into the D1-like (D_{1R} and D_{5R}) and D2-like (D_{2R}, D_{3R}, and D_{4R}) DAR families. The D1-like receptors couple to the G_{s/olf} proteins to activate adenylyl cyclase-mediated formation of cAMP, while the D2-like receptors couple to the G_{i/o} proteins to inhibit adenylyl cyclase (Missale et al., 1998; Sibley and Monsma Jr, 1992). Several studies, however, have proposed DAR-mediated signaling pathways that do not involve activation of either G_{i/o} or G_{s/olf} proteins.

The first evidence for alternate signaling pathways came from multiple studies reporting “D_{1-like}” receptor stimulation of intracellular calcium mobilization suggested to be a result of G_{q} mediated activation of phospholipase C (PLC) (Mahan et al., 1990; Pacheco and Jope, 1997; Undie and Friedman, 1990; Wang et al., 1995). Subsequently, it was shown that \textit{in vitro} cell cultures co-expressing the D_{1R} and D_{2R} could couple to intracellular calcium mobilization through the G_{q}-PLC-diacylglycerol pathway (Lee et al., 2004; Rashid et al., 2007a). This calcium response required both co-expression and co-activation of both receptor subtypes. This led to the proposal of a “non-canonical” mechanism for DAR-mediated signaling wherein the D_{1R} forms a heteromeric complex with the D_{2R} and induces PLC-mediated intracellular calcium mobilization (Hasbi et al., 2011; Lee et al., 2004; Rashid et al., 2007b). The precise mechanism for this type of signaling and its prevalence \textit{in vivo}, however, remains unclear.

\textit{In vivo}, there is evidence for (Aizman et al., 2000; Ariano et al., 1997; Lee et al., 2004; Lester et al., 1993; Surmeier et al., 1992, 1996) and against (Bertran-Gonzalez et al., 2008; Gerfen et al., 1990; Hersch et al., 1995; Le Moine and Bloch, 1995; Le Moine et al., 1991) the existence of neural cells co-expressing both D_{1R} and D_{2R}. Interestingly, some neurons which
appear to co-express D₁R and D₂R have neuronal projections that only express D₁R or only D₂R (Lee et al., 2004). This, along with the different methods of detection and visualization, may partially explain the incongruent reports of D₁R and D₂R co-localization. However, several recent studies using confocal FRET techniques argue for direct demonstration of the existence of D₁-D₂ heteromers in 10-20% of the cell bodies and pre-synaptic terminals of medium spiny neurons within the nucleus accumbens (Hasbi et al., 2009; Perreault et al., 2011, 2012a), and the two DARs have been shown to co-internalize following selective activation of either receptor (O’Dowd et al., 2005; So et al., 2005).

Interestingly, several agonists of the benzazepine family seem to exhibit differential effects on the D₁R monomer versus the proposed D₁-D₂ heteromer (Rashid et al., 2007b). One such compound, SKF83822, has been proposed to selectively activate D₁R-mediated cAMP production while having no effect on calcium mobilization (Rashid et al., 2007a, 2007b). In contrast, another benzazepine, SKF83959, has been proposed to selectively activate the heteromer-mediated calcium release and have no effect on cAMP production (Hasbi et al., 2011; Rashid et al., 2007a, 2007b). More recent studies have used this finding to interpret the results of systemic SKF83959 injections in mice which resulted in increased Ca²⁺/calmodulin-dependent kinase II phosphorylation and increased brain-derived neurotrophic factor expression in striatal neurons (Hasbi et al., 2009; Ng et al., 2010). It was also shown that, when injected into rats, expression of glutamate decarboxylase-67 and the vesicular glutamate transporters 1 and 2 in striatal neurons was altered by SKF83959 (Perreault et al., 2012b), which, again, was interpreted to be due to selective D₁-D₂ heteromer activation.

In the current study, we further investigate the biology and pharmacology of the proposed D₁-D₂ heteromer and the mechanism of calcium mobilization in heterologous expression systems. While we find that co-activation of both D₁R and D₂R protomers is required for calcium mobilization to occur, there appear to be multiple mechanisms besides G₉ activation through which this pathway is elicited. We also studied the functional characteristics
of SKF83959, to determine its viability as a heteromer-selective \textit{in vivo} ligand, and found that it was significantly less selective than previously appreciated. In fact, we were not able to provide evidence for selective activation of the D$_1$-D$_2$ heteromer. These results indicate that D$_1$Rs and D$_2$Rs can synergize to induce calcium mobilization, although the mechanisms of activation are multiple and complex and there is not, as of yet, a selective pharmacology.
Materials and Methods

Materials—HEK293-tsa201 (HEK293T) cells were a gift from Dr. Vanitha Ramakrishnan. A D1R expressing stable cell line was purchased from Codex Biosolutions, Inc. (Gaithersburg, MD). [3H]-N-methyl-SCH23390 (80.5 Ci/mmol) and [3H]-N-methylspiperone (85.5 Ci/mmol) was obtained from PerkinElmer Life Sciences (Waltham, MA). Cell culture media and reagents were purchased from MediaTech/Cellgro (Manassas, VA). Cell culture flasks and materials and all assay plates were purchased from Greiner Bio-One (Monroe, NC). SKF83959 and SKF83822 were purchased from Tocris Bioscience/RD Systems (Minneapolis, MN). All other compounds and buffer components were purchased from Sigma-Aldrich (St Louis, MO), except where indicated.

Cell Culture and Transfection—HEK293T cells and D1R CODEX cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with a final concentration of 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, and 10 μg/mL gentamicin. Cells were incubated at 37°C, 5% CO2, and 90% humidity. They were passaged and plated mechanically using calcium-free Earle’s balanced salt solution (EBSS) and pelleted by centrifugation at 1000 rpm for 10 min. For transfection studies, HEK293T cells were seeded in 150 mm plates at 10 × 10^6 cells per plate. After 24 hr, cells were transfected according to the manufacturer’s recommendations using Clontech’s CalPhos™ transfection kit (Clontech Laboratories, Inc., Mountain View, CA). The DAR plasmid constructs were FLAG-tagged rat D1R, D2S, or D2L in the pCD-SRα vector (Monsma et al., 1990; Takebe et al., 1988; Zhang et al., 1994) and D4R in pcDNA3.1(+) vector (Schetz and Sibley, 2001). Additional experiments were done using the Gq protein in the pcDNA3.1(+) vector (Missouri S&T cDNA Resource Center, Rolla, MO) and various functionally dominant negative GRK2 mutants: GRK2 C-terminus 495-689 in pcDNA3(+), GRK2 K220R in pcDNA3(+), and empty pcDNA3.1(+).
(Freedman et al., 1995; Koch et al., 1994). For all transfections, 5 μg of each DNA construct was used to transfected cells, with the exception of D₁R, in which 10 μg was used.

**Radioligand Binding Assays**—48 hr after transfection, cells were dissociated from plates using calcium-free Earle’s balanced salt solution (EBSS), and intact cells were collected by centrifugation at 900 × g for 10 min. Cells were resuspended and lysed using 5 mM Tris-HCl and 5 mM MgCl₂ at pH 7.4 at 4°C. Cell lysate was pelleted by centrifugation at 20,000 × g for 30 min and resuspended in 5 mM Tris-HCl at pH 7.4. 100 μL cell lysate (containing 8 μg protein for D₂R assays or 10 μg protein for D₁R assays) was incubated for 90 min at room temperature with various concentrations of [³H]-N-methyl-SCH23390 (D₁R binding) or [³H]-N-methylspiperone (D₂R binding) in a final reaction volume of 250 μL. Non-specific binding was determined in the presence of 4 μM (+)-butaclamol. Bound ligand was separated from the unbound by filtration through a PerkinElmer Unifilter-96 GF/C 96 well micro-plate using the PerkinElmer Unifilter-96 Harvester, washing 3 times, 1 mL per well in ice-cold assay buffer. After drying, 50 μL of liquid scintillation cocktail (MicroScint PS, Perkin Elmer, Waltham, MA) was added to each well, plates were sealed, and analyzed on a PerkinElmer Topcount NXT™. For competition binding assays, a fixed concentration of 0.5 nM [³H]N-methyl-SCH23390 was incubated with various concentrations of SKF83959, and the remainder of the assay was performed as described above. Kᵢ values were calculated from observed IC₅₀ values using the Cheng-Prusoff equation and a Kₐ value of 0.5 nM for SCH23390, as determined in independent saturation isotherms (data not shown). Expression of the D₄R was determined in an identical assay format as that for the D₂R.

**Competition Radioligand Binding Screen**—A primary, single-point, radioligand competition binding assay was performed to assay for radioligand binding inhibition by SKF83959 (10 μM).
Forty-three GPCRs and neurotransmitter-related proteins were screened in the primary assay using radioligands with known binding properties. Percent inhibition was calculated by subtracting percent specific binding in the presence of the test compound from the percent specific binding in the absence of the test compound, (N=4). Receptors whose corresponding radioligands had greater than 50% inhibition at 10 μM SKF83959 underwent secondary radioligand competition binding assays to generate full competition curves. K_i determinations and receptor binding profiles were provided by the National Institute of Mental Health (NIMH) Psychoactive Drug Screening Program (PDSP), Contract # HHSN-271-2008-00025-C. The NIMH PDSP is directed by Dr. Bryan L. Roth (University of North Carolina, Chapel Hill, NC) and by Project Officer Jamie Driscol (NIMH, Bethesda, MD). For experimental details including radioligands used and associated K_d values for each individual receptor, please refer to the PDSP website http://pdsp.med.unc.edu/.

**Calcium Mobilization Assays**—HEK293T cells were transiently transfected as described. 24 hr after transfection, cells were plated in 384-well, optical, clear bottom, black walled plates (20 μL/well, 30,000 cells/well; Greiner Bio-one, Monroe, NC). 48 hr following transfection, cells were incubated for 60 min at room temperature in the dark with Fluo-8 NW calcium dye and an extracellular signal quencher to block any signal from extracellular calcium (Screen Quest™ Fluo-8 NW Calcium Assay Kit, AAT Bioquest, Inc., Sunnyvale, CA), as recommended by the manufacturer. The plates were then treated with various concentrations of antagonist or agonists (diluted in the presence of 0.2 mM sodium metabisulfite) as indicated in the results and figure legends. For agonist reads, plates were read in real time kinetically (every 0.6 sec) by recording a baseline read for 14 sec prior to addition of an agonist compound and then continually measured for 2 min after agonist addition. For antagonist reads, plates were read in real time kinetically (every 0.6 sec) by recording a baseline reading for 20 sec prior to addition of that antagonist. Then, three min later, agonist compound was added and the plates were read
for an additional 3 min. All compound additions were done in unison using the 384-tip onboard robotics on an FDSS μCell (Hamamatsu, Bridgewater, NJ), and plates were continuously read using the FDSS μCell from the bottom throughout the assay with an excitation wavelength of 480 nm and an emission wavelength of 540 nm. Data were recorded and quantified as maximum minus minimum (max-min) RFU within the assay window using FDSS software. Data are expressed as a percentage of the control max-min RFU for given studies as indicated in the figure legends. In these experiments, D₁R and D₂R receptor expression levels typically varied between 1-3 pmol/mg protein. We found that co-expressing both receptors sometimes affected their expression compared to expressing them alone (data not shown). However, this did not affect the calcium mobilization response, which, while not studied in detail, appeared to simply require a minimum level of dual receptor expression.

Statistical analysis—Data are expressed as a percentage of control values for individual experiments. Non-linear regression of all data was conducted on GraphPad Prizm 5.01 (GraphPad Software, Inc., La Jolla, CA). Results are expressed as mean ± S.E.M.

See supplemental materials and methods section for additional procedures.
Results

Previous studies have suggested that the D₁-D₂ receptor complex may signal as a heteromer and have implicated SKF83959 as a compound that may selectively activate this signaling complex (Lee et al., 2004; Rashid et al., 2007a, 2007b; So et al., 2005). However, these findings have not been corroborated and the mechanisms by which the D₁-D₂ receptor complex signals remain unclear. To investigate the apparent ability of D₁-D₂ receptor oligomerization to alter the G protein coupling of component receptors, we first transiently expressed the D₁R either alone or concurrently with either the short (D₂S) or long (D₂L) isoforms of the D₂R and measured intracellular calcium mobilization via kinetic fluorescence imaging. Preliminary co-immunoprecipitation experiments revealed that D₁-D₂ hetero-oligomers were indeed capable of forming under these expression conditions (Supplemental Fig. 1). When cells were transfected with the D₁R and D₂LR or the D₁R with D₂SR, a clear dose-dependent activation of calcium mobilization was observed in response to DA (Fig. 1). Importantly, we observed no difference in coupling efficacy or agonist potency between the short and long isoforms of the D₂R. However, when cells were transfected with any of the subtypes alone, the receptors failed to couple to calcium mobilization (Fig. 1). These data suggest that expression and activation of both the D₁R and D₂R are essential for coupling to calcium mobilization and signaling.

To further investigate that the activation of both receptor subtypes is required to stimulate calcium mobilization, we utilized receptor subtype selective antagonists. Concentration response inhibition curves for the D₁R-selective (SCH23390) and the D₂R-selective (sulpiride) antagonists were generated for cells transfected with the D₁R and D₂R (Figs. 2A and 2B). Cells were simultaneously stimulated with 1 μM DA and examined for calcium mobilization. We observed complete inhibition of the calcium signal with either SCH23390 or sulpiride treatment. The potencies of the antagonists (SCH23390 IC₅₀ ~8.0 nM, sulpiride IC₅₀ ~0.7 nM) are consistent with their known affinities for their selective subtypes as determined in our laboratory.
(data not shown) as well as other groups (Millan et al., 2001; Seeman and Van Tol, 1993). More importantly, complete inhibition of the calcium response is seen at antagonist concentrations that have no effect on the opposite receptor subtype. Thus, selectively blocking DA activation of either receptor subtype is sufficient to prevent calcium mobilization further suggesting that both receptor protomers must be activated for this signaling to occur.

While the studies employing subtype selective antagonists suggested that both D1R and D2R are required for calcium signaling, it might be possible that stabilizing one subtype into an inactive state within a heteromer might alter the conformation of the corresponding partner. Thus, to further elucidate the coupling mechanism, subtype-selective agonists were used to determine if indeed activation of both protomers are required for calcium mobilization. As seen in Fig. 3, concurrent administration of a D1R-selective (SKF83822) and a D2R-selective (quinpirole) agonist to cells co-transfected with D1R and D2R resulted in a calcium mobilization response that nearly matched that of DA. In contrast, when D1R plus D2R co-transfected cells were stimulated with quinpirole alone, no calcium mobilization was observed. Furthermore, when the co-transfected cells were stimulated with SKF83822, no calcium mobilization was seen at concentrations selective for D1R. A small response was observed at 10 μM, but this was at a concentration where SKF83822 loses receptor subtype selectivity and can begin to stimulate the D2R as well. Previous studies showed that SKF83822 has an affinity for D1R in the ~2 nM range and D2R in the ~200 nM range (O’Sullivan et al., 2004). Experiments done in our lab have demonstrated a D2R affinity that is greater than 10 μM (data not shown), supporting the idea that the SKF83822-mediated calcium response seen at high concentrations is due to non-selective receptor activation. In addition, when cells were transfected with any of the subtypes individually, no signal was seen from any of the agonists (data not shown). Taken together, these data indicate that stimulation of both receptor subtypes is necessary for calcium mobilization.
Previous studies suggested that SKF83959 may be a D₁-D₂ heteromer-selective compound and a significant calcium response to this ligand has been reported in cells co-expressing the D₁R and D₂R (Beaulieu and Gainetdinov, 2011; Lee et al., 2004; Rashid et al., 2007a, 2007b). This compound has also been reported to have seemingly paradoxical effects on the D₁R, exhibiting both antagonist and agonist properties depending on the system (Cools et al., 2002; Panchalingam and Undie, 2001; Zhang et al., 2005). In our current studies, we treated D₁R and D₂R co-transfected cells with SKF83959 and, surprisingly, were unable to elicit a calcium response (Fig. 4A). Furthermore, when SKF83959 was added in concert with the D₂R selective agonist quinpirole, we were still unable to observe a significant calcium response. It should be noted that SKF83959 consistently failed to stimulate calcium mobilization even when this experiment was performed using different lots of compound from different vendors on separate days, as well as with different drug solvents (data not shown). We also had one lot of compound chemically analyzed to verify its purity (data not shown). To demonstrate that the SKF83959 compound was pharmacologically active in our hands, we performed two separate experiments. In Fig. 4B, we stimulated calcium mobilization with DA and then dose-dependently added either the D₁R-selective antagonist SCH23390 as a control (see Fig. 2A) or SKF83959 to see if it might function as an antagonist in this system. In fact it did, exhibiting even higher potency than SCH23390, although its efficacy of antagonism was less, exhibiting a maximum inhibition of ~50%. Finally, we performed a radioligand binding competition assay with SKF83959 and cells transfected with the D₁R (Fig. 4C). SKF83959 was able to potently and fully compete for radioligand binding to the D₁R. These experiments (Figs. 4B, C) demonstrate that SKF83959 is active in binding to the monomeric D₁R as well as active as a partial antagonist of the calcium response observed in D₁R and D₂R co-transfected cells. In contrast, it does not appear to function as an agonist with respect to stimulating calcium mobilization in the D₁R and D₂R co-transfected cells.
Given the apparent discrepancies of our findings with some previous studies (Hasbi et al., 2011; Lee et al., 2004; Rashid et al., 2007b), and the possibility that SKF83959 may not be as selective as previously thought, we sought to screen its selectivity against various GPCRs. This was accomplished through collaboration with the NIMH Psychoactive Drug-Screening Program (http://pdsp.med.unc.edu). For the primary screen, a single-point radioligand binding competition experiment was performed with 10 μM SKF83959 as a competitor against an appropriate receptor-specific radioligand of known properties. Forty-three GPCRs and signaling proteins were screened this way and twenty of them resulted in >50% inhibition at 10 μM SKF83959 (Table 1). In contrast, twenty-three GPCR targets were found to have <50% inhibition at 10 μM SKF83959 and were therefore considered relatively “inactive/low affinity” for SKF83959 (Supplemental Table 1). The twenty “active” receptors/proteins underwent secondary radioligand competition binding experiments to generate full competition curves for SKF83959 and Kᵢ values for these receptors were determined and shown in Table 1. Of note is that the serotonin 5-HT2A, 5-HT2B, 5-HT2C, 5-HT5A and 5-HT6 receptors, the adrenergic α2A, α2B and α2C receptors, the D₁, D₂, and D₅ receptors, and the serotonin transporter all have nanomolar Kᵢ values. SKF83959 demonstrated very high (sub-100 nM) affinity for four of these GPCRs: the serotonergic receptor subtypes 5-HT2C, the adrenergic receptor subtype α2C, the D₁, and D₅ receptor subtypes, and the serotonin transporter. Notably, SKF83959 has also recently been shown to be a potent allosteric modulator of the sigma-1 receptor (Guo et al., 2013). Taken together, these data indicate that SKF83959 has significantly high affinity for a wide number of receptors and thus caution should be taken when interpreting in vivo experimentation and the selectivity of this agent.

While D₁ and D₂ receptors appear capable of signaling through calcium mobilization when both receptors are stimulated, the mechanism of transduction remains unclear. To better understand the mechanisms involved, we tested the hypothesis that the receptors, perhaps
within the context of a heteromer, may switch G protein-coupling selectivity and gain the ability to activate \( \alpha_q \). We first examined this possibility by over-expressing \( \alpha_q \) in cells expressing the D_1R+D_2R. Interestingly, the resulting DA-stimulated calcium signal was increased by 200% when compared to cells transfected with the D_1R+D_2R alone (Fig. 5A). Expression of only the \( \alpha_q \) protein in the absence of either receptor did not enable the ability of DA to stimulate calcium mobilization (Fig. 5A). In parallel studies, we examined how over-expression of \( \alpha_q \) with the D_1R or D_2R alone could couple to intracellular calcium mobilization. While cells transfected with D_1R or D_2R alone did not give a calcium response, consistent with Fig. 1, when \( \alpha_q \) was over-expressed, the D_1R was able to elicit a DA-stimulated calcium signal in the absence of the D_2R (Fig. 5B), although the calcium response was not as large as that seen with the D_1R+D_2R+\( \alpha_q \) transfection (cf. Fig. 5A and 5B). No such phenomenon was observed with the D_2R. Taken together, these data suggest that the \( \alpha_q \) protein may be involved in calcium mobilization mediated by a D_1-D_2 heteromer, however, this interpretation is complicated by the fact that over-expression of \( \alpha_q \) can also lead to monomeric D_1R coupling.

Given our results with \( \alpha_q \) over-expression, we re-evaluated SKF83959-stimulation of calcium mobilization under these conditions in the D_1R and D_2R co-expressed cells. We found that with \( \alpha_q \) over-expression, SKF83959 is able to stimulate calcium mobilization in a manner similar to that of DA (Fig. 6A), whereas it is unable to stimulate such a response in cells lacking \( \alpha_q \) over-expression (Fig. 4 and 6A). Interestingly, SKF83959 was also able to stimulate calcium mobilization in cells expressing the D_1R and over-expressing \( \alpha_q \), but not D_1R alone (Fig. 6A). These results led us to test the antagonist sensitivity of the SKF83959 responses as shown in Fig. 6B. We found that the D_1R-selective antagonist, SCH23390, could completely ablate SKF83959 stimulation of calcium mobilization in both D_1R+\( \alpha_q \) transfected as and D_1R+D_2R+\( \alpha_q \) transfected cells. However, in contrast to what we observed for DA stimulation of D_1R+D_2R co-transfected cells, the D_2R-selective antagonist, sulpiride, was unable to block SKF83959 stimulation of calcium mobilization. These results suggest that over-expression of
Gqα enables SKF83959 to stimulate monomeric D₁R present in the D₁R and D₂R co-transfected cells, rather than enabling it to gain function as a D₁-D₂ heteromeric-selective agonist.

While the extant hypothesis, which our over-expression data support, is that Gq is central to the stimulation of calcium mobilization, the central question is if direct coupling with a D₁-D₂ heteromer may be involved. An alternative hypothesis is that the D₁R and D₂R signal through downstream pathways that converge on the Gq protein and/or other components of the calcium mobilization process. In order to test whether D₁-D₂ synergistic signaling is independent of Gι or Gs protein function, we interfered with the activity of Gι and Gs via treatment with toxins. D₁R and D₂R co-transfected cells were incubated overnight in media containing pertussis toxin (PTX) to inhibit Gι protein function (Namkung et al., 2009) or cholera toxin (CTX) to interfere with Gs protein function (Cour et al., 2011). Cells were then assayed for calcium mobilization in response to DA stimulation. We found that treatment with either CTX or PTX drastically, but not entirely, reduced the calcium response (Fig. 7). These data support the involvement of D₁R-Gs and D₂R-Gι mediated mechanisms that majorly contribute to the calcium response in the D₁R and D₂R co-transfected cells.

Another possibility, however, may be that general Gι-Gq “crosstalk” is occurring after receptor activation, which leads to PLC activation. Multiple cases of Gι-Gq cross-talk in other receptor systems and cell types have been documented (Carroll et al., 1995; Okajima et al., 1989; Rebres et al., 2011; Toms and Roberts, 1999), and Gι-Gq cross-talk in the D₁-D₂ receptor system could account for the PTX sensitivity of the calcium signal. In this model, any Gi-linked GPCR, not just the D₂R would be able to support a Gq-mediated calcium response. In order to test this possibility, we used the D₄R, a Gi-linked DAR, which has not been found to form hetero-oligomers with the D₁R (González et al., 2012). We co-transfected the D₁R and D₄R and compared the DA response to that in the D₁R+D₂R transfected cells (Fig. 8). In fact, the D₄R was shown not to support a calcium response in the presence of co-expressed D₁R, indicating that non-specific Gι-Gq crosstalk, at least as previously described (Carroll et al., 1995; Okajima
et al., 1989; Rebres et al., 2011; Toms and Roberts, 1999) does not explain the D₁-D₂ heteromer-mediated calcium response.

The potential involvement of multiple Gₐ-proteins led us to also investigate other mechanisms by which D₁R and D₂R activation could stimulate calcium mobilization. Notably, Gβγ subunits have been shown to increase cytoplasmic calcium concentrations by stimulating PLCβ (Beaulieu and Gainetdinov, 2011). A recent publication found that the ghrelin receptor-D₂R dimer-linked calcium response was PTX sensitive, required PLC activity, and could be ablated by sequestering the Gβγ subunits (Kern et al., 2012). To see if Gβγ plays a role in the D₁-D₂ heteromer-mediated calcium release, we co-transfected the D₁R and D₂R with two different functionally dominant negative G-protein receptor kinase 2 (GRK2) mutants. The mutants we used were GRK2 K220R and the GRK2 C-terminal 495-689 peptide fragment (GRK2 c-term), both of which are unable to phosphorylate GPCRs, but can bind to, and sequester Gβγ subunits (Freedman et al., 1995; Koch et al., 1994). We found that over-expression of GRK2 K220R was able to completely ablate DA-stimulated calcium mobilization in the D₁R and D₂R co-transfected cells (Fig 9A). Similarly, over-expression of GRK2 c-term drastically reduced, but did not completely ablate, the DA-stimulated calcium response (Fig. 7B). These data suggest that the observed calcium mobilization occurring in response to D₁R and D₂R activation is largely dependent on free Gβγ subunits.
Discussion

Receptor oligomers of many different GPCR types have been proposed to form homo- or hetero-oligomers with biochemical and functional characteristics that are unique to their oligomeric conformations (Ferre et al., 2009). These GPCR oligomers have been found not only to occur within a type of GPCR, but also across different classes, families, types, and subtypes (Prinster et al., 2005). In addition to signaling, internalization and degradation of GPCRs in homo- and hetero-oligomers has been found to differ from their monomeric activities (Ferre et al., 2009; Milligan, 2004; Missale et al., 2010; Prinster et al., 2005; Terrillon and Bouvier, 2004). Like previously described receptor oligomers, it has been shown that the D1R and D2R can co-IP with each other (Lee et al., 2004; Pei et al., 2010; Supplemental Fig. 1), and fluorescence imaging has shown that the two receptors co-internalize when one or the other receptor is stimulated (Dziedzicka-Wasylewska et al., 2006; Łukasiewicz et al., 2009; O'Dowd et al., 2005, 2012; So et al., 2005). We have demonstrated that the calcium response is unique to cells that co-express both D1 and D2 DARs and that the DARs must be co-stimulated, as an antagonist to either receptor blocks the transduction. However, the mechanism of action and whether heteromers versus homomers form the functional units for calcium signaling remains unclear.

It has been suggested that the co-activation of the D1-D2 complex causes a conformational change that results in the direct interaction between the C-terminus of the D1R and the third intracellular loop (ICL3) of the D2R (O'Dowd et al., 2012). The ICL3 is the only region of difference between D2LR and D2SR, and there is evidence that it results in differences in the G protein coupling and signaling capabilities of each D2R isoform (Kendall and Senogles, 2011). Recently, it was proposed that the ICL3 of D2LR, but not the D2SR, could form a complex with the D1R (Pei et al., 2010), but the findings were based on the use of GST and TAT-fused D2R ICL3 fragments, which may not accurately mimic native receptor conformations and interactions. Later, it was shown that both D2R splice isoforms were able to co-internalize with...
the D₁R (O’Dowd et al., 2012). Our results show that both D₂SR and D₂LR can couple with the D₁R to mobilize calcium (Fig. 1), and we have found that this is also true for both human (data not shown) and rat DARs. We have also confirmed that both receptors must be expressed in the same cell and co-activated to induce a calcium response in HEK293T cells.

Our data also suggest that G₉ protein signaling may play a role in the calcium response elicited by the D₁-D₂ complex. This was demonstrated by observing increased calcium mobilization in response to DA in cells transfected with the D₁R and D₂R plus G₉α. However, we also observed that the D₁R alone may couple to G₉α when the alpha subunit is expressed in significantly high amounts. This is likely due to the D₁R having a relatively low affinity for G₉α, however, it may activate G₉-mediated calcium mobilization under conditions where G₉ expression is very high. This is also supported by the enhanced calcium response we observe when the D₁R and D₂R are co-expressed in the presence of high levels of G₉ protein, where the D₁R is the protomer within the heteromer that likely activates G₉α (Rashid et al., 2007b). In this model, it is hypothesized that the D₂R allosterically modulates the D₁R (Hasbi et al., 2011; Rashid et al., 2007b). We believe, however, that the enhanced calcium mobilization seen in the D₁R+D₂R+G₉α transfected cells is not solely due to D₁R monomer activation of G₉, as the degree of calcium mobilization (300% of control, Fig. 5A) is twice that seen in the D₁R-G₉α transfected cells (Fig. 5B). Interestingly, another study has also reported D₁R-mediated calcium release from internal stores in mouse Ltk- cells transfected with the human D₁R (Liu et al., 1992), indicating that this is not an event particular to our experimental paradigm. Thus, while G₉ may play a role in the apparent ability of the D₁-D₂ heteromer to couple to calcium signaling, this may be dependent on the level of G₉ protein expression, either on a total cellular basis, which would thus be cell-type dependent, or this signaling may be localized to specific membrane microdomains (see below).

It has also been suggested that SKF83959 may act as a D₁-D₂ heteromer-selective agonist, and it has been used as a putative heteromer-selective probe in vivo. However, these
studies are not without controversy as SKF83959 has a history of unusual pharmacology. Undie et al. found that SKF83959 inhibited D1R-stimulated cAMP formation and also induced striatal intracellular calcium mobilization in rats and monkeys (Panchalingam and Undie, 2001). It lacked the side effects typical to D1R agonists that stimulate cAMP production but paradoxically seemed to cause typical D1R agonist-like behaviors in rats (Perreault et al., 2010), and is an effective anti-parkinsonian agent in MPTP-lesioned monkeys unresponsive to L-DOPA (Andringa et al., 1999). In our hands SKF83959 did not stimulate a calcium response in cells transfected with both the D1R and D2R, despite the fact that it was active in binding to the D1R. In fact, it appeared to act as an antagonist of the DA-stimulated calcium response in D1R and D2R co-transfected cells. In contrast, when Gqα was over-expressed, SKF83959 stimulated a calcium response in cells co-transfected with the D1R and Gqα as well as cells co-transfected with the D1R, D2R and Gqα. However, we observed that while the D1R-selective antagonist, SCH23390, completely blocked the SKF83959-stimulated calcium response in both transfection conditions, the D2R-selective antagonist, sulpiride, was ineffective in the D1R and D2R co-transfection condition. This contrasts with sulpiride’s ability to completely block DA-stimulated calcium mobilization in the D1R and D2R co-transfected cells (cf. Figs. 2B and 6B). This suggests that SKF83959 is not activating the D1-D2 heteromer, but rather is activating only D1R monomers that exist in the D1R and D2R co-transfected cells. This could be explained by the functionally-selective, or biased agonism, properties of SKF83959 in that it can selectively activate D1R-Gq signaling, provided that there is sufficient Gqα present, but our current results do not support its ability to activate the D1-D2 heteromer.

It has also been proposed that D1-D2 heteromer activation via SKF83959 in vivo and in vitro results in increased calcium/calmodulin-dependent protein kinase IIα levels in the striatum and nucleus accumbens, further resulting in enhanced brain-derived neurotrophic factor expression and increased neuronal maturation and differentiation (Hasbi et al., 2009; Ng et al., 2010; Perreault et al., 2012b; Rashid et al., 2007a). Given that our experiments indicated that
SKF83959 could not induce \( \text{D}_1 \)-\( \text{D}_2 \) heteromer-selective calcium mobilization in a controlled cell environment, we conducted a single-point competition-binding screen against an array of forty-three GPCRs and additional signaling proteins (Table 1 and Supplemental Table 1). We observed that SKF83959 demonstrated considerably high affinity for multiple receptors and other signaling proteins, and we conducted secondary competition binding experiments on the ones for which it showed the highest affinity. Surprisingly, SKF83959 showed nanomolar affinities for many different GPCRs including several serotonergic, adrenergic, dopaminergic, and muscarinic receptor subtypes (Table 1). This, as well as our functional data, questions whether or not SKF83959 may be useful as a selective probe to study \( \text{D}_1 \)-\( \text{D}_2 \) heteromer, or even \( \text{D}_1 \)-like receptor signaling \textit{in vivo}.

Our data also suggested that calcium signaling through the \( \text{D}_1 \)-\( \text{D}_2 \) receptor complex is largely sensitive to \( \text{G}_i \) and \( \text{G}_s \) inhibition by PTX and CTX, respectively. This led us to investigate additional hypotheses for the mechanism of \( \text{D}_1 \)-\( \text{D}_2 \) calcium signaling. Recently, Kern et al. (2012) showed that the ghrelin receptor could hetero-oligomerize with the \( \text{D}_2 \)R. This heteromer induced calcium release from internal cellular stores in a PLC dependent and PTX-sensitive manner, and seemed to require \( \text{G}_{\beta\gamma} \) subunit activation. Previous studies have shown that GRK2 can bind to and sequester \( \text{G}_{\beta\gamma} \) subunits (Koch et al., 1994) and catalytically inactive GRK2 mutants that retain \( \text{G}_{\beta\gamma} \) binding have been used as tools to block \( \text{G}_{\beta\gamma} \) signaling without the complication of added receptor desensitization (Freedman et al., 1995; Koch et al., 1994). Our data demonstrated that the catalytically inactive GRK2 K220R mutant completely ablated the DA-stimulated calcium response in the \( \text{D}_1 \)-R and \( \text{D}_2 \)-R transfected cells while GRK2 c-term (a truncated GRK2 protein that only includes the \( \text{G}_{\beta\gamma} \) binding domain) largely decreased the calcium response. Since activated \( \text{G}_{\beta\gamma} \) subunits can stimulate PLC\( \beta \) activity (Camps et al., 1992), our results are consistent with the hypothesis that the DA-stimulated calcium response significantly involves \( \text{G}_{\beta\gamma} \) activation of PLC\( \beta \). Additionally, the N-terminal RGS domain of GRK2...
has been shown to facilitate weak GTPase-activating protein-like activity on G_q, inhibiting PLC activation. This may explain the difference in degree of calcium signal inhibition between the GRK2 K220R mutant and the truncated GRK2 c-term mutant (Carman et al., 1999). Therefore, the activation of PLC\(\beta\) may be G_{q\alpha} as well as G_{\beta\gamma} dependent and largely due to synergistic crosstalk between the D_1R and D_2R.

Fig. 10 represents several hypothetical signaling pathways for D_1-D_2 receptor-calcium signaling in HEK293 cells. Pathway A represents D_1-D_2 heterodimer activation of G_q leading to G_{q\alpha} activation of PLC\(\beta\), as has been hypothesized in the literature (Rashid et al., 2007b). Pathway B represents G_{\beta\gamma} activation of PLC\(\beta\), where free beta/gamma subunits could arise through activation of either G_s, G_i or G_q. Pathway C represents co-activation of D_1R and D_2R monomers and cross-talk between G_s and G_i protein-mediated downstream signaling pathways ultimately leading to PLC\(\beta\) activation. Given that PTX and CTX can nearly eliminate the DA-stimulated calcium signaling, we believe that Pathway A is largely inoperative in our system under basal conditions. Pathway C could readily account for the requirement for dual receptor activation, but the fact that G_{\beta\gamma} sequestration largely eliminates the DA calcium response would suggest that Pathway B is critically important. The PTX/CTX results further implicate G_s or G_i, however, the requirement for dual receptor activation in Pathway B is not completely clear. Certainly, additional work will be required to answer these questions, however, it is clear from these studies that D_1-D_2 receptors can dually activate calcium signaling through more than a single mechanism.

One additional consideration for D_1-D_2-calcium signaling, which does not necessarily exclude the possibility of heteromer formation, may involve aggregation of the two DARs and their associated proteins in lipid rafts. Lipid rafts are a well-known but poorly understood platform for modulating certain protein-protein interactions in neurons as well as affecting GPCR ligand sensitivity, membrane trafficking, and signaling (Allen et al., 2006; Björk and
Svenningsson, 2011; Celver et al., 2012; Kong et al., 2011; Korade and Kenworthy, 2008; Sebastião et al., 2011). Lipid rafts would readily enable crosstalk between the D1R and D2R, and could assist in the multi-faceted signaling profile of the D1-D2 receptor complex. In addition, differences in lipid raft composition, cell background, and assay detection may explain some of the differences observed between our data and the data generated by other groups. Despite the seeming complexity of the D1-D2 receptor signaling mechanisms, it may yet be useful to study how synergistic concurrent activation of the D1R and D2R may induce effects not seen when either receptor is expressed alone. This can be examined by co-expressing mutants of the the D1R and D2R which have been reported to be unable to form dimers (O’Dowd et al., 2012), and studying the effect of co-activation on the generation of a calcium signal. Additionally, a compound that can selectively bias both receptors towards a conformation that promotes PLC activation may be useful in providing a clearer understanding of the DAR system in vivo.
**Authorship Contributions**
Participated in research design: Chun, Free, Doyle, Sibley, Rankin, Huang
Conducted experiments: Chun, Doyle, Rankin, Huang
Contributed new reagents or analytic tools: Free
Performed data analysis: Chun, Free, Doyle, Rankin, Huang
Wrote or contributed to the writing of the manuscript: Chun, Free, Sibley, Rankin, Huang
References:


Legends for Figures

Figure 1. Agonist-induced calcium mobilization in DA receptor transfected cells.

HEK293T cells were transiently transfected with D1R, D2LR, D2SR, D1R+D2LR, or D1R+D2SR as indicated and described in the methods. 24 hr later, cells were plated in 384-well plates and assayed the following day for calcium mobilization following stimulation by DA (D1R+D2LR EC50=73.8 nM, D1R+D2SR EC50=58.2 nM). Data are representative of three independent experiments done with the same assay conditions on different days. Data are expressed as percentage of control, normalized to the maximum signal seen via DA stimulation of D1R+D2LR transfected cells. Error bars indicate S.E.M. from multiple wells within the representative experiment.

Figure 2. Inhibition of D1R+D2LR-mediated calcium mobilization by either D1R- or D2R-selective antagonists.

HEK293T cells were transfected with D1R+D2LR as described and 24 hr later were plated in 384-well plates. Cells were incubated with the indicated concentrations of the D1R-selective antagonist SCH23390 (A) or the D2R-selective antagonist sulpiride (B), and then stimulated with an ~EC80 of DA (1 μM; SCH23390 IC50=8.0 nM, sulpiride IC50=0.7 nM). Data are expressed as a percentage of the control (10 μM) DA response and are representative of two independent experiments performed with the same assay conditions on different days. Error bars indicate S.E.M. from multiple wells within the representative experiment.

Figure 3. Stimulation of D1R+D2LR-mediated calcium mobilization by either D1R- or D2R-selective agonists.

HEK293T cells were transfected with D1R+D2LR as described, plated 24 hr later in 384-well plates, and assayed for calcium accumulation the following day. Cells were stimulated with one of the following agonists as indicated: DA, the D1R-selective agonist SKF83822, the D2R-selective agonist quinpirole, or both SKF83822 and quinpirole (D1R+D2LR EC50=610.8 nM) combined. Control cells expressing the D1R, D2SR, or D2LR individually did not
show a significant calcium response to concurrent agonist administration. Data are expressed as a percentage of control maximum DA stimulated response and are representative of two independent experiments performed with the same assay conditions on different days. Error bars indicate S.E.M. from multiple wells within the representative experiment.

**Figure 4. Pharmacological characterization of SKF83959 on D1R+D2LR-mediated calcium mobilization.** HEK293T cells were transfected with D1R+D2LR as described, plated 24 hr later in 384-well plates, and assayed for calcium accumulation the following day. A; Cells were stimulated with one of the following conditions as indicated: DA, SKF83959, the D2R-selective agonist quinpirole, or both SKF83959 and quinpirole combined. B; Cells were incubated with SKF83959 or the D1R-selective antagonist SCH23390, then stimulated with an ~EC₈₀ of DA (1 μM). Data are expressed as a percentage of control maximum DA-stimulated response and are representative of 2-3 independent experiments performed with the same assay conditions on different days. Error bars indicate S.E.M. from multiple wells within the representative experiment. C; HEK293 cells stably transfected with D1R (Codex Biosolutions, Inc., Gaithersburg, MD) were grown and membranes harvested according to the materials and methods. Membranes were incubated with various concentrations of SKF83959 and 0.5 nM [³H]-SCH23390 as indicated. Graph is representative of two independent experiments done on different days. Data are expressed as specific binding in units of fmol/mg. Kᵢ value was calculated using the Cheng-Prushoff equation and a radioligand Kᵢ value of 0.5 nM as determined via saturation binding isotherms (data not shown). Average Kᵢ for SKF83959 on D₁R was 2.6 nM ± 0.7.

**Figure 5. Influence of G₉₉ protein over-expression on D₁R+D₂LR-mediated calcium mobilization.** A; HEK293T cells were transfected with D₁R+D₂LR with and without G₉₉, or with G₉₉ alone (D₁R+D₂R EC₅₀=168.3 nM, EC₉₀=100%; D₁R+D₂R+G₉₉ EC₅₀=16.8 nM,
EC<sub>max</sub>=300.1%). B; HEK293T cells were transfected with D<sub>1</sub>R+D<sub>2</sub>L,R, D<sub>1</sub>R, or D<sub>2</sub>R with and without G<sub>qα</sub> (D<sub>1</sub>R+G<sub>q</sub> EC<sub>50</sub>=10.3 nM, EC<sub>max</sub>=152.2%). 24 hr later cells were plated in 384-well plates and assayed the following day for calcium mobilization following stimulation by the indicated concentrations of DA. Data are expressed as a percentage of control maximum DA stimulation for D<sub>1</sub>R+D<sub>2</sub>L,R alone and are representative of 2-3 independent experiments performed with the same assay conditions on different days. Error bars indicate S.E.M. from multiple wells within the representative experiment.

Figure 6. SKF83959 stimulates D<sub>1</sub>R-dependent calcium mobilization in the presence of G<sub>qα</sub>. HEK293T cells were transfected with D<sub>1</sub>R+D<sub>2</sub>L,R, G<sub>qα</sub>, or with G<sub>qα</sub> alone as described, plated 24 hr later in 384-well plates, and assayed for calcium accumulation the following day. A; Cells were stimulated with SKF83959. The line at 100% denotes the maximal DA response of D<sub>1</sub>R+D<sub>2</sub>L,R cells. B; Cells were incubated with the D<sub>1</sub>R-selective antagonist SCH23390 (1 μM) or the D<sub>2</sub>R-selective antagonist sulpiride (1 μM), and then stimulated with an ~EC<sub>80</sub> of SKF83959 (100 nM). Error bars indicate S.E.M. from multiple wells within the representative experiment, which was replicated twice with similar results.

Figure 7. G protein dependency of D<sub>1</sub>R+D<sub>2</sub>L,R-mediated calcium mobilization. HEK293T cells were transfected with D<sub>1</sub>R+D<sub>2</sub>L,R. Cells were incubated over night in 1 μg/mL pertussis toxin (PTX) or 1 μg/mL cholera toxin (CTX). 48 hr post-transfection, cells were assayed for calcium mobilization via stimulation with the indicated concentrations of DA (CTX EC<sub>max</sub>=14%, inhibition=86% control, PTX EC<sub>max</sub>=24%, inhibition=76% control). Data are expressed as a percentage of control maximum DA stimulation seen in untreated D<sub>1</sub>R+D<sub>2</sub>L,R cells, and are representative of 2-3 independent experiments performed with the same assay conditions on different days. Error bars indicate S.E.M. from multiple wells within the representative experiment.
Figure 8. Dopamine does not elicit a calcium response in cells co-expressing the D₁R and D₄R. HEK293T cells were transiently transfected with D₁R+D₂LR or D₁R+D₄R, as indicated and described in the Materials and Methods. 24 hr later cells were plated in 384-well plates and assayed for calcium mobilization through stimulation by the indicated concentrations of DA. Data are expressed as a percentage of control maximum DA stimulation seen in cells transfected with D₁R+D₂LR only (EC₅₀ = 162.0 nM), and are representative of 2-3 independent experiments done with the same assay conditions on different days. Expression of the D₄R was confirmed using radioligand binding assays as described in the Materials and Methods and was similar to that of the D₂R. Error bars indicate S.E.M. from multiple wells within the representative experiment.

Figure 9. GRK2 influence on DA-mediated D₁R+D₂LR calcium mobilization. HEK293T cells were transiently transfected with D₁R+D₂LR, and either empty pcDNA vector the GRK2 catalytically inactive mutant GRK2 K220R (A; D₁R+D₂R EC₅₀=269.1 nM), or the GRK2 C-terminal 495-689 fragment (B; GRK2 c-term; D₁R+D₂R EC₅₀=90.4 nM, Eₘₐₓ=100% control; D₁R+D₂R+pcDNA EC₅₀=188.5 nM, Eₘₐₓ=106%; D₁R+D₂R+GRK2 c-term EC₅₀=288.1 nM, Eₘₐₓ=30% control, 70% inhibition), as indicated and described in the methods. 24 hr later cells were plated in 384-well plates and assayed following day for calcium mobilization following stimulation by the indicated concentrations of DA. Data are expressed as a percentage of control maximum DA stimulation seen in cells transfected with D₁R+D₂LR only, and are representative of 2-3 independent experiments done with the same assay conditions on different days. Error bars indicate S.E.M. from multiple wells within the representative experiment.
Figure 10. Various mechanisms of PLCβ activation that may occur when the D1R and D2R are co-expressed and co-activated.
### Tables

#### Table 1: SKF93959 competition binding experiments against various GPCRs.

<table>
<thead>
<tr>
<th>Target</th>
<th>SKF83959 $K_i$ (nM)</th>
<th>S.E.M.</th>
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<tbody>
<tr>
<td>5-HT1A</td>
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<td>352.3</td>
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<tr>
<td>5-HT2A</td>
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<tr>
<td>5-HT2B</td>
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<td>145.1</td>
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<tr>
<td>5-HT5A</td>
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</tr>
<tr>
<td>5-HT6</td>
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<td>α1A</td>
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<td>154.5</td>
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<tr>
<td>α1D</td>
<td>1115.5</td>
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</tr>
<tr>
<td>α2A</td>
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<tr>
<td>D2R</td>
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<tr>
<td>SERT</td>
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$K_i$ values were derived from radioligand binding competition curves generated against each of the above targets (N=2) as described in the Materials and Methods section. Abbreviations are as follows: 5-HT1A, serotonergic receptor subtype 1A; 5-HT2A, serotonergic receptor subtype 2A; 5-HT2B, serotonergic receptor subtype 2B; 5-HT2C, serotonergic receptor subtype 2C; 5-HT5A, serotonergic receptor subtype 5A; 5-HT6, serotonergic receptor subtype 6; α1A, α-adrenergic receptor subtype 1A; α1D, α-adrenergic receptor subtype 1D; α2A, α-adrenergic receptor subtype 2A; α2B, α-adrenergic receptor subtype 2B; α2C, α-adrenergic receptor subtype 2C; M4, muscarinic receptor subtype 4; M5, muscarinic receptor subtype 5; SERT, serotonin transporter.
Figure 4
Figure 5

A

Calcium Mobilization (% Control) vs. Dopamine (M)

B

Calcium Mobilization (% Control) vs. Dopamine (M)

Legend:
- $D_1R + D_2LR$
- $D_1R + D_2LR + G_q$
- $G_q$
- $D_1R$
- $D_1 + G_q$
- $D_2LR$
- $D_{2L} + G_q$
Figure 6

A

![Graph showing changes in calcium mobilization](image)

B

![Bar chart comparing SKF83959 effects](image)
Figure 7

[Graph showing calcium mobilization (% control) against dopamine (M) for different conditions: D1R+D2LR, D1R+D2LR, PTX, and D1R+D2LR, CTX.]
Figure 8

The graph shows the calcium mobilization (% Control) in response to different concentrations of dopamine. The x-axis represents the concentration of dopamine (M), ranging from $10^{-10}$ to $10^{-5}$. The y-axis represents calcium mobilization (% Control) ranging from 0 to 125.

Two conditions are compared:

- **D$_1$R+D$_2$R**: Represented by filled squares. The calcium mobilization increases significantly with increasing dopamine concentrations. The curve is upward-sloping, indicating a positive correlation.

- **D$_1$R+D$_4$R**: Represented by open circles. The calcium mobilization remains relatively constant across the range of dopamine concentrations, indicating little to no effect.

The error bars indicate the variability of the data points.
Supplemental Materials

D$_1$-D$_2$ Dopamine Receptor Synergy Promotes Calcium Signaling via Multiple Mechanisms

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Running Title:

D1-D2 Receptor Synergy and Ca^{2+} Signaling

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Abbreviations:

Co-IP: Co-immunoprecipitation
D_{1}R: D_{1} dopamine receptor subtype
D_{2}R: D_{2} dopamine receptor subtype
D_{2L}R: D_{2} long splice variant
D_{2S}R: D_{2} short splice variant
DA: Dopamine
DAG: Diacylglycerol
DAR: Dopamine receptor
EC50: 50% excitatory concentration
GRK2: G protein receptor kinase 2
GPCR: G protein-coupled receptor
IC_{50}: 50% inhibitory concentration
PKC: Protein kinase C
PLC: Phospholipase C
Supplemental Table 1: List of various GPCR targets with low/no affinity for SKF83959.

5-HT1B
5-HT1D
5-HT1E
5-HT3
α1B
β1
β2
β3
BZP Rat brain
DAT
DOR
GABAA
H1
H3
H4
KOR
M1
M2
M3
MOR
NET
σ1
σ2

Receptors showed less than 50% inhibition of binding by 10 μM SKF83959, and therefore were classified as having low to no affinity for the compound. Percent inhibition was calculated by subtracting percent specific binding in the presence of the test compound from the percent specific binding in the absence of the test compound, (N=4). The National Institute of Mental Health’s Psychoactive Drug Screening Program, as referenced in Table 1, generously provided the binding profiles. Abbreviations are as follows: 5-HT1B, serotonergic receptor subtype 1B; 5-HT1D, serotonergic receptor subtype 1D; 5-HT1E, serotonergic receptor subtype 1E; 5-HT3, serotonergic receptor subtype 3; α1B, α-adrenergic receptor subtype 1B; β1, β-adrenergic receptor subtype 1; β2, β-adrenergic receptor subtype 2; β3, β-adrenergic receptor subtype 3; BZP Rat Brain Site: allosteric benzodiazepine binding site on GABA<sub>A</sub> receptor; DAT, dopamine transporter; DOR, δ-opioid receptor; GABA<sub>A</sub>, ionotropic GABA receptor; H1, histamine receptor.
subtype 1; H3, histamine receptor subtype 3; H4, histamine receptor subtype 4; KOR, κ-opioid receptor; M1, muscarinic receptor subtype 1; M2, muscarinic receptor subtype 2; M3, muscarinic receptor subtype 3; MOR, μ-opioid receptor; NET, norepinephrine transporter; σ1, sigma receptor subtype 1; σ2, sigma receptor subtype 2
Supplementary Figure 1: Co-immunoprecipitation of the D₁R and D₂L R. Small arrows indicate the location of the D₂L R in lane 7 and the D₁R in lanes 3 and 5. The filled arrow indicates a non-specific background band as it is observed in non-transfected cells. HEK293T cells were transfected with either the FLAG-tagged D₂L R, the non-tagged D₁R, the D₁R with a vector that expressed only the FLAG peptide (Tag2B), or the D₁R with the FLAG-tagged D₂L R, as indicated on the blots. Proteins were extracted and lysates were either electrophoresed or immunoprecipitated (IP) using anti-FLAG agarose beads, and immunoblotted (IB) as indicated in the Supplemental Materials and Methods section. IBs were probed using either a D₂L/S R primary antibody or a D₁R primary antibody. Lanes 1, 3, and 6 were loaded with whole cell lysate, while the remaining lanes underwent IP prior to being loaded on the gel.

In this experiment, the D₂R is immunoprecipitated using an anti-FLAG antibody and appears in lane 7 as multiple glycosylated protein bands. The D₁R appears as a single glycosylated
protein band of ~60 kDa that is co-immunoprecipitated with the D$_2$R, as shown in lane 5. In contrast, the D$_1$R does not co-immunoprecipitate with a peptide containing just the FLAG sequence (Tag2B), as shown in lane 4.
Supplemental Materials and Methods:

Cell Culture and Transfection—HEK293T cells were cultured in Dulbecco’s modified essential medium (DMEM) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 50 units/mL penicillin, 50 μg/mL streptomycin, and 10 μg/mL gentamycin. Cells were grown at 37°C in 5% CO₂ and 90% humidity. Untagged rat D₁R (Monsma et al., 1990) in pcDNA3 and FLAG-tagged rat D₂L R (Namkung and Sibley, 2004) were expressed singly or dually in HEK293T cells. Tag2B in pCMV (Stratagene, La Jolla, CA) was used as a negative control for FLAG-tagged D₂L R. HEK293T cells were transfected using the calcium phosphate precipitation method for each experiment as follows: 5 million cells were seeded in 150 mm culture dishes, then transfected 24 hr later with 15 μg of DNA of expression construct. After 24 hr, the media was exchanged and experiments performed the following day.

Immunoprecipitation and Gel Electrophoresis—Cells were removed from culture dishes and collected by centrifugation (300 × g). They were then resuspended in 1 mL of solubilization buffer (50 mM HEPES, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 150 mM NaCl, 50 mM NaF, 40 mM sodium pyrophosphate, and Complete-Mini; Roche Applied Science, Indianapolis, IN) protease inhibitor mixture and incubated on an orbital shaker at 4°C for 1 hr. The lysates were transferred to 1.5 mL Axygen tubes and microfuged at 26,000 × g for 40 min. The lysates were pre-cleared using Protein G-agarose beads on an orbital shaker at 4°C for 3 hr then microfuged for 5 min at 1000 × g. Lysates transected with FLAG-D₂L R were transferred to 100 μL of anti-FLAG M2-agarose beads (Sigma-Aldrich, St. Louis, MO) equilibrated in solubilization buffer and rotated on an orbital shaker at 4°C overnight. Lysates of untransfected cells or cells transfected with non-tagged D₁R were removed from the Protein G-agarose pellets and stored at 4°C overnight. The anti-FLAG M2-agarose beads were collected via centrifugation and washed three times by resuspension and recentrifugation in solubilization buffer. The agarose
was then subjected to a final wash in 1× TE buffer, pH 7.4 plus protease inhibitors. Proteins were eluted from the beads using 70 μL of NuPAGE-lithium dodecyl sulfate sample buffer (Invitrogen, Carlsbad, CA) at 37°C for 1 hr, then microfuged at 2500 × g for 2 min to remove agarose beads and 30 μL of each supernatant was loaded on duplicate gels. 20 μL of lysate from non-transfected cells or cells transfected with nontagged D1R was mixed with 40 μL of LDS buffer, mixed, then divided in half and loaded in duplicate gels. Proteins were resolved on duplicate 4–12% BisTris NuPAGE gels in MOPS buffer (Invitrogen, Carlsbad, CA) for 1 hr at 195 V, constant voltage.

**Western Blotting and Chemiluminescent Development**—Proteins separated by PAGE were transferred onto polyvinylidene difluoride membranes for 1 hr at 30 V constant voltage. Membranes were blocked in blocking solution (1% BSA, 0.01% Tween 20 in TBS) for 1 hr at room temperature prior to incubation with the primary antibody. Primary antibodies used in this study include the following: rat monoclonal anti-D1R dopamine receptor (clone 1-1-F11 S.E6, catalog number D-187, Sigma-Aldrich, St. Louis, MO) and rabbit polyclonal anti-D2L/SR (catalog number AB5084P, Chemicon, Temecula, CA). Primary antibody solutions were diluted 1:10,000 for D1R primary and 1:1000 for D2L/SR primary in blocking solution. Each PVDF membrane was exposed to a single primary antibody solution (D1R or D2L) overnight at 4°C on orbital shaker. Primary antibody solutions were removed and each membrane was washed three times, 5 min each in TBST. 1:10,000 dilutions in blocking solution were prepared for each HRP-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA; anti-rat for the D1R and anti-rabbit for the D2L). Membranes were incubated in appropriate secondary antibody solution for 1 hr at room temperature on an orbital shaker. Membranes were washed three times, 5 min each in TBST. Proteins were visualized via the SuperSignal West Pico Chemiluminescent Substrate Kit or the SuperSignal West Dura Chemiluminescent Substrate (Pierce, Rockford, IL) according to the manufacturer's instructions, and images were recorded on film.
Supplemental References:
