Supplemental Materials

D_1-D_2 Dopamine Receptor Synergy Promotes Calcium Signaling via Multiple Mechanisms

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Running Title:

D_1-D_2 Receptor Synergy and Ca^{2+} Signaling

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Abbreviations:
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Co-IP: Co-immunoprecipitation
D_1R: D_1 dopamine receptor subtype
D_2R: 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

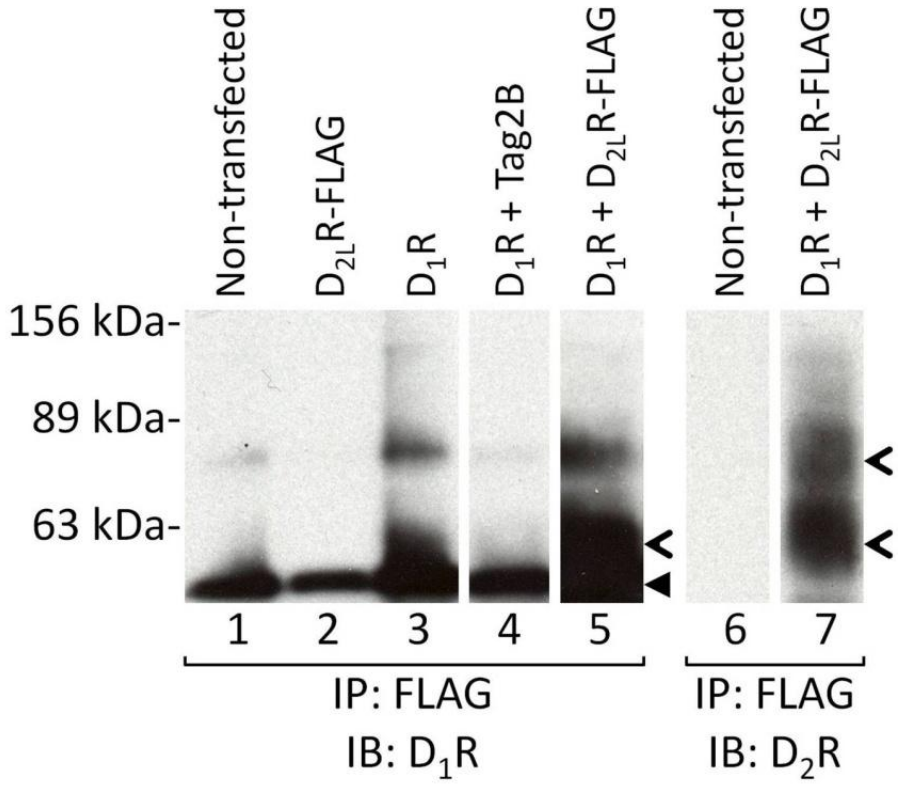
Supplemental Table 1: List of various GPCR targets with low / no affinity for SKF83959.

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 compund, (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 $GABA_A$ receptor; DAT, dopamine transporter; DOR, δ-opioid receptor; $GABA_A$, 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

Supplemental Figure 1:



Supplementary Figure 1. Co-immunoprecipitation of the D_1R and $D_{2L}R$. Small arrows indicate the location of the $D_{2L}R$ in lane 7 and the D_1R 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 $D_{2L}R$, the non-tagged D_1R, the D_1R with a vector that expressed only the FLAG peptide (Tag2B), or the D_1R with the FLAG-tagged $D_{2L}R$, 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 $D_{2L/S}R$ primary antibody or a D_1R 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 D_2R is immunoprecipitated using an anti-FLAG antibody and appears in lane 7 as multiple glycosylated protein bands. The D_1R appears as a single glycosylated

protein band of ~60 kDa that is co-immunoprecipitated with the D_2R, as shown in lane 5. In contrast, the D_1R 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% CO_2 and 90% humidity. Untagged rat D_1R (Monsma et al., 1990) in pcDNA3 and FLAG-tagged rat $D_{2L}R$ (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 $D_{2L}R$. 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 $\times$ *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 $\times$ 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 $\times$ g. Lysates transfected with FLAG-$D_{2L}R$ 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 D_1R 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_x 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 $_x$ 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 D_1R 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-D_1R dopamine receptor (clone 1-1-F11 S.E6, catalog number D-187, Sigma-Aldrich, St. Louis, MO) and rabbit polyclonal anti-$D_{2L/S}R$ (catalog number AB5084P, Chemicon, Temecula, CA). Primary antibody solutions were diluted 1:10,000 for D_1R primary and 1:1000 for $D_{2L/S}R$ primary in blocking solution. Each PVDF membrane was exposed to a single primary antibody solution (D_1R or $D_{2L}R$) 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 D_1R and anti-rabbit for the $D_{2L}R$). 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:

Monsma, F.J., Mahan, L.C., McVittie, L.D., Gerfen, C.R., and Sibley, D.R. (1990). Molecular cloning and expression of a D1 dopamine receptor linked to adenylyl cyclase activation. PNAS *87*, 6723–6727.

Namkung, Y., and Sibley, D.R. (2004). Protein kinase C mediates phosphorylation, desensitization, and trafficking of the D2 dopamine receptor. J. Biol. Chem. *279*, 49533–49541.