KI-09-13-1439

ONLINE SUPPLEMENT

The Cooperative Roles of the Dopamine Receptors, D_1R and D_5R, on Renal Sodium Transport Regulation

[1]John J. Gildea, [1]Ishan T. Shah, [1]Jonathan A. Israel, [2]Christoph Enzensperger, [1]Robert Van Sciver, [1]Helen E. McGrath, [3]Pedro A. Jose, and [1]Robin A. Felder

[1]The University of Virginia Health System, Department of Pathology, Charlottesville, VA
[2]Institut für Pharmazie, Lehrstuhl für Pharmazeutische/Medizinische Chemie, FriedrichSchiller-Universität Jena, Jena, Germany
[3]University of Maryland School of Medicine, Departments of Medicine and Physiology, Baltimore, MD

Short title: Cooperative role of dopamine D_5R in sodium transport

Correspondence to be sent to:

Robin A. Felder, Ph.D.
The University of Virginia
P.O. Box 801400
Charlottesville, VA 22908
rfelder@virginia.edu
Ph 434-466-1131
Fax 434-924-5718

Expanded Materials and Methods

Immunofluorescent Staining of Human and Rat Renal Cortex for D_1R or D_5R Localization:

Kidneys from human and Sprague-Dawley rat were removed and decapsulated and the outer 1 mm of cortical tissue was excised and washed in PBS 3 times. The tissues were fixed with 4% paraformaldehyde in PBS for 2 hours at room temperature, and the fixative was then inactivated with three, 5 minute washes in TBS (20 mmol/L Tris pH 7.4, 150 mmol/L NaCl). The tissues were embedded in OCT and snap frozen in liquid nitrogen. Eight-micron frozen sections were cut using a Leica cryostat, attached to superfrost plus slides (Fisher) and allowed to dry for 10 minutes. The slides were rehydrated in PBS, permeablized with 1% SDS for 5 minutes, and washed three times in PBS-T (PBS plus 0.2% Tween 20) before the slides were blocked overnight using 2% nonfat dry milk. The antibodies used for co-localization were directly labeled with Alexa 488 (D_1R) and Alexa 555 (D_5R) and diluted 1:100 in blocking buffer. Antibodies were incubated for 2 hours at room temperature and washed 5 times in PBS-T and mounted in Fluoromount G.

Live Sensitized Emission FRET Measurements:

Images were captured under identical conditions and the automated spectral bleedthrough calculations performed according to the Slidebook 4.2 FRET Module macro. In the live HEK293 FRET experiments, the cells were transfected with D_5R or D_1R cDNA alone or together and were captured using the CyPet (excitation filter 427/10 nm – emission filter 472/30 nm), Ypet (excitation filter 504/12 nm – emission filter 542/27 nm), and FRET channels (excitation filter 427/10 nm – emission filter 542/27 nm). The spectral bleedthrough was automatically subtracted using the bleedthrough correction FRET macro. The cells were also transfected separately with D_1R and D_5R and the next day were mixed together into the same well to test that the algorithm works as expected.

Immunofluorescent Staining of Human RPTCs for Cell Surface D_1R or D_5R and Fixed Cell Sensitized Emission FRET Measurements:

Controls for non-specific fluorescent binding and fluorescent secondary antibody staining were performed and threshold levels for background staining established. Exposure times were identical for each cell type across each individual antibody and the fluorescence levels shown in the image are above these established background levels. The specifics of the staining procedure were: cells were placed on collagen IV-coated glass bottom 96-well plates and washed twice with phosphate-buffered saline (PBS). They were fixed in PBS containing 1% paraformaldehyde for 2 minutes and washed with tris-buffered saline (TBS) three times for 5 minutes. The fixed cells were then blocked overnight in PBS containing 5% BSA in PBS. All samples were incubated for 1 hour in PBS 1% BSA at a 1:100 dilution with gentle rocking at room temperature, followed by washing (three times, 5 minutes each wash) in PBS. The cells were imaged using an Olympus IX81 automated multi-well spinning disk confocal microscope.

NHE3-mediated Sodium Influx Assay:

Cell and Drug Preparation*:*

RPTCs were cultured in 96-well glass bottom collagen-coated Matrical plates (Spokane, WA) at 37°C until they reached 50% confluence. The cells were serum-starved overnight prior to loading with a sodium ion indicator, sodium benzofuran isophthalate (SBFI, 5 µmol/L, Molecular Probes, Eugene, OR) with 0.04% Pluronic F-127 for 2 hours in PBS with calcium and magnesium. The cells were washed twice and allowed to recover at 37°C in serum-free media for 30 minutes. They were then washed two more times with PBS and incubated at room temperature. The cells were placed under the microscope and XYZ positions marked for imaging. A single baseline image was acquired, and then ouabain (100 µmol/L), a cardiac glycoside which increases intracellular sodium concentration by blocking sodium efflux via inhibition of NaKATPase activity, was added to each well. Vehicle or the drugs (as detailed in Table S1) were added into the wells in addition to the ouabain.

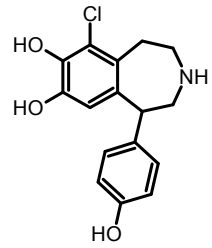
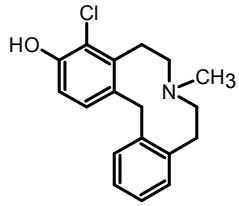
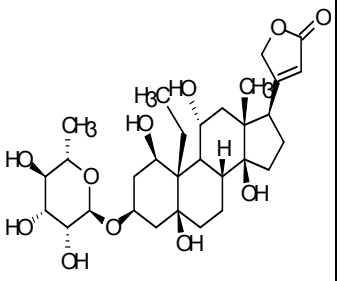
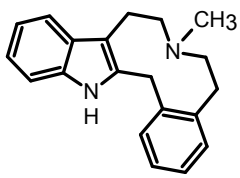
Microscopy:

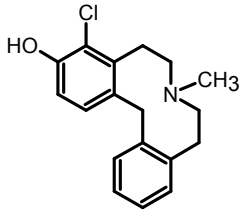
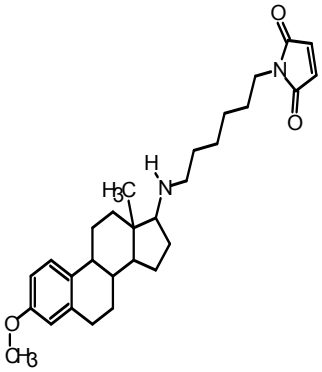
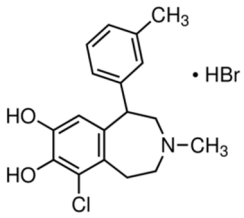
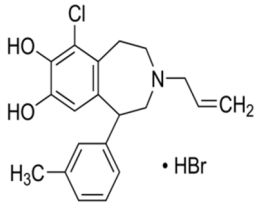
Images were collected on an Olympus IX-81 inverted epifluorescence microscope with a UV-specific 20x UAPO objective and Hamamatsu ER CCD camera. Slidebook Software package (Intelligent Imaging Innovations, Inc., Denver, CO) integrated all of the hardware, as well as provided for image acquisition and processing. An automated XY stage and piezo-Z allowed precise capture of images from multiple wells of a 96-well microplate under the same conditions. Time-lapse data were simultaneously captured from up to 12 wells per experiment, which allowed the comparison of data from several cell lines with several drug treatment combinations. Cells with sufficient SBFI were selected during image analysis in order to obtain quantifiable data. Cells with poor loading were deemed unhealthy and thus not representative of normal sodium transport activity. Less than 10% of cells in our experiments were poorly loaded with dye. Time-lapse ratiometric images were acquired every three minutes, starting from time 0 in 3 separate wells per group with 20 cells per well measured.

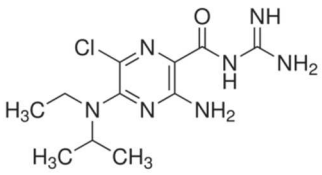
NaKATPase-mediated Sodium Efflux Assay:

Cells were loaded with a sodium ion indicator, sodium benzofuran isophthalate (SBFI, 5 µmol/L, Molecular Probes, OR), washed and incubated for 30 minutes in potassium-free HEPES media (20 mmol/L HEPES pH 7.4, 130 mmol/L NaCl, 1 mmol/L CaCl, 1 mmol/L MgCl) to raise the intracellular sodium concentration. The drugs, which were added at this point for 10 minutes, are listed in Table S1, with all details included there. Before imaging, EIPA (10 µmol/L final concentration, a selective inhibitor of the Na+/H+ exchanger 3 (NHE3)) and KCl (2.7 mmol/L final concentration) were added to all wells as 10x stock. Changes in intracellular sodium concentration were measured by live multiwell ratiometric fluorescence imaging of SBFI and internally calibrated using the ratio imaging module of Slidebook™ version 4.2 as is used in the sodium influx assay described above.

Table S1. Pharmacological Agents

Full Name	Abbreviation	Action	Source	Concentration
Fenoldopam mesylate 	FEN	D_1R/D_5R agonist	Corlopam, Hospira, Inc.	1 μmol/L
SKF38393 	SKF38393	D_1R/D_5R agonist	Sigma, D047	1 μmol/L
Ouabain 	OUB	NaKATPase inhibitor	Sigma, O3125	100 μmol/L
LE300 	LE	D_1R/D_5R antagonist	Tocris Bioscience, 1674	10 μmol/L

LE-PM436 	LE-PM436	D_5R selective antagonist	Christoph Enzensperger #	1 nmol/L
* U73122 	U73122	PLC inhibitor	EMD, 662035	10 µmol/L
SKF83959 	SKF83959	PLC-specific D_1R/D_5R activator	Sigma, S2816	1 µmol/L
SKF83822 	SKF83822	cAMP-specific D_1R/D_5R activator	Sigma SML0513	1 µmol/L

** EIPA 	EIPA	NHE3 Inhibitor	Sigma A3085	10 µmol/L

*1-[6-[[(17β)-3-Methoxyestra-1,3,5(10□)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione

**5-(N-ethyl-N-isopropyl) amiloride

The rationale for creating the first high affinity D_5R antagonist was previously described **(1)**. Briefly, LE300 (7-methyl-6,7,8,9,14,15-hexahydro-5*H*-indolo[3,2-*f*][3]benzazecine (**2**) is recognized as a potent D_1R antagonist in renal tissue. The dibenz[*g,j*]-1-oxa-4-azacycloundecenes, in which one benzene is substituted by one +M and −I substituent, showed some selectivity toward D_5R within the D_1-like receptor family. Therefore, the introduction of a chlorine atom as an additional +M and −I substituent into the substituted benzene ring of dibenz[*d,g*]azecines proved to be a successful strategy. Also the nonsubtype-selective D_1R/D_5R antagonist SCH 23390 ((5*R*)-8-chloro-3-methyl-5-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepin-7-ol) bears a chlorine substituent and a hydroxy group at the condensed benzene ring. 3-Hydroxy-7-methyl-5,6,7,8,9,14-hexahydrodibenz[*d,g*]azecine served as the starting material for the introduction of additional chlorine atoms into the hydroxy-substituted benzene ring by a reaction with sulfonyl dichloride in glacial acetic acid yielding the 4-chloro- and the 2,4-dichloro-substituted compounds 4-chloro-3-hydroxy-dibenz[*d,g*]azecine (LE-PM 436). This compound proved to be a subnanomolar ligand toward the D_1R (>30-fold selectivity $D_1R > D_2R$) with an even higher affinity toward D_5R, showing a K_i (D_5R) of 57 pM and therefore representing the most potent D_5R antagonist; 10−15-fold selectivity $D_5R > D_1R$ along with picomolar affinity.

1. Mohr P., Decker, M., Enzensperger C., and Lehmann J. 2006. Dopamine/serotonin receptor ligands. 12(1): SAR studies on hexahydro-dibenz[d,g]azecines lead to 4-chloro-7-methyl-5,6,7,8,9,14-hexahydrodibenz[d,g]azecin-3-ol, the first picomolar D5-selective dopamine-receptor antagonist. *Journal of Medicinal Chemistry*. 49(6): 2110-2116.

2. Höfgen, B.; Decker, M.; Mohr, P.; Schramm, A. M.; Rostom, S. A. F.; El-Subbagh, H.; Schweikert, P. M.; Rudolf, D. R.; Kassack, M. U.; Lehmann J. Dopamine/serotonin receptor ligands. 10:□ SAR studies on azecine-type dopamine receptor ligands by functional screening at human cloned D_1, D_{2L}, and D_5 receptors with a microplate reader based calcium assay lead to a novel potent D_1/D_5 selective antagonist. *J. Med. Chem.***2006**, *49*, 760−769.

FIGURE S1

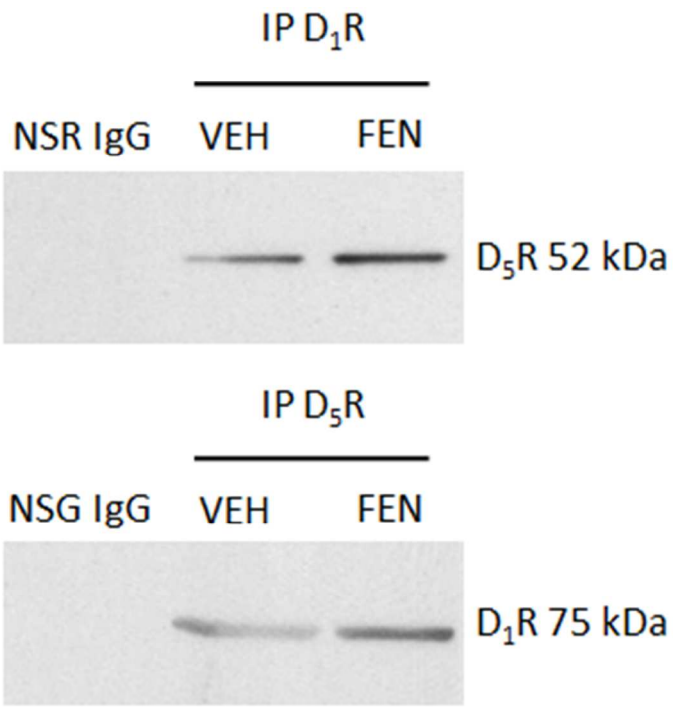


Figure S1. Increase in D_1R and D_5R Co-immunoprecipitation after D_1-like receptor agonist stimulation with fenoldopam (FEN). Renal proximal tubule cell lysates were immunoprecipitated with either a rabbit polyclonal anti-D_1R antibody or an equal amount of non-specific rabbit IgG (NSR IgG) and western blotted for D_5R using a goat polyclonal antibody. The reciprocal immunoprecipitation, using a goat polyclonal antibody against the D_5R or non-specific goat IgG (NSG IgG), and western blot using a D_1R rabbit polyclonal antibody is also shown in the lower blot. Adequate washing is evidenced by lack of immunoreactivity in the non-specific IgG lanes. An increase in immunoreactivity in the FEN lane suggests an increase in association after agonist stimulation.

FIGURE S2

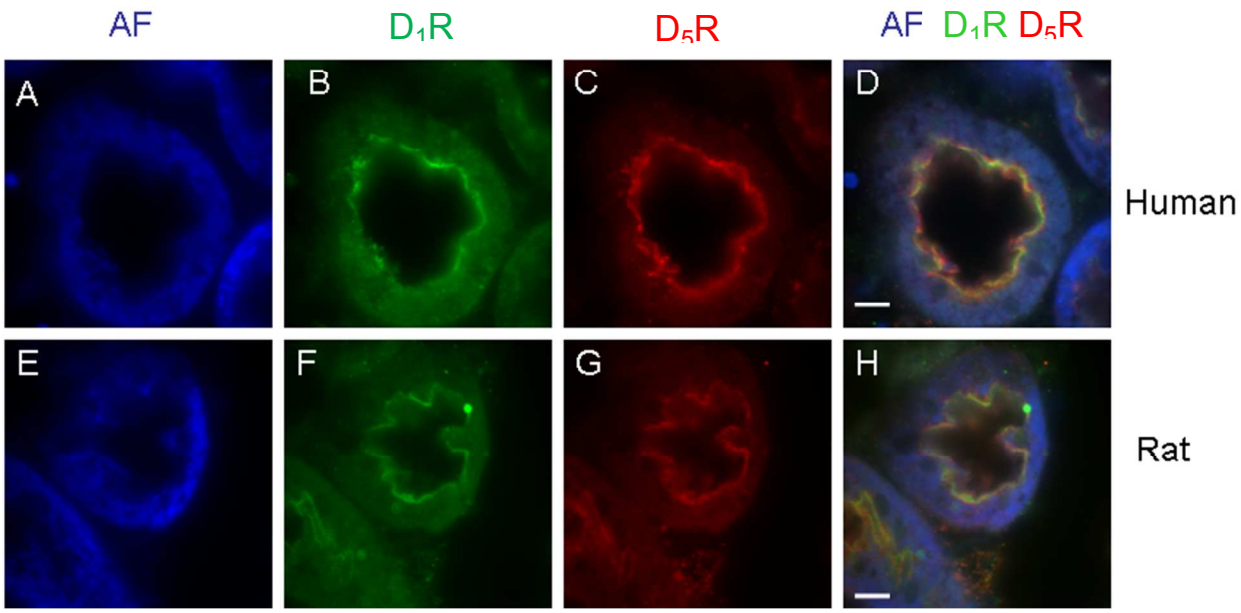


Figure S2. D_1R and D_5R Co-localization in Human and Rat Renal Proximal Tubules. Human (top row) and Sprague Dawley rat (bottom row) renal cortices were fixed and flash-frozen sections were used for immunofluorescent staining. The first column in blue (A and E) is an autofluorescent (AF) image used to delineate the transition between cytoplasm and brush border. The second column is the immunolocalization of the D_1R primarily in a subapical position (B and F) at the base of the microvilli (green). The third column is the immunolocalization of the D_5R also at a subapical area (C and G, red). The composite image shows the co-localization of the D_1R and D_5R precisely at the transition of cytoplasm and brush border (D and H, yellow). The white bar in D and H represents 10 microns.

FIGURE S3

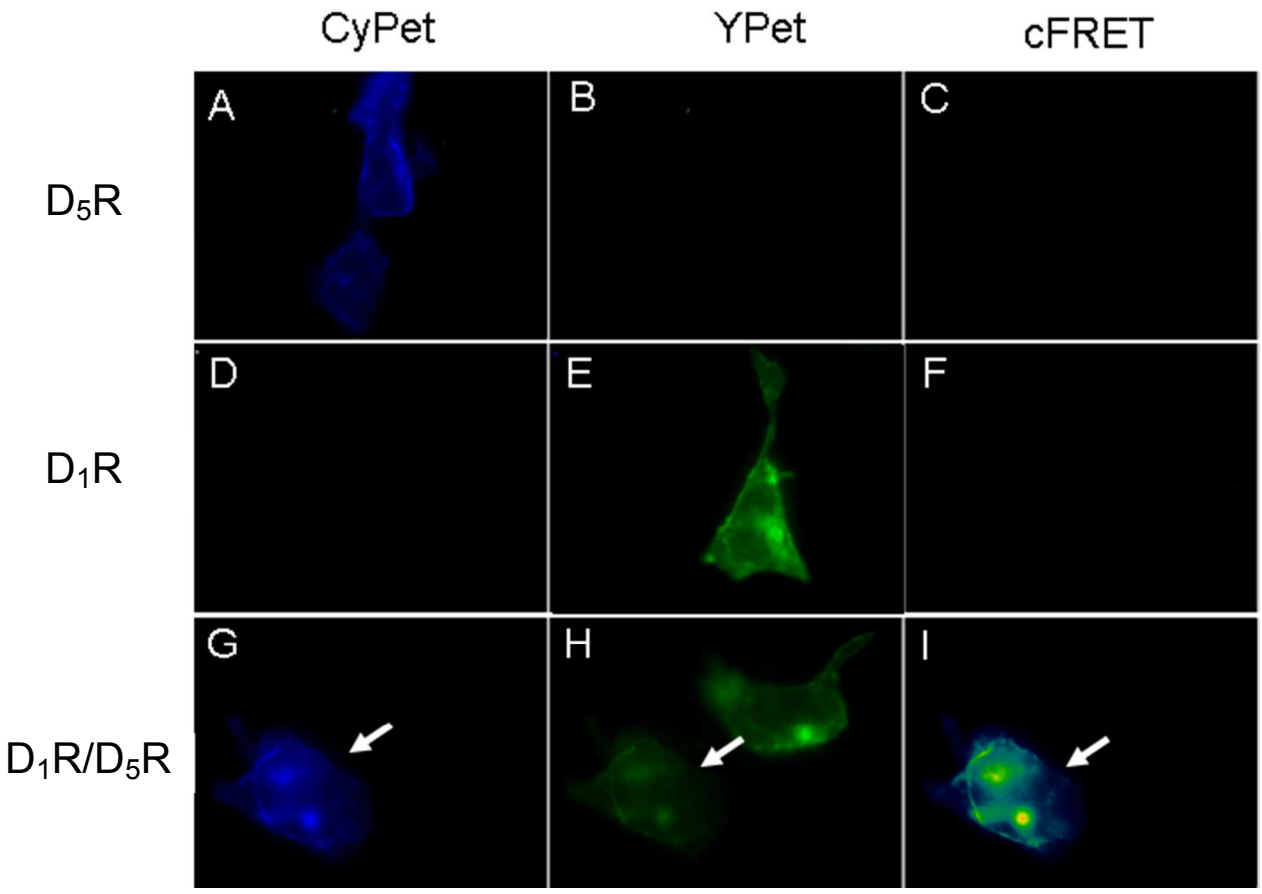


Figure S3. Live-cell FRET Measurement of D_1R -YPet and D_5R -CyPet Fusion Proteins in Transiently Transfected HEK293 Cells. The first row of images indicates that when D_5R-CyPet plasmid was transfected into HEK293 cells, a clear signal was measured in the CyPet channel (A); there was no spectral bleedthrough into the YPet channel (B) and there was no measurable signal in the corrected FRET (cFRET) channel (C), as expected. The second row of images indicates that the same was true for the D_1R-YPet-transfected HEK293 cells. There was a clear signal measured in the YPet channel (E); there was no spectral bleedthrough into the CyPet channel (D) and no measurable signal in the cFRET channel (F). In the bottom row, when the HEK293 cells were co-transfected with D_5R-CyPet and D_1R-Ypet, a clear signal was measured in the CyPet (G, arrow) and YPet channels (H, arrow), and a clear cFRET signal was detected only in the co-transfected cell (I, arrow). Images were collected using a 100x oil immersion objective lens.

FIGURE S4

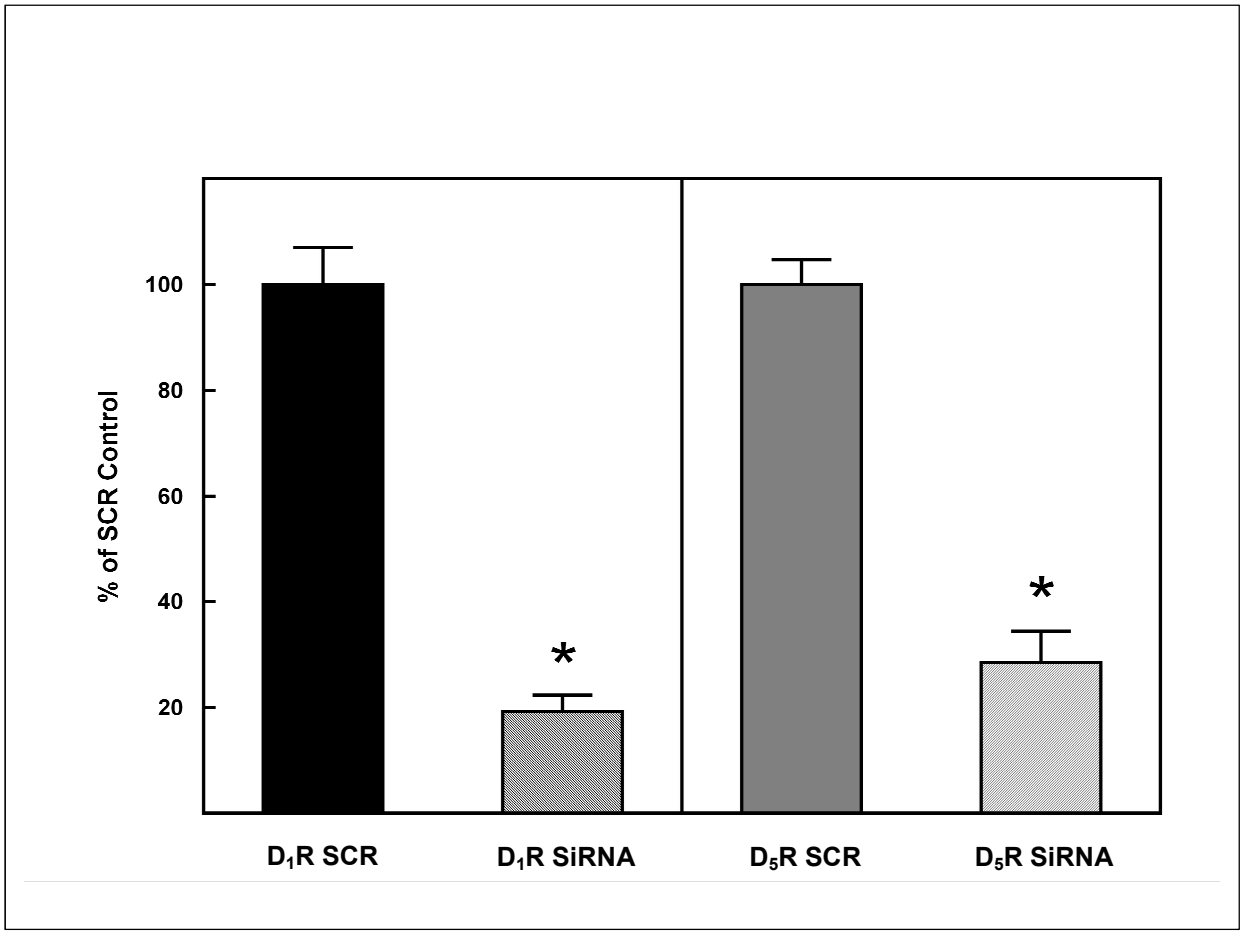


Figure S4. D_1R and D_5R Immunofluorescent Staining in RPTCs Transfected with D_1R and D_5R siRNA or Scrambled Control (SCR). Cells were transfected with either D_1R or D_5R specific siRNA (100 nmol/L) or SCR control and 48 hrs later were fixed and stained for either D_1R or D_5R. Both D_1R and D_5R siRNAs markedly decreased their respective expression levels compared to SCR control ($P<0.001$ vs SCR, N=4).