Supporting Information

Crossreceptor Interactions between Dopamine D_{2L} and Neurotensin NTS_1 Receptors Modulate Binding Affinities of Dopaminergics

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Figure 1. Emission spectra of the fluorescence detected co-immunoprecipitation of D_{2L}-NTS1 heteromer.

Immunoprecipitation with mouse anti-D_{2L}-antibody resulted in different intensities of the CFP emission spectra of the investigated probes. The negative controls of lysis buffer (A, black curve), the NTS1-CFP complex (B, green curve) and the co-expression of D_{2L} with NTS1 and cytosolic CFP (C, purple curve), exhibited no fluorescence due to the absence of a fluorophore or the lack of precipitation by the antibody or the combination of both circumstances.

The co-expression of D_{2L}-CFP and NTS1 (D, blue curve) transiently expressed in HEK293 cells displayed after IP with the anti-D_2-antibody the most intense CFP-emission spectra.

Figure 1.

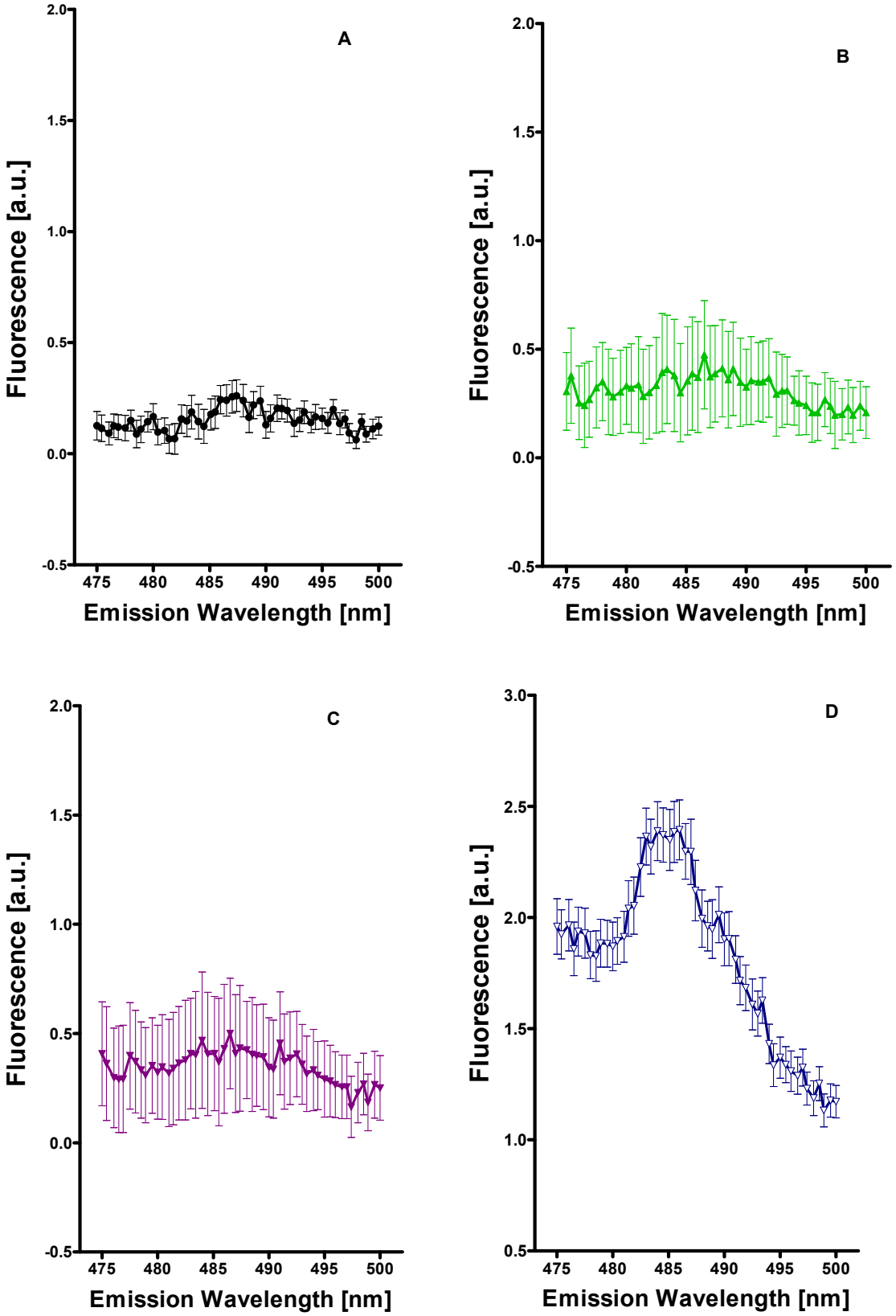

A
B
C
D
Fluorescence [a.u.]
Emission Wavelength [nm]
475
480
485
490
495
500

Figure 2..

Western blot was performed with the mouse anti-D_2-antibody of the whole cell lysates of HEK293 cells transiently expressing dopamine D_{2L} or NTS_1 receptor in comparison to mock transfected cells. D_{2L} expressing lysate revealed a strong band at 100 kDa of the D_{2L} receptor dimer and a weak band for the glycosylated D_{2L} monomer. Mock transfected and NTS_1 expressing cells demonstrated a weak band at 100 kDa of the endogenously expressed D_2 receptor in HEK293 cells (Atwood B. K, Lopez J., Wager-Miller J., Mackie K., Straiker A. (2011) Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. *BMC Genomics* 12, 1-14.).

Labeling of endogenously expressed dopamine D_{2L} receptor in HEK 293 cells by western blotting was accounted of low relevance for the fluorescence-detected co-immunoprecipitation due to the missing fluorophore at the endogenous D_{2L}.

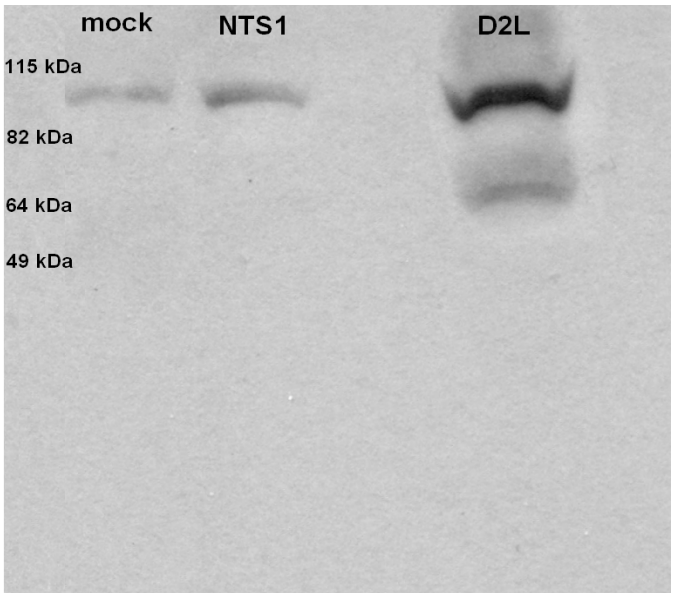


Table 1. Influence of neurotensin on [^{3}H]spiperone binding at the D_{2L}-NTS_1-heteromer compared with singly expressed D_{2L} receptor membranes and the mixture of mono-expressed D_{2L} and NTS_1.[a]

	Co- expression of D_{2L} and NTS_1		D_{2L}		Mixture of singly expressed NTS_1 and D_{2L}	
	K_D [nM]	B_{max} [fmol/mg]	K_D [nM]	B_{max} [fmol/mg]	K_D [nM]	B_{max} [fmol/mg]
control	0.36 ± 0.05	2900 ± 290	0.40 ± 0.005	2200 ± 370	0.32 ± 0.01	1700 ± 350
+ NT	0.23 ± 0.07	2900 ± 600	0.70 ± 0.12	2300 ± 390	0.54 ± 0.03	1630 ± 230

[a]The K_D-values of the three different systems were determined on membrane preparations of transiently transfected HEK293 cells of the coepxression D_{2L}-NTS1, singly expressed D_{2L} and the mixed single expressions of D_{2L} and NTS1 using [^{3}H]spiperone for the saturation experiments. Experiments were performed in absence and presence of 10 µM NT. Data were derived from 4-15 individual experiments each done in triplicates.

Table 2. Modulation of K_D values of [^{3}H]7-OH-DPAT at the D_{2L}-NTS_1-heteromer in presence of different concentrations of NT and NT(8-13) and SR48692. Data were derived from 4 to 25 individual experiments each done in triplicates Significances were calculated in an unpaired *t*-test compared to control (* and ** $p<0.001$ and $p< 0.005$, respectively).

	Co- expression of D_{2L} and NTS_1			Co- expression of D_{2L} and NTS_1	
	K_D [nM]	B_{max} [fmol/mg]		K_D [nM]	B_{max} [fmol/mg]
Control	7.2 ± 0.62	550 ± 43	7-OH-DPAT +100 nM NT(8-13)	125 ± 16 *	380 ± 90
7-OH-DPAT + 100 nM NT	62 ± 11 *	420 ± 100	7-OH-DPAT +10 nM NT(8-13)	47 ± 14 **	410 ± 65
7-OH-DPAT +10 nM NT	21 ± 4.2 *	410 ± 65	7-OH-DPAT +1 nM NT(8-13)	23 ± 2.1 *	500 ± 54
7-OH-DPAT + 400 nM SR48692	6.9 ± 0.51	600 ± 78	7-OH-DPAT + 400 nM SR48692 and 100 nM NT	8.9 ± 0.61	480 ± 75

Figure 3. Binding curve of homologous competition experiments using [3H]7-OH-DPAt in presence of 10 μM neurotensin. Curves with error bars representing the SEM derived from 6 individual experiments performed in triplicate.

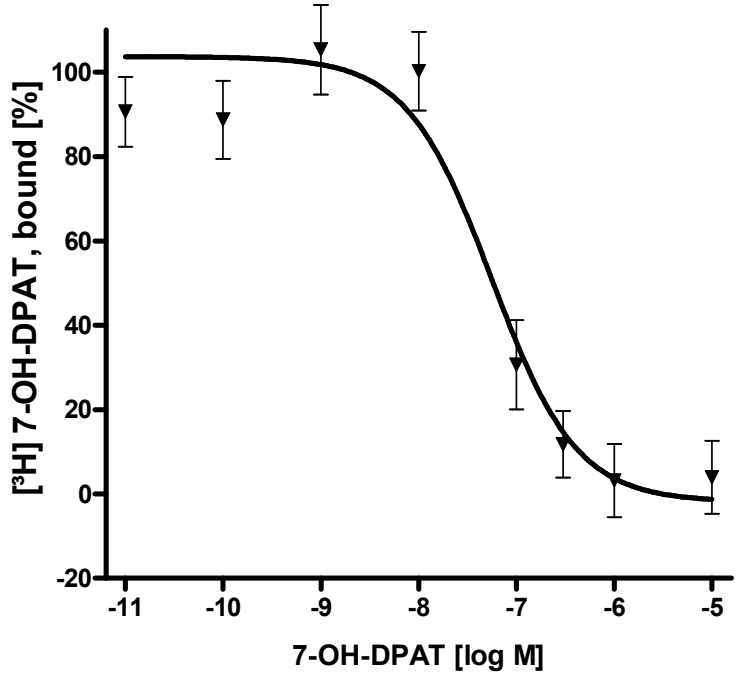


Figure 4. Binding curve of homologous competition experiments using [3H]7-OH-DPAt in presence of 10 nM neurotensin. Curves with error bars representing the SEM derived from 5 individual experiments performed in triplicate.

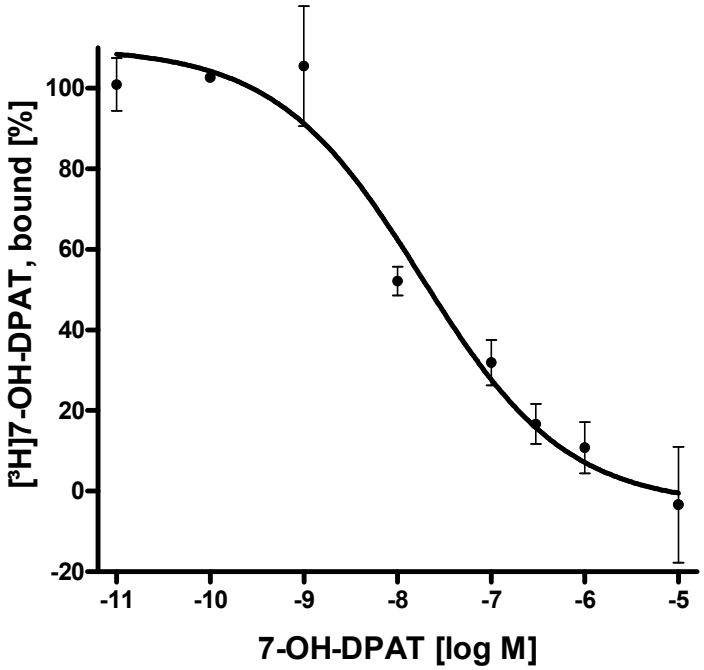


Table 3. [^{3}H]Neurotensin binding remained unchanged in presence of dopamine D_2 receptor agonist and antagonists. Influence of dopaminergics on [^{3}H]neurotensin binding at the D_{2L} -NTS1-heteromer compared with singly expressed NTS_1 receptor membranes and the mixture of mono-expressed D_{2L} and NTS_1.[a]

	Co- expression of D_{2L} and NTS_1		NTS_1		Mixture of singly expressed NTS_1 and D_{2L}	
	K_D [nM]	B_{max} [fmol/mg]	K_D [nM]	B_{max} [fmol/mg]	K_D [nM]	B_{max} [fmol/mg]
Control	1.6 ± 0.19	3700 ± 690	1.6 ± 0.16	4800 ± 490	1.3 ± 0.64	2400 ± 350
+ 7-OH-DPAT	2.6 ± 0.18	5900 ± 330	n.d.		n.d.	
+ spiperone	1.1 ± 0.14	3800 ± 460	n.d.		n.d.	

[a]The K_D-values were determined on membrane preparations of transiently transfected HEK293 cells of the coepxressed D_{2L}-NTS_1, singly expressed NTS_1 and the mixed single expressions of D_{2L} and NTS1 using [^{3}H]neurotensin for the saturation experiments. Experiments were performed in absence and presence of 1 µM spiperone and 140 nM 7-OH-DPAT, respectively. Data were derived from 3-12 individual experiments each done in triplicates. n.d. not determined

Figure 5. To control the membrane localization of transiently transfected HEK293 cells co-expressing the eYFP-tagged D_{2L} receptor (yellow) and eCFP-labelled NTS_1 mutant receptors, confocal microscopy with CFP and YFP emission and excitation wavelengths were performed. The D_{2L}-YFP construct (yellow, left) was shown to be co-localized in the plasma membrane with (A) NTS_1W129A-eCFP (right, cyan) and (B) NTS_1W134A-eCFP.

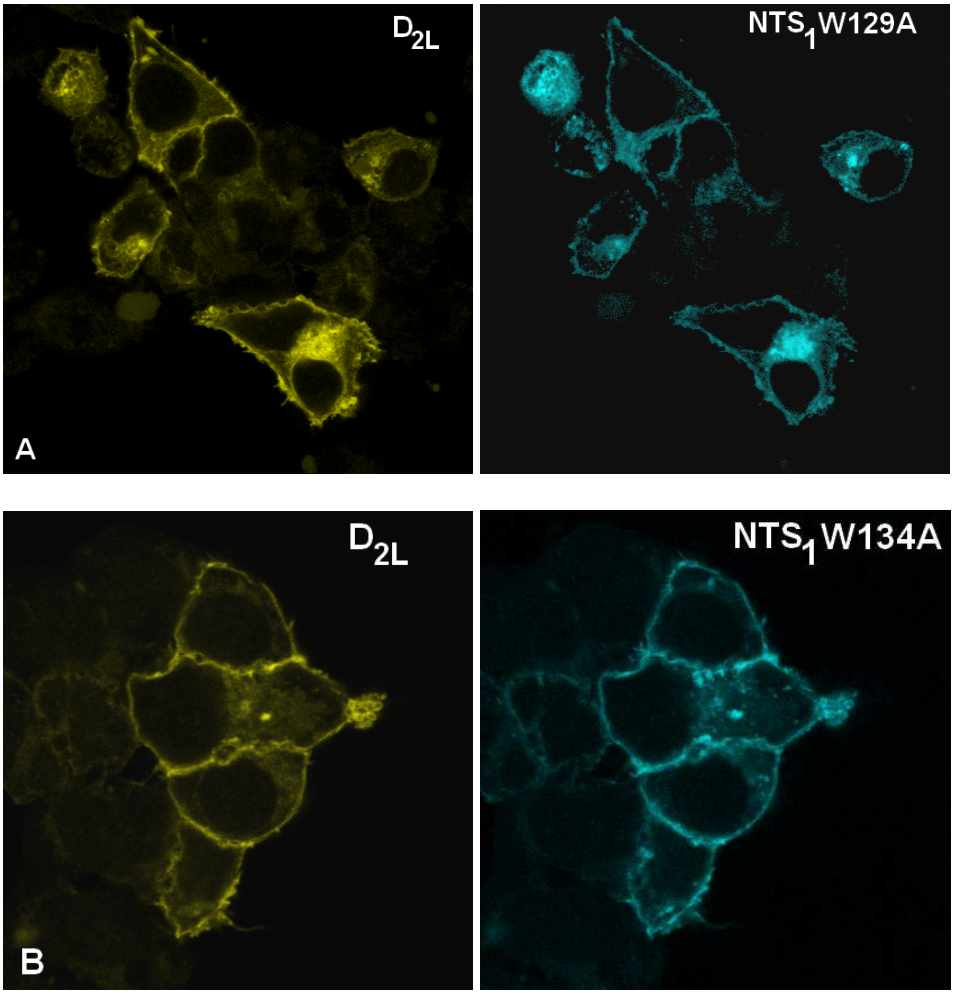
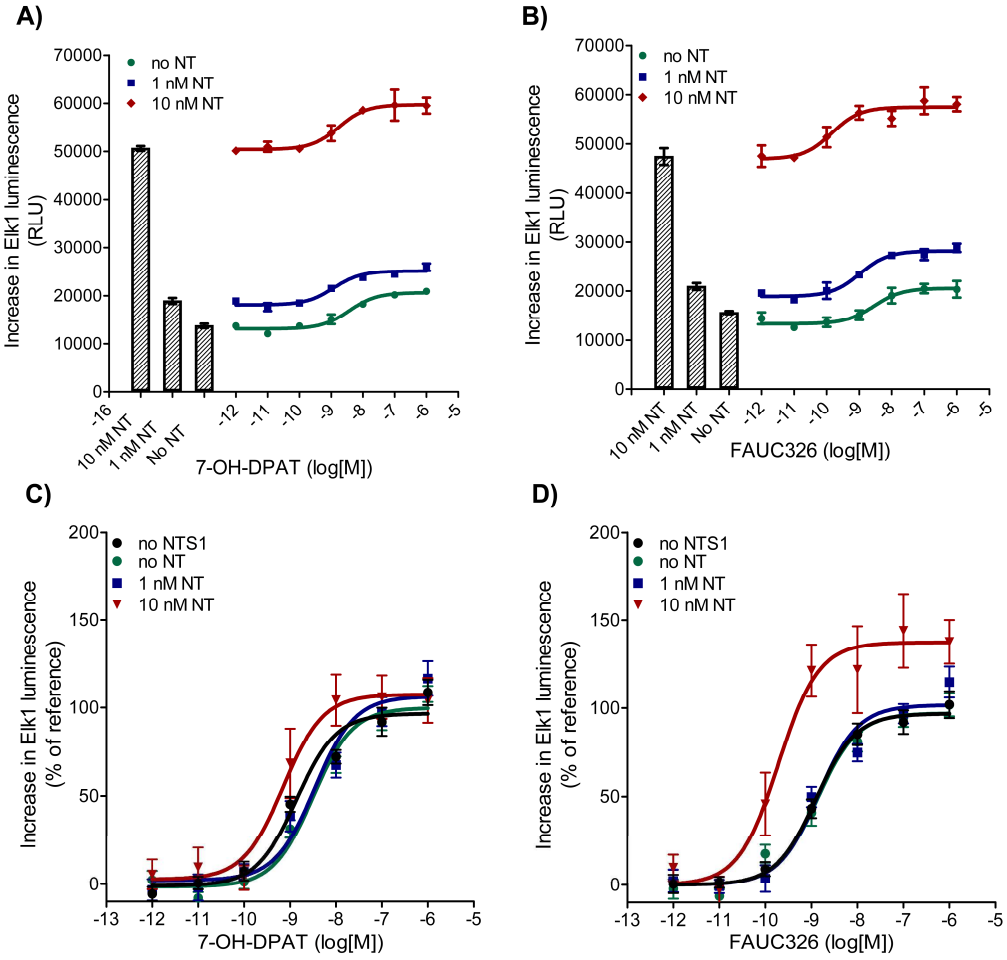


Table 4. Receptor binding data for the test compound FAUC326 at the porcine dopamine D_1, serotonin 5-HT_{1A}, 5-HT_2 and adrenergic α_1 as well as the human D_{2L}, D_{2S}, D_3 and D_4 receptors. Mean K_i values with SEM are derived from 2 to 7 experiments each done in triplicates.

		K_i-values (nM) ± SEM							
		[^{3}H] SCH23390	[^{3}H]spiperone				[^{3}H] WAY 100635	[^{3}H] ketanserin	[^{3}H] prazosin
		pD_1	hD_{2L}	hD_{2S}	hD_3	$hD_{4.4}$	$p5\text{-}HT_{1A}$	$P5\text{-}HT_2$	$p\alpha_1$
FAUC 326	$K_{0,5}$	6400 ± 180					6,5 ± 0,94	53 ± 4,9	100 ± 26
	$K_{i\ high}$		0,64 ± 0,11	0,33 ± 0,05	0,90 ± 0,20	2,8 ± 0,70			
	$K_{i\ low}$		30± 2,3	14 ± 2,0	16 ± 2,7	64 ± 15			

Figure 6. Neurotensin positively modulates the signaling of D2L in the coexpression with NTS1. A) Stimulation of transiently transfected HEK cell coexpressing D2L and NTS1 receptors with 7-OH-DPAT (A) and FAUC326 (B) at discrete concentrations of neurotensin (NT). The data of representative experiment performed in triplicate are shown. The normalized and pooled data of 4-6 experiments performed in triplicates are shown for 7-OH-DPAT (C) and FAUC326 (D), were the basal value defined 0% and the increase over the basal for dopaminergic in the absence of NT 100%.



Inhibition of cAMP accumulation assay. Bioluminescence based cAMP-Glo™ assay (Promega) was performed according to the manufacturers instructions. Briefly, CHO cells expressing D_{2L} wild receptor were seeded into a white 96-well plate (10 000 cells/well) 24 hr prior to the assay. Cells were first briefly washed with Krebs-Ringer Buffer (pH 7.4) to remove traces of serum and were incubated with various concentrations of substances in the presence of 20 μM forskolin in Krebs Ringer Buffer that contained 500 μM IBMX and 100 μM Ro 20-1724, pH 7.4. After 15 min of incubation at room temperature, the cells were lysed with cAMP-Glo lysis buffer. After lysis, the kinase reaction was performed with a reaction buffer containing PKA. At the end of the kinase reaction an equal volume of Kinase-Glo reagent was added. The plates were read with a luminescence protocol on a microplate reader Victor^{3}V (Perkin-Elmer). The experiments were performed two to nine times per compound with each concentration in duplicate.

PhosphoERK1/2 ELISA assay. The PathScan phospho-p42/44 MAPK (Thr202/Tyr204) Sandwich ELISA (Cell Signaling) was performed according to manufactures instructions. Briefly, $6x10^6$ CHO cells that expressed D_{2L} receptor were seeded in a 100 mm plate. The next day, cells were washed once with the serum free media and incubated in the presence of serum free media for additional 24 hr. On the day of the experiment, the medium was removed and replaced with the serum-free media containing various concentrations of the test substances as indicated and incubated for 5 min at 37^{o}C. The wash with ice-cold PBS and the addition of the lysis buffer stopped the reaction. The plates were kept on ice, cells scraped, briefly sonicated (UP50H, Hielscher Ultrasound Technologies) and centrifuged at 15 000 g for 10 min. The supernatant was promptly diluted with the sample dilutent and incubated overnight at 4^{o}C in the well. After intensive washing steps, the detection of the phosphorylated ERK1/2 followed. The absorbance was read at 450 nm within 2 min after addition of STOP solution on Victor^{3}V (Perkin Elmer) microplate reader. The experiment was performed four times per compound.

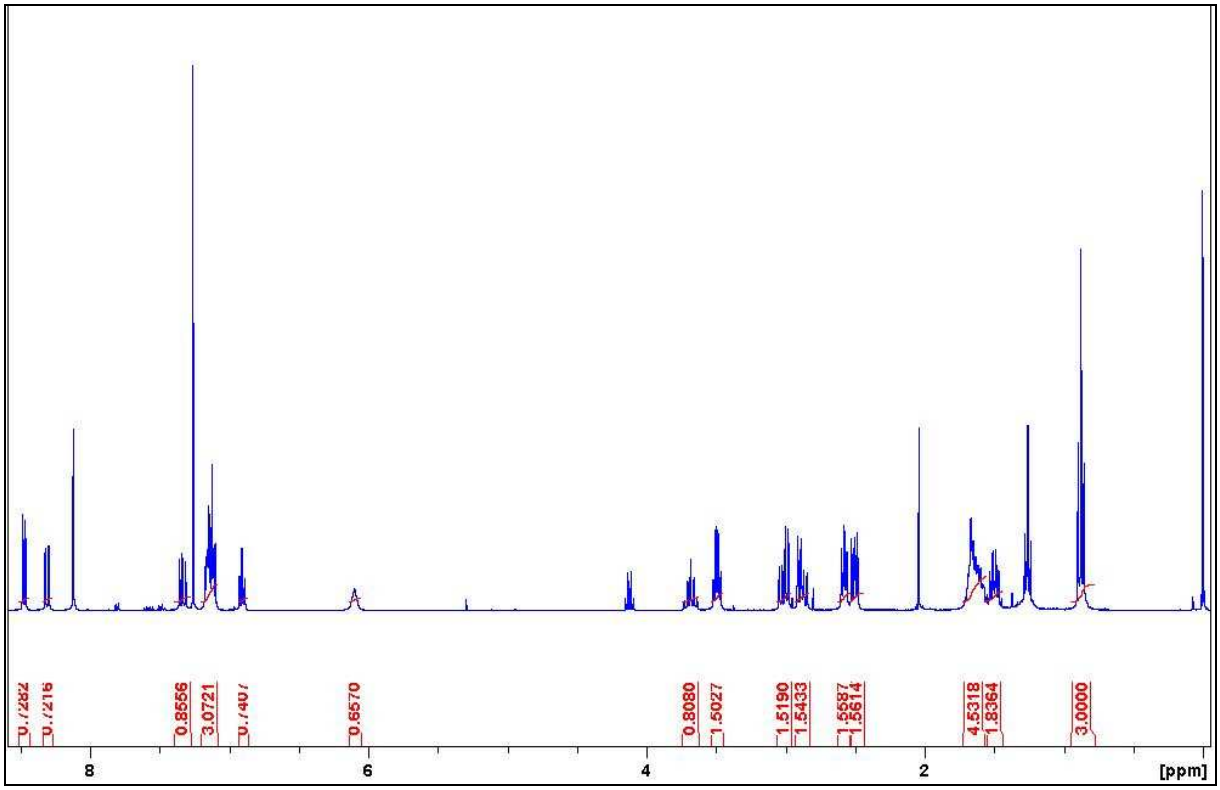
Luciferase Reporter Gene Assay. The luciferase reporter gene assay was performed according to the manufacturer's instructions (PathDetect Elk1 Trans-Reporting System, Stratagene, USA). A day before the transfection, HEK 293 cells were seeded in the 10 cm tissue culture dish. The cells were transfected with the TransIT-293 transfection reagent (Mirus Bio Corporation, Madison, WI) and 2.5 μg D_{2L}, 2.5 μg NTS1, 5 μg reporter pFR-Luc, and 0.5 μg pFA2-Elk1 fusion *trans*-activator plasmid. Twenty-four hours after transfection, cells were trypsinized and washed once to remove FBS. Cells were resuspended in DMEM-F12 supplemented with 1% FBS, 2 mM L-glutamine, and 1% Pen-Strep and seeded into white half area 96-well plates (40000 cells/well). The test compounds were added at the indicated concentrations at a final volume of 50 μL and incubated for 24 h at 37°C and 5% CO_2. To quantify an increase in luciferase expression, 50 μL of Bright-Glo reagent (Promega, Mannheim, Germany) was added to each well. After 3 min of incubation at RT and constant agitation to achieve complete cell lysis, luminescence was measured with a multiplate reader (Viktor^{3}V, Perkin-Elmer, Rodgau Germany).

The data obtained from the luciferase reporter gene assay were normalized (using the treatment of coexpressed receptors with 7-OH-DPAT and FAUC326 and in the absence of NT as reference to set 0% and 100%), pooled and 95% confidence interval for EC_{50} values and efficacy estimated.

N-[(N'-Indan-2-yl-N'-propyl)-4-aminobutyl]pyrazolo[1,5-*a*]pyridine-3-carboxamide (FAUC 326)

^{1}H NMR:

^{1}H (360 MHz $CDCl_3$)



^{13}C NMR:

^{13}C (150 MHz $CDCl_3$)