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

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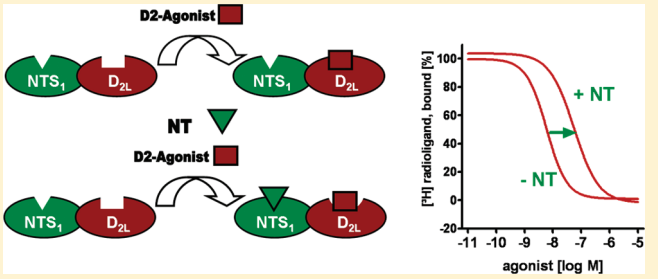
Supporting Information

ABSTRACT: Dopaminergic systems have been described to functionally interact with the neuromodulatory peptide neurotensin. Employing fluorescence detected coimmunoprecipitation and radioligand binding experiments, we herein demonstrate that coexpression of dopamine D_{2L} receptor and the neurotensin receptor subtype NTS_1 leads to physical interaction and the formation of heteromers in transfected human embryonic kidney 293 cells. In this in vitro system, a trans-inhibitory effect on the agonist binding affinity of D_2 was observed in presence of neurotensin. To correlate between the functional properties of dopaminergic agents and the magnitude of neurotensin-induced modulation of D_{2L} binding affinities in cells coexpressing D_{2L} and NTS_1, a structurally diverse set of dopamine receptor agonists, partial agonists, and antagonists was tested. Ligand specific profiles indicating substantial bias between ligand efficacy and transmodulation were discovered, suggesting a heteromerization-based functional selectivity. In the presence of neurotensin, the novel D_2 agonist FAUC 326 displayed a 34-fold decrease of binding affinity in cells coexpressing D_{2L} and NTS_1.



KEYWORDS: Dopamine D_{2L} receptor, neurotensin receptor 1, NTS_1, G-protein coupled receptor, GPCR, coexpression, intramembrane receptor−receptor interaction, negative cooperativity, binding affinity, coimmunoprecipitation, dimer, heteromer

The ability to form spatial complexes allowing molecular interactions between receptor protomers facilitates cooperative effects and tissue specific control of neuronal activity. Thus, heterodimerization is discussed as an integral feature of G-protein coupled receptor (GPCR) mediated signal transduction.[1−3] Intramembrane receptor−receptor interactions of associated protomers may modify binding and activating properties of GPCR ligands.[4−7] Using Förster resonance energy transfer (FRET) technique, a recent study on the interactions between α_{2A}-adrenergic and μ-opioid receptors demonstrated a trans-inhibitory effect between the protomers. In detail, morphine binding to the μ-opioid receptor triggered the conformational changes in the norepinephrine-occupied α_{2A}-adrenergic receptor leading to the inhibition of its signaling.[8] Physical interactions between δ- and μ-opioid receptors were suggested to modulate μ-mediated tolerance and dependence[9] when the development of bivalent ligands was constituted as a useful approach to investigate changes in receptor properties as a consequence to dimerization.[10,11] Heterodimerization is also a highly relevant phenomenon for dopamine receptor mediated signaling.[12,13] Demonstrating the development of a new complex with enhanced functional activity through hetero-oligomerization, the physical interaction between the dopamine D_{2L} and the somatostatin SST5 receptor was investigated via radioligand binding studies, functional assays, and FRET microscopy.[14] The hetero-oligomerization of D_{2L} and SST5, known for their colocalization in the central nervous system (CNS), led to a synergistic effect on binding and signaling as molecular cross-talk and the correlation between activated receptor function and oligomerization was stressed.

Dopaminergic systems have been also described to functionally interact with the neuromodulatory peptide neurotensin (NT, pE-L-Y-E-N-K-P-R-R-P-Y-I-L)[15] which is suggested to play a role in the pathophysiology of brain diseases including schizophrenia, Parkinson's disease. and Morbus Alzheimer.[16−18] Thus, neurotensin was shown to negatively alter binding affinity of the dopamine receptor agonist [^{3}H]*N*-*n*-propyl-nor-apomorphine in specific brain areas.[19−25] However, biomolecular interactions and a putative heteromer formation between dopamine receptors and neurotensin receptors have not been investigated yet.

Employing fluorescence detected coimmunoprecipitation and radioligand binding experiments, we herein demonstrate that coexpression of dopamine D_{2L} receptor and the neurotensin receptor subtype NTS_1 in human embryonic kidney cells (HEK293 cells) leads to physical interaction at a molecular level. In this in vitro system, a trans-inhibitory effect on the agonist binding affinity of D_2 was observed in the presence of neurotensin. A biochemical fingerprint[26] of the D_{2L}-NTS_1 heteromer was explored by investigating the effect of both neurotensin and NTS_1 on the D_2 receptor binding properties of different

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dopaminergics. Mutagenesis studies on NTS_1 in extracellular loop 1 (EL1) were used to investigate that the trans-modulatory effect is exerted by a specific receptor−agonist interaction. Structurally diverse ligands were investigated to determine the relationship between the intrinsic activity of dopaminergics and the modulatory effect of NTS_1−neurotensin binding on the affinity of dopaminergics.

RESULTS AND DISCUSSION

Colocalization and Heteromer Formation. As an important necessity for the functional interaction between receptor heteromers, a sufficient expression of the interaction partners in the cell membrane is prerequisite. To control the membrane expression of D_{2L} and NTS_1, fusion proteins C-terminally tagged by eYFP and eCFP,[27] respectively, were employed. The cellular distribution was monitored by confocal microscopy with appropriate excitation and emission wavelengths for the two fluorescent proteins. The fluorescently tagged NTS_1 and D_{2L} receptors coexpressed in HEK293 cells displayed colocalization of both GPCRs in the plasma membrane, thus allowing the formation of a new functional entity (Figure 1).

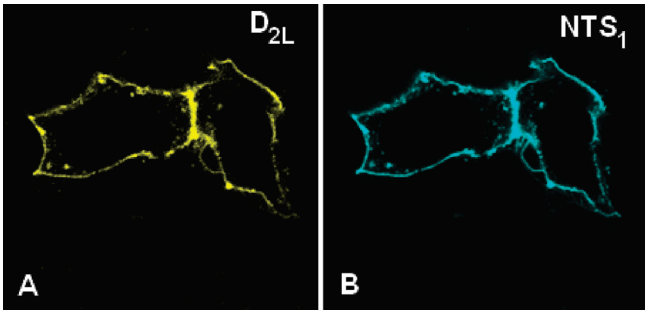
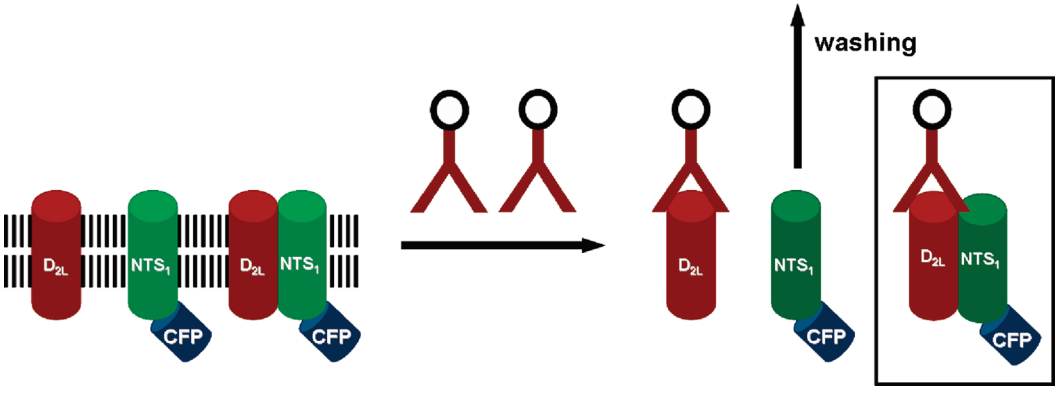


Figure 1. D_{2L} and NTS_1 receptors colocalize in the plasma membrane. To determine the cellular distribution of D_{2L} and NTS_1 receptors in the HEK293 cells, the cells were transiently transfected with the eYFP-tagged D_{2L} and eCFP-labeled NTS_1 receptors. The images were acquired by confocal microscopy. The D_{2L}-eYFP construct (left, A) was shown to be colocalized with NTS_1-eCFP (right, B) in the plasma membrane.

Coimmunoprecipitation followed by Western blotting is a general approach to investigate the formation of physically interacting receptor heteromers.[28−30] To circumvent cross-reactions that were associated with only moderate specificity of a commercially available NTS_1 antibody, we investigated the formation of D_{2L}-NTS_1 heteromers by immunoprecipitation with a D_{2L} antibody and detection of the coprecipitated system by fluorescence spectroscopy. The specificity of the antibody for the D_{2L} receptor was proven by Western blotting of HEK cells transiently expressing D_{2L} receptor revealing a band at 100 kD that corresponds to the dimeric D_{2L} receptor.[31] For the fluorescence-assisted coimmunoprecipitation, a C-terminally eCFP-tagged NTS_1 was cotransfected with the D_{2L} receptor in HEK293 cells. After lysis, precipitation with the D_{2L}-antibody-bead complex, and washing, heteromer formation was evaluated by detecting the emission spectrum of the eCFP-derived fluorophore (Scheme 1). The quantification of the emission spectra was performed by calculating the area under the curves (AUC).

To determine the maximal fluorescence intensity obtained by the fluorescence-detected coimmunoprecipitation, the fluorophore was covalently attached to the D_{2L} receptor (D_{2L}-eCFP). This construct was transiently coexpressed with unlabeled NTS_1 in HEK cells. Because we used an antibody specifically recognizing D_{2L}, the detected signal was independent from physical interaction between D_{2L} and NTS_1 and thus represents the maximal expected signal (Figure 2A). To ensure that the increase in the detected fluorescence could be assigned to the physical interaction between D_{2L} and NTS_1-eCFP, the basal fluorescence of lysis buffer and the protein A/G agarose beads was measured. In contrast to the intensity found for the expression of the D_{2L}-eCFP receptors, these controls displayed fluorescence curves with substantially lower intensity (4−10%) (Figure 2B). The expression of cytosolic eCFP together with D_{2L} and NTS_1 did not result in an increased intensity of a fluorescence signal. We observed an increase in the fluorescence intensity solely by the probe containing coexpressed D_{2L} and NTS_1-eCFP. Coexpression of D_{2L} and NTS_1-eCFP represented 67% of the signal obtained for the immobilized D_{2L}-eCFP receptor. Thus, a stable physical interaction between the two receptor protomers and the formation of a stable D_{2L}-NTS_1 heteromer is inferred by the above-mentioned data.

Scheme 1. General Principle of Fluorescence-Detected Coimmunoprecipitation[a]



[a] The D_{2L} receptor and the NTS_1-eCFP construct were transiently transfected in HEK293 cells. After cell lysis, coimmunoprecipitation of the fluorescently labelled NTS_1 with the D_{2L} receptor bound by the anti-D_{2L}-antibody bead complex resulted in an isolation of a fluorescence-emitting complex.

Modulation of Ligand Binding Affinity across the Receptor Dimer Interface. Investigating neuronal tissue, a large body of evidence indicated a negative cooperativity of NT on the receptor binding of dopamine[32] and the D_2 agonist *N*-*n*-propyl-nor-apomorphine[19,21,23] apparent in a 20−50% increase of K_i or K_D values, respectively. To better understand the molecular origins of these observations, a recombinant in vitro test system was established expressing the long splice variant of the human dopamine D_2 receptor (D_{2L}) and the human neurotensin receptor 1 (NTS_1). To achieve approximately equimolar expression levels of receptors, transient transfection of HEK293 cells with equal amounts of cDNAs for D_{2L} and NTS_1 was performed. The radioligand displacement studies were run on membrane preparations from cells expressing either D_{2L} only, NTS_1 only or D_{2L} and NTS_1 coexpressed receptors. Binding properties of the transiently expressed NTS_1 receptors were determined using [^{3}H]neurotensin. The dopamine receptor agonist 7-OH-DPAT and the antagonist spiperone were not able to alter the NTS_1 affinity of neurotensin in the membrane preparation of D_{2L} and NTS_1 coexpressing cell line. Thus, the D_{2L} receptor in active or inactive conformation bound by agonist or antagonist, respectively, is not able to disturb the recognition of the NTS_1 agonist binding site by its ligand neurotensin.

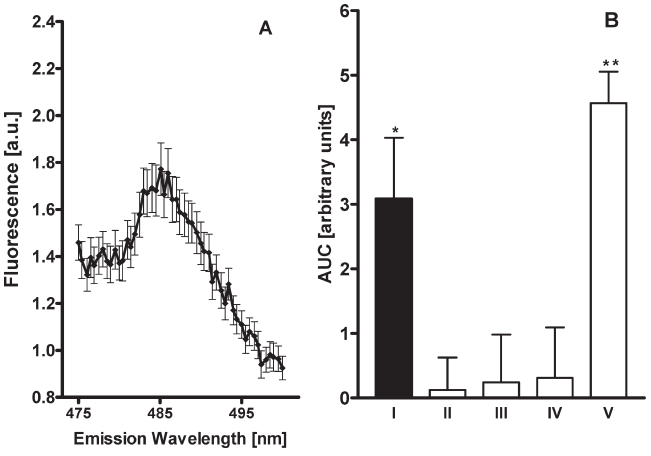


Figure 2. Fluorescence-detected coimmunoprecipitation assay indicated a stable physical interaction between the receptor NTS_1-eCFP and D_{2L} protomers. (A) Emission spectrum from 475 to 500 nm determined after excitation at 430 nm of lysed HEK293 cells coexpressing D_{2L} and the NTS_1-eCFP fusion protein. (B) Area under the curve with error bars were calculated from the measured eCFP emission spectra (from 480 to 490 nm) after immunoprecipitation with mouse anti-D_2 antibody of six independent experiments of each sample of the (I) coexpression of D_{2L} and NTS_1-eCFP; (II) control consisting of lysis buffer with protein A/G agarose beads; (III) single expression of NTS_1-eCFP; (IV) coexpression of D_{2L} with NTS_1 and cytosolic eCFP; and finally (V) coexpression of D_{2L}-eCFP and NTS_1. (* $p < 0.01$ and ** $p < 0.001$ compared to control in ANOVA followed by Tukey test).

To determine modulatory effects of neurotensin on D_{2L} receptor agonist affinity, K_D values of the D_2 agonist [^{3}H]7-OH-DPAT were determined. 7-OH-DPAT is structurally very similar to the parent neurotransmitter dopamine and was therefore expected to simulate the natural receptor ligand interactions most appropriately. Employing the test system expressing D_{2L} and NTS_1, homologous competition experiments resulted in a K_D value of 7.2 nM (Table 1), which was very similar to the dissociation constants that we obtained from a membrane preparations of D_{2L} expressing cell line and from a mixture of membrane preparations from cells containing singly expressed D_{2L} or NTS1 receptors (8.4 and 7.9 nM, respectively). Homologous competition experiments with membrane preparations of a coexpressed cell line revealed a K_D value of 57 nM after addition of neurotensin (10 μM). This indicates an 8-fold decrease of binding affinity. Highly similar B_{max} values confirmed that the portion of receptors in the high affinity state was not influenced by the affinity modifying neurotensin-induced effect. Singly expressed D_{2L} receptors and mixed membrane preparations singly expressing D_{2L} or NTS_1 did not show any alterations of affinity and receptor density after the addition of neurotensin. To investigate if neurotensin can modulate dopamine receptor antagonist binding,[33] saturation experiments were performed using the D_2 antagonist [^{3}H]spiperone (Supporting Information). The data displayed that the affinity of D_2 antagonists remained unchanged.

Aiming to better simulate physiological conditions, lower concentrations of neurotensin were investigated for its ability to influence the agonist binding of [^{3}H]7-OH-DPAT at the D_{2L}-NTS_1 heteromer. We observed an 8-fold and a 3-fold increase of K_D values of [^{3}H]7-OH-DPAT after incubation with 100 and 10 nM NT, respectively (Figure 3). Because the neurotensin concentration of 10 μM did not result in a stronger effect than 100 nM, we concluded that the neurotensin induced effect is saturable.

Because the C-terminal hexapeptide NT(8−13) is considered as an endogenously active form of neurotensin,[16,34] the influence of NT(8−13) on D_2 agonist binding was evaluated. Application of 1, 10, and 100 nM NT(8−13) induced a 3-fold, 6-fold, and 17-fold reduction of 7-OH-DPAT binding affinity, respectively, which is in accordance with recent investigations using rat neostriatum.[35] To corroborate that the negative cooperative effect depends on a specific NTS_1−agonist interaction, the influence of the nonpeptidic NTS_1 ligand SR48692[36,37] on [^{3}H]7-OH-DPAT binding was investigated. As expected,[36] NTS_1 binding of the nonpeptidic antagonist did not alter the agonist binding properties of [^{3}H]7-OH-DPAT. The parallel application of the agonist neurotensin and the antagonist SR48692, both used in concentrations of 100 and 400 nM, respectively, abolished the

Table 1. Influence of Neurotensin on [^{3}H]7-OH-DPAT Binding Shown as K_D Values ± SEM [nM][a]

	coexpression 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]
7-OH-DPAT	7.2 ± 0.62	550 ± 43	8.4 ± 0.65	530 ± 190	7.9 ± 0.74	790 ± 150
7-OH-DPAT + NT[b]	57 ± 7.5[c]	610 ± 110	8.9 ± 0.57	340 ± 90	9.2 ± 0.80	850 ± 290

[a] K_D values derived from 12−25 individual experiments each done in triplicate were determined on membrane preparations of transiently transfected HEK293 cells using the radioloigand [^{3}H]7-OH-DPAT. [b] Experiments were performed in the presence of 10 μM neurotensin. [c] Significances were calculated in an unpaired *t* test compared to control * $p < 0.001$.

neurotensin-induced decrease of dopamine agonist binding. Obviously, neurotensin induced transmodulation depends on a receptor−ligand interaction at the primary binding site, and SR48692 was able to inhibit an agonist−dependent conformational change at the D_{2L}−NTS_1 interface.

To confirm that the allosteric effect across the receptor−receptor interface is exerted by specific agonist binding, further control experiments were performed. In earlier studies, we described two mutations in the extracellular loop of the NTS_1 (W129A and W134A) displaying a complete loss of agonist binding of [^{3}H]NT(8−13) but exhibiting retention of antagonist binding of [^{3}H]SR48692.[38] As evident from the images acquired by confocal microscopy, these mutant NTS_1 receptors were properly expressed and colocalized with the D_{2L} receptor in the plasma membrane of transiently transfected HEK cells. For coexpressed D_{2L}/NTS_1W134A as well as the D_{2L}/NTS_1W129A receptors, no binding of [^{3}H]neurotensin was detectable. The D_{2L} affinities of spiperone and 7-OH-DPAT in the absence and presence of 100 nM neurotensin remained unchanged in these systems (Table 2).

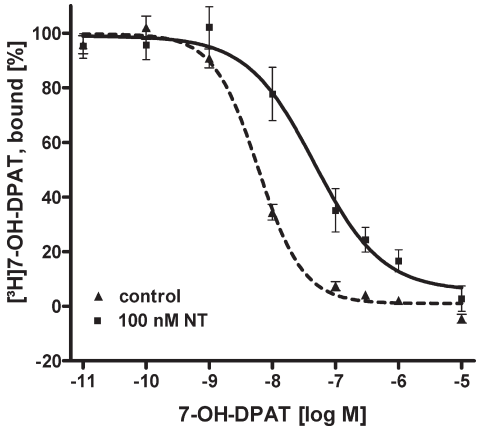


Figure 3. Right-shifted curves of [^{3}H]7-OH-DPAT binding of Table 1 at the D_{2L}-NTS_1 heteromer and a Hill slope of −0.8 demonstrate the negative cooperativity induced by neurotensin. Homologous competition curves with error bars representing the SEM are shown.

Based on the obtained experimental data, we concluded that the long splice variant of the D_2 receptor and the neurotensin receptor NTS_1 form a heterodimer with novel functional properties; the binding affinity of the D_2 receptor agonist decreases only in the presence of the NTS_1 receptor agonists neurotensin and NT(8−13). Thus, the negative cooperative effect of neurotensin depends on a specific NTS_1−ligand interaction at the orthosteric neurotensin binding site, obviously leading to cross-receptor interactions within the heterodimer that modulate D_{2L} agonist binding.

Transmodulatory Effects on Diverse Dopamine Receptor Ligands. To correlate between the functional properties of dopaminergic agents in D_{2L} expressing cells and the magnitude of neurotensin-induced modulation of D_{2L} binding affinities in cells coexpressing D_{2L} and NTS_1, a structurally diverse set of dopamine receptor ligands consisting of aminoindanes, phenylpiperazines, and phenylpiperidines with full agonist, partial agonist, and antagonist properties, respectively, was tested and compared to the endogenous ligand dopamine and the reference agonist 7-OH-DPAT (Figure 4). Ligand efficacy was determined

Table 2. Binding Data of Coexpressed NTS_1 Mutant Receptors with D_{2L} Display No Susceptibility toward the Application of Neurotensin[a]

	[^{3}H]neurotensin		[^{3}H]spiperone		[^{3}H]7-OH-DPAT		[^{3}H]7-OH-DPAT with 100 nM neurotensin	
	K_D	B_{max}	K_D	B_{max}	K_D	B_{max}	K_D	B_{max}
D_{2L}-NTS_1W129A	no specific binding		0.10 ± 0.002	4400 ± 360	4.3 ± 0.15	420 ± 43	6.2 ± 0.61	320 ± 55
D_{2L}-NTS_1W134A	no specific binding		0.08 ± 0.001	4100 ± 380	4.0 ± 0.01	480 ± 70	4.1 ± 0.42	300 ± 60

[a] K_D values ± SEM [nM] and B_{max} values ± SEM [fmol/mg] were determined in 3−4 individual experiments each done in triplicate.

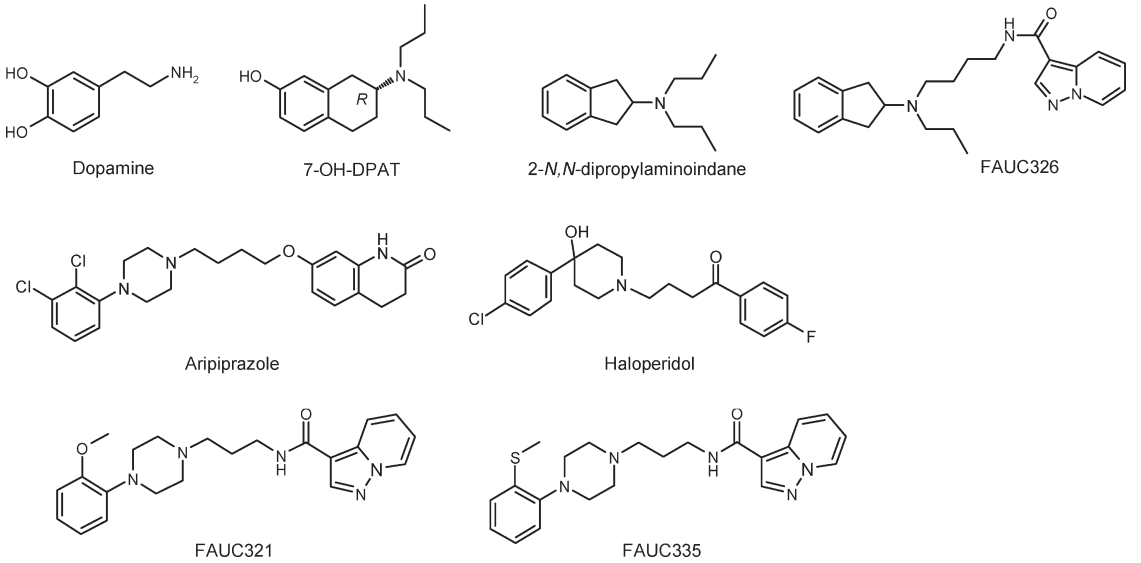


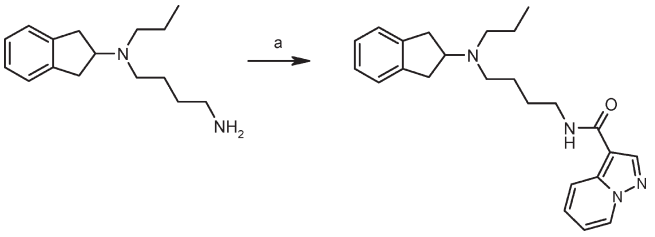
Figure 4. Chemical structures of the ligands investigated in this study.

Table 3. Affinities on the D_{2L}-NTS_1-Heteromer and Functional Properties at the D_{2L} Receptor of the Dopamine Receptor Agonists and Antagonists

	K_i [nM][a]	K_i [nM] + 100 nM NT[a]	ratio $K_{i+NT}/K_{i\ -NT}$[b]	cAMP efficacy in % (pEC50)[d]	ERK1/2 efficacy in % (pEC50)
dopamine	8.8 ± 1.2	82 ± 3.3	9.3	111 ± 5 (7.7 ± 0.10)	106 ± 4 (7.8 ± 0.11)
7-OH-DPAT	7.2 ± 0.62	62 ± 11	8.6	84 ± 4 (8.6 ± 0.11)	98 ± 2 (9.0 ± 0.08)
aripiprazole	1.7 ± 0.13	2.3 ± 0.05	1.3	62 ± 5 (7.5 ± 0.15)	62 ± 5 (6.9 ± 0.17)
haloperidol	0.68 ± 0.04	1.4 ± 0.21	2.0	−8 ± 1 (8.7 ± 0.27)	n.a.
2-*N*,*N*-dipropylaminoindane	5.0 ± 1.8	22 ± 5.2	4.4	89 ± 10 (7.9 ± 0.8)	n.d.
FAUC 326	0.32 ± 0.07	11 ± 2.4	34	114 ± 10 (8.7 ± 0.91)	95 ± 4 (9.0 ± 0.9)
FAUC 335	0.38 ± 0.09	4.7 ± 1.3	12	46 ± 4 (7.9 ± 0.19)	55 ± 4 (7.5 ± 0.11)
FAUC 321	0.86 ± 0.12	3.2 ± 0.33	3.7	35 ± 2 (8.1 ± 0.17)	55 ± 3 (8.4 ± 0.14)

[a] The affinities of investigated substances were determined on membrane preparations of transiently transfected HEK293 cells coexpressing D_{2L} and NTS_1 using [3H]7-OH-DPAT for competition experiments. Data are derived from 3−8 individual experiments each done in triplicate. [b] The ratio $K_{i+NT}/K_{i\text{-}NT}$ indicates the changes in affinity. [c] K_D value in [nM]. [d] Functional data of test compounds presented as *p*EC50 values and efficacies (%) were determined by measuring the D_{2L} receptor mediated the inhibition of cAMP accumulation and in the stimulation of ERK1/2 phosphorylation. Pooled data of 3−9 experiments performed in triplicate are shown as mean values ± SEM. Legend: n.a., not available; n.d., not determined.

Scheme 2[a]



[a] Reagents and conditions: (a) pyrazolo[1,5-a]pyridine-3-carboxylic acid, TBTU, DIPEA, CH_2Cl_2, 4 h, RT.

by measuring the ability of the test compound to modulate the D_{2L} receptor mediated inhibition of cAMP accumulation and the stimulation of extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation. As a representative for dopamine D_2 receptor antagonists, we chose the classical antipsychotic drug haloperidol. Haloperidol behaved as an antagonist in both functional assays and was not able to significantly alter binding affinity of the D_{2L} receptor in our D_{2L}/NTS_1 coexpressing system (Table 3). From the family of phenylpiperazines, the atypical antipsychotic drug aripiprazole and two structurally related congeners FAUC 321 and FAUC 335 were evaluated.[39,40] In fact, analysis of the biological data revealed that the test compounds behaved very differently. The 2,3-dichlorophenyl-piperazine aripiprazole, which is known for its partial agonist activity at pre- and postsynaptic D_2 receptors[41,42] and for functional selective behavior,[43] displayed a maximal efficacy of 62% in the functional assays, but it was not able to significantly modulate D_2 binding affinity in the D_{2L}/NTS_1 coexpressing cell line. The 2-methoxyphenylpiperazine derivative FAUC 321 exhibited only 35% and 55% in the functional assays, but it was able to reduce dopamine receptor binding 3.7-fold in the coexpressed system. This effect was even stronger for the thiomethyl-substituted analogue FAUC 335 when a ratio of dopamine receptor affinities of 12 indicated a heterodimer-specific allosteric effect that was superior to the full agonist dopamine ($K_{i+NT}/K_{i\text{-}NT}$ = 9.3). This shows that, even within one family of compounds, ligand efficacy and the heteromer-promoted affinity modulation do not correlate with each other. Whereas aripiprazole and FAUC 335 will have very similar biological activity in D_{2L} expressing tissue of the CNS, the two test compounds will behave differently in brain areas coexpressing D_{2L} and NTS_1 leading to a heteromerization-based functional selectivity. Besides this, subtle structural variations can cause substantially different modulatory potencies.

Finally, we evaluated 2-*N*,*N*-dipropylaminoindane[44] and its long-chain analogue FAUC 326 for their biological profiles. As a derivative of the full agonist 2-*N*,*N*-dipropylaminoindane,[45] FAUC 326 was newly synthesized for this study because we intended to further improve D_2 receptor binding affinity by formally attaching an affinity-generating appendage to one of the propyl substituents,[46] which should lead to an energetically favorable interaction with the lipophilic microdomain of the D_2 receptor according to our very recent docking studies[46] (Scheme 2).

The full agonists 2-*N*,*N*-dipropylaminoindane and FAUC 326 showed significantly distinct modulatory effects on D_2 binding in the coexpressing cell line. Whereas neurotensin was able to reduce the binding affinity of 2-*N*,*N*-dipropylaminoindane by a factor of 4.4, a 34-fold decrease of binding affinity was observed for the novel D_2 agonist FAUC 326.

For the characterization of the influence of NT on the signaling of D_2 receptor, we have chosen the firefly (*Photinus pyralis*) luciferase based PathDetect Elk1 gene reporter assay. The transcription factor Elk-1 is phosphorylated and activated by p42/p44 MAPK.[47] Employing a D_{2L}-NTS_1 coexpressing cell line, D_{2L} promoted signaling of 7-OH-DPAT and FAUC 326 was observed in a dose dependent manner when coapplication of neurotensin (10 nM) led to a 5- to 6-fold improvement of EC_{50} values. Further investigations will be necessary to decipher and to better understand the functional system.

CONCLUSION

Employing fluorescence detected coimmunoprecipitation and radioligand binding experiments, we herein demonstrate that coexpression of dopamine D_{2L} receptor and the neurotensin receptor subtype NTS_1 leads to physical interactions at a molecular level in transfected human embryonic kidney 293 cells. In this in vitro system, a trans-inhibitory effect on the agonist binding affinity of D_2 was observed in the presence of neurotensin. To correlate between the functional properties of dopaminergic agents and the magnitude of neurotensin-induced modulation of D_{2L} binding affinities in cells coexpressing D_{2L}

and NTS_1, a structurally diverse set of dopamine receptor agonists, partial agonists, and antagonists was tested, indicating that significant intrinsic activity is necessary. However, ligand specific profiles indicating substantial bias between ligand efficacy and transmodulation were discovered. In the presence of neurotensin, the novel D_2 agonist FAUC 326 displayed a 34-fold decrease of binding affinity in cells coexpressing D_{2L} and NTS_1. The physiological implication of different NT and NTS1 concentrations depending on the brain region may lead to an individual in vivo activity for drugs. Whereas aripiprazole and FAUC 335 will have very similar biological activity in D_{2L} expressing tissue of the CNS, the two test compounds will behave differently in brain areas coexpressing D_{2L} and NTS_1. Such a heteromerization-based functional selectivity might play an important role in neuropathophysiology of Parkinson's disease. With regard to the increased NT brain levels in the substantia nigra of parkinsonian patients,[48] the transmodulatory effects described might have an impact of the therapy with classical dopamine receptor agonists. More efficacious drugs for the treatment of Parkinson's disease are suggested to have low sensitivity toward NT-induced cross-receptor interactions. The D_{2L}-NTS_1 heteromer should be considered as a drug target for the development of novel anti-Parkinson drugs.

METHODS

Materials. All cell culture materials were purchased from Invitrogen LifeTechnologies (Karlsruhe, Germany). [^{3}H]Spiperone (102−114 Ci/mmol) and [^{3}H]7-OH-DPAT (157−163 Ci/mmol) were purchased from GE Healthcare (Freiburg, Germany), and [^{3}H]neurotensin (100−112 Ci/mmol) was purchased from PerkinElmer (Rodgau, Germany). cAMP-Glo assay was purchased from Promega (Mannheim, Germany). Dopamine (3,4-dihydroxyphenethylamine), spiperone, haloperidol, 7-OH-DPAT (*R*-(+)-7-hydroxy-2-(N,N-di-*n*-propylamino)tetraline hydrobromide), aripiprazole, and other substances were purchased from Sigma (Steinheim, Germany), unless otherwise stated.

Expression Vectors. The wild type $hNTS_1$ and hD_{2L} cDNA was purchased from the UMR cDNA Resource Center and subcloned into a pcDNA^{TM}3.1(+) eukaryotic expression vector (Invitrogen, Karlsruhe, Germany) using *EcoR1/Xba1* restriction sites.[38,46] Oligonucleotidic primers were purchased from Biomers.net (Ulm, Germany). Restriction enzymes were purchased from New England Biolabs (Frankfurt am Main, Germany). NTS_1-eCFP-construct was done by the PCR-based mutagenesis method described by Ko and Ma[49] with primers for the insertion of the *Sap1* restriction site and the removal of the stop codon. NTS_1-cDNA was modified using the start primer CGACTCACTATAGGGAGACCCAA and the reverse end primer AAGCTCTTCATGCGTA-CAGCGTCTCGCGGGT. The eCFP-cDNA fragment was obtained by the use of AAGCTCTTCTGCAATGGTGAGCAAGGGCGAG and GCAACTAGAAGGCACAGTCGAGG as start and end primers, respectively. The final cloning in the pcDNA^{TM}3.1 vector was performed using *Nhe1/Sap1/Apa1* restriction sites. PCR for the D_{2L}-eYFP construct was performed using the D_{2L} cDNA with the forward primer GCTAGCGTTTAAACTTAAGCTTGG and the reverse oligonucleotide CCTCTAGACTCGCCGCGGCAGTGGAG and for the eYFP fragment the primers GCTAGCGCTACCGCGGGGCAGCAATG and CAGCTTGAGTAGCCCCTCTAGAT-TACTAGGCGGCGGTCA. The D_{2L} PCR product was digested with the restriction enzymes *HindIII* and *SacII*. *SacI* and *XbaI* were utilized for the eYFP PCR fragment. Both digested fragments were ligated in the vector template of the wild type D_{2L} receptor digested with *HindIII* and *XbaI*. Fidelity of PCR amplification and introduction of the fluorescence protein tags in the receptor cDNAs were confirmed by sequencing by LGC Genomics (Berlin, Germany)

Cell Culture. Human embryonic kidney cells (HEK293) were grown in DMEM-Ham's F12 medium (1:1), supplemented with 10% fetal calf serum, 100 U/mL penicillin G, 100 μg/mL streptomycin, and glutamine (2 mM). All cells were grown at 37 °C under a humidified atmosphere with 5% CO_2.

Confocal Microscopy. For the preparation of the microscope slides, the coverslips were coated using 0,01% Poly-L-Lysin-Solution (Sigma Adrich, Steinheim, Germany) and HEK293 cells were grown in 6-well plates for 24 h with 10 000 cells/well. Double transfections were done with 2 μL of cDNA per well at a 1:1 ratio of the cDNAs using TransIT-293 transfection reagent (MoBiTec, Göttingen, Germany).

After 48 h cells, were fixed in 4% paraformaldehyde for 30 min and mounted on slides using a PBS/glycerol mixture 1:9 as mounting medium.[50] Slides were sealed using colorless nail polish to avoid desiccation. The images were acquired using a Leica TCS SP5 confocal microscope (63× oil objective) of Prof. T. Stamminger at the Institute for Clinical & Molecular Virology (University of Erlangen-Nuremberg, Germany). Images of eCFP were acquired with laser excitation at 405 nm (20% power) and emission collection from 470 to 490 nm. eYFP samples were recorded with excitation by an argon laser at 514 nm (30% power), and the emission was collected from 510 to 530 nm. Fluorescence bleedthrough was minimized by adjusting the pinhole and photomultiplier tubes and confirmed through sequential laser scans.

Fluorescence-Detected Coimmunoprecipitation. HEK293 cells were transiently transfected with 24 μg of DNA per Petri dish (145 × 25 mm) using TransIT-293 transfection reagent (Mirus Bio Corporation). Two days after transfection, the cells were washed once with PBS. The cells (1.5×10^6) were then abraded with a cell scraper into 800 μL of IP-lysis buffer (10× cell lysis buffer, Cell Signaling with 0.5% sodium deoxycholate) and sonicated on ice (10 strokes, 60% amplitude, 30 kHz). The lysate was incubated for 1 h at 4 °C, and insoluble material was removed by centrifugation at 15 000*g* for 4 min at 4 °C. The supernatant was precleared using 10 μL of protein A/G Plus agarose beads (0.5 mL agarose/2.0 mL, Santa Cruz Biotechnologies, Santa Cruz) for 2 h at 4 °C. The beads were removed by a centrifugation step at 3000*g* and 4 °C for 30 s, and the supernatant was incubated for 2 h at 4 °C with 1.4 μg of the mouse anti-D_2 antibody B-10 (Santa Cruz Biotechnologies, Santa Cruz) against amino acids 1−50 at the N-terminus of the dopamine receptor. After the addition of 25 μL of protein A/G Plus agarose beads, the mixture was incubated overnight at 4 °C under constant rotation and subsequently centrifuged at 3000 g for 30 s at 4 °C. The pellet was washed three times with ice-cold IP-lysis buffer. The beads were resuspendend in 200 μL lysis buffer and transferred in black 96-well plates. The fluorescence emission spectra of the samples were determined using a fluorescence spectrophotometer Cary Eclipse (Agilent, Darmstadt). The eCFP was excited at 430 nm (slit 5 nm) and the emission spectra from 475 to 500 nm were recorded.

Membrane Preparations. For the membrane preparations HEK293 cells were transiently transfected with 24 μg DNA per Petri dish (145 × 25 mm) of the cDNA encoding the proteins of dopamine D_{2L}, of the NTS_1 constructs or a mixture of both cDNAs using TransIT-293 transfection reagent according to the protocol given by the manufacturer. Transfected cells were cultivated for 48 h and then harvested. To harvest the cells, the medium was removed and cells were washed once with phosphate buffered saline. Then the cell material was abraded with a cell scraper and resuspended in 10 mL of harvest buffer (10 mM Tris-HCl, 0.5 mM, EDTA, 5.4 mM KCl, and 140 mM NaCl, pH 7.4) into a centrifuge tube. After centrifugation at 220*g* for 8 min the cellular pellet was resuspended in 5 mL of homogenate buffer (50 mM Tris-HCl, 5 mM EDTA, 1.5 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM KCl, and 120 mM NaCl, pH 7.4). Cells were used directly or stored at −80 °C. After thawing or directly, the cells were homogenized using a Polytron instrument (20 000 rpm, 5 times for 5 s each in an ice bath) and

pelleted at 50 000*g* for 18 min. The supernatant was discarded, and the membrane pellet was resuspended in binding buffer (50 mM Tris, 1 mM EDTA, 5 mM $MgCl_2$, 100 μg/mL bacitracin, 5 μg/mL soybean trypsin inhibitor, pH 7.4) and homogenized with a Potter-Elvehjem homogenizer. Membrane homogenates were stored in small aliquots at −80 °C. Protein concentration was determined by the method of Lowry et al.,[51] using bovine serum albumin as a standard.

Homologous and Nonhomologous Competition Experiments with [^{3}H]7-OH-DPAT. To determine the binding at the high affinity binding site of the D_{2L} receptor, the agonist (*R*)-7-OH-DPAT (specific activity 157−163 Ci/mmol) was used as tritiated radioligand to determine the K_D and B_{max} values in homologous competition experiments or K_i values of different test compound in nonhomologous competition experiments. All assays were performed in 24-well plates at a total volume of 500 μL. The [^{3}H]7-OH-DPAT was utilized as 1.5 nM solution for the labeling of 150 μg/mL protein per well. Varying concentrations of unlabeled 7-OH-DPAT or test compounds (0.01 to 100 000 nM) were added to the radioligand. To determine the unspecific binding, 10 μM 7-OH-DPAT was used. Total binding was determined in the absence of test compound. Competition experiments were performed in the absence and presence of NTS_1 ligands. After the addition of the membrane homogenates, the mixture was incubated for 1 h at 37 °C. The assay was stopped by rapid filtration through GF/B filters precoated with 0.3% polyethylenimine. Filters were washed five times with ice-cold Tris-EDTA buffer (50 mM Tris, 1 mM EDTA pH 7.4), dried at 50 °C, sealed with MeltiLex solid scintillator (PerkinElmer, Rodgau, Germany), and radioactivity counted in a MicroBeta Trilux (PerkinElmer, Rodgau, Germany).

Saturation Experiments Using [^{3}H]Spiperone and [^{3}H]-Neurotensin. Membrane preparations of coexpressed or singly expressed dopamine D_{2L} and NTS_1 receptors expressing HEK 293 cells were incubated in 96-well plates with 10 different concentrations (0.005−2 nM) of the tritiated D_2 antagonist spiperone (specific activity 102−114 Ci/mmol) or [^{3}H]neurotensin (specific activity 100−112 Ci/mmol). Nonspecific binding was defined in the presence of 10 μM haloperidol or neurotensin, and total binding was measured in absence of any competing drug. To investigate the influence of different NTS_1 or D_{2L} receptor ligands, either substance or buffer was added to the reaction mixture. After addition of membrane preparations with protein concentrations of 40 μg/mL, the assay mixture was incubated for 30−60 min at 37 °C and stopped by rapid filtration and further proceeded as described above for [^{3}H]7-OH-DPAT.

Functional Assays. Inhibition of cAMP accumulation assay and phosphoERK1/2 ELISA assay were performed as previously described.[45] A detailed description is available in the Supporting Information.

Data Analysis. Fluorescence-detected coimmunoprecipitation assay was analyzed by calculating the area under the curve from 480 to 490 nm using PRISM (GraphPad Software, San Diego, CA). Data are presented as mean ± SEM. Statistical analysis of single group comparisons was performed using an unpaired *t* test. Significances was set as $P < 0.05$.

Analysis of the saturation experiments were performed using a nonlinear regression analysis of the data for the determination of K_D and B_{max} values using PRISM. The resulting competition curves were analyzed by nonlinear regression using the algorithms in PRISM (GraphPad Software, San Diego, CA). The data were initially fitted using a sigmoid model and an IC_{50} value, representing the concentration corresponding to 50% of maximal inhibition. Data were then calculated for a one-site model using the program PRISM. IC_{50} values were transformed to K_i values according to the equation of Cheng and Prusoff.[52] Data are presented as mean ± SEM. Statistical analysis of single group comparisons was performed using an unpaired *t* test. Significances was set as $P < 0.05$.

Chemistry. *N-[(N′-Indan-2-yl-N′-propyl)-4-aminobutyl]pyrazolo-[1,5-a]pyridine-3-carboxamide (FAUC326).* To a solution of pyrazolo-[1,5-a]pyridine-3-carboxylic acid (36.8 mg, 0.23 mmol) in dried CH_2Cl_2 (4 mL), DIPEA (150 μL, 0.91 mmol) was added. The mixture was cooled to 0 °C before a solution of TBTU (113.8 mg, 0.35 mmol) in dried DMF (1 mL) was added. Then, a solution of *N*-indane-2-yl-*N*-propyl)butane-1,4-diamine[45] (104.9 mg, 0.43 mmol) in CH_2Cl_2 (5 mL) was added dropwise. The mixture was stirred for 4 h at room temperature before aqueous $NaHCO_3$ was added. The aqueous layer was extracted several times with CH_2Cl_2, and the combined organic layers were dried ($MgSO_4$) and evaporated. The residue was purified by flash chromatography (hexane/EtOAc 1:2 + 0.5% NMe_2Et) to give FAUC326 as a colorless oil (37.4 mg, 38%): IR 3433 s, 2931 m, 2866 w, 1639 m, 1554 m, 1277 m, 744 m cm^{-1}. ^{1}H NMR (360 MHz, $CDCl_3$) δ 0.87 (t, J = 7.4 Hz, 3 H), 1.44 −1.56 (m, 2 H), 1.56−1.72 (m, 4 H), 2.45−2.54 (m, 2 H), 2.54−2.62 (m, 2 H), 2.88 (dd, J = 15.4 Hz, 8.7 Hz, 2 H), 3.01 (dd, J = 15.4 Hz, 7.7 Hz, 2 H), 3.41−3.57 (m, 2 H), 3.60−3.74 (m, 1 H), 6.04−6.17 (m, 1 H), 6.86−6.96 (m, 1 H), 7.08−7.18 (m, 4 H), 7.33 (ddd, J = 8.9 Hz, 6.8 Hz, 1.1 Hz, 1 H), 8.12 (s, 1 H), 8.47 (ddd, J = 6.9 Hz, 1.0 Hz, 1.0 Hz, 1 H), 8.47 (ddd, J = 6.9 Hz, 1.0 Hz, 1.0 Hz, 1 H). ^{13}C NMR (90 MHz, $CDCl_3$) δ 11.9, 20.2, 25.0, 28.0, 36.4, 39.4, 50.9, 53.4, 63.0, 107.0, 113.5, 119.6, 124.4, 126.2, 128.8, 140.1, 140.6, 141.8, 163.2. EI-MS m/z 390. Anal. ($C_{24}H_{30}N_4O \cdot 1.5H_2O$) C, H, N. Purity 100% (HPLC).

■ ASSOCIATED CONTENT

Supporting Information. Western blot, emission spectra of fluorescence detected co-immunoprecipitation, confocal images of the coexpression of D_{2L} and NTS_1 as well as receptor binding data for the test compound FAUC 326 at various receptors, binding data and curves of homologous competition experiments using [^{3}H]7-OH-DPAT in the presence of different NTS1 ligands, saturation experiments using [^{3}H]spiperone and [^{3}H]neurotensin, experimental description of the used functional assays, dose response curves of MAPK-driven luciferase reporter gene assay, and analytical data of FAUC326. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

S.K. conducted experiments and contributed to the writing of the manuscript. T.N. performed confocal microscopy experiments and functional assays and contributed to the writing of the manuscript. P.G. designed the research programme, and contributed to the writing of the manuscript.

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■ ABBREVIATIONS

GPCR, G-protein coupled receptor; FRET, Förster resonance energy transfer; D_{2L}, dopamine D_{2L} receptor; NTS_1, neurotensin

receptor 1; HEK, human embryonic kidney cells; eCFP, enhanced cyan fluorescent protein; eYFP, enhanced yellow fluorescent protein; AUC, area under the curve; K_D, equilibrium dissociation constant; B_{max}, the maximum of specific binding; EC_{50}, half maximal effective concentration; IP, immunoprecipitation; nd, not determined; 7-OH-DPAT, 7-hydroxy-*N*,*N*-dipropyl-2-aminotetraline; SR48692, 2-([1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl)-1H-pyrazole-3-carbonyl]amino)admantane-2-carboxylic acid; ERK, extracellular-signal-regulated kinases; cAMP, cyclic adenosine monophosphate; DA, dopamine; EDTA, ethylendiaminetetraacetic acid; Tris, tris-(hydroxymethyl)aminomethane; PBS, phosphate-buffered saline; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide

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