DOI: 10.1002/cmdc.201100499

Selective Agonists for Dopamine/Neurotensin Receptor Heterodimers

Susanne Koschatzky and Peter Gmeiner*[a]

The neuromodulatory peptide neurotensin has been described to functionally interact with dopaminergic pathways of the human brain. We employed radioligand binding studies to investigate the physical interaction between co-expressed dopamine D_{2L} or D_3 and neurotensin NTS_1 or NTS_2 receptors. Substantial cross-inhibitory effects of both receptor subtypes NTS_1 and NTS_2 on the agonist binding of D_{2L} or D_3 were detected in the presence of neurotensin. To identify ligand-specific modulation and subtype-dependent differences, the novel dopamine receptor agonists **5** and **6** bearing the 7-OH-DPAT pharmacophore were synthesized. Exceptional ligand specificity was observed for D_3–NTS_2 co-expression, which gave a 20-fold decrease in affinity for biphenylcarboxamide **5** in the presence of neurotensin. Comparing the binding properties of dopaminergic compounds in the presence of neurotensin, dopamine receptor subtype-selective profiles of the cross-inhibitory effect of neurotensin were observed.

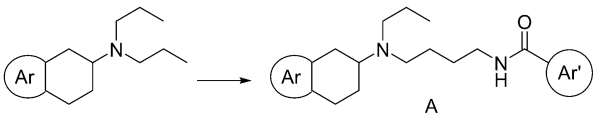
Introduction

The mechanistic understanding of G-protein-coupled receptors (GPCRs) to function as monomers has been extended by their ability to form homodimers, heterodimers, or higher-order oligomers.[1] Molecular interactions in these spatial complexes lead to modulation of ligand pharmacology, signal transduction, and cellular trafficking and thus may explain the observation of tissue-selective GPCR activity in many cases.[2]

Such a functional interaction between the neuromodulatory peptide neurotensin (NT, pE-L-Y-E-N-K-P-R-R-P-Y-I-L) and dopaminergic pathways in the CNS has been described to influence the pathophysiology of brain diseases including schizophrenia and Alzheimer's disease.[3] Previous studies of the impact of NT on the binding properties of dopamine and [^{3}H]*N*-*n*-propylnorapomorphine in dopamine D_2 receptor-rich brain areas reported a negative cooperative effect of the neuropeptide on dopamine receptor agonist properties.[4] Using the preferential dopamine D_3 receptor agonist [^{3}H]7-OH-DPAT (7-hydroxy-*N*,*N*-dipropyl-2-aminotetralin) to label subcortical limbic areas of the rat brain, NT was demonstrated to induce a 20% decrease in binding affinity and a slight increase in the number of available binding sites.[5] Given the enhanced influence of NT on D_3 receptor-controlled mesolimbic rather than nigrostriatal dopamine pathways, these findings indicate that the D_3 receptor subtype plays a crucial role in dopamine–neurotensin interactions.

Taking advantage of fluorescence-detected co-immunoprecipitation, we recently demonstrated a physical interaction and the formation of heterodimers between D_{2L} and the neurotensin receptor NTS_1.[6] By the use of a recombinant in vitro test system, a cross-inhibitory effect on the agonist binding affinity of D_2 was observed in the presence of NT. Structurally diverse ligands were investigated to determine the relationship between the intrinsic activity of dopaminergics and the modulatory effect of NTS_1–NT binding on the affinity of dopaminergics. To learn more about the tissue-selective modulation of the dopaminergic system, NT-induced modulation of D_3 receptors co-expressed with NTS_1 or NTS_2 and the D_{2L}–NTS_2 interaction should be investigated.

With the use of HEK293 cell lines co-expressing D_3–NTS_1, D_3–NTS_2, and D_{2L}–NTS_2, herein we report the binding profiles of dopamine and the dopamine receptor agonist 7-OH-DPAT and their dimer-specific modulation in the presence of neurotensin.[6] Binding-deficient NTS_1 mutants were used to investigate the role of the active receptor conformation of NTS_1 as a prerequisite for the trans-modulatory effect between the D_3 receptors and NT receptors. Based on recent investigations revealing that *N*-arylamidobutyl-substituted dopaminergics have excellent D_3 receptor binding and activating properties, we synthesized the 7-OH-DPAT congeners of type A (Scheme 1).[6,7] Employing these novel test compounds, the influence of structural changes on the NT-induced decrease in affinity was investi-



Scheme 1. Structural evolution of type A 7-OH-DPAT congeners.

[a] *S. Koschatzky, Prof. Dr. P. Gmeiner*
Department of Chemistry and Pharmacy
Emil Fischer Center, Friedrich Alexander University
Schuhstr. 19, 91052 Erlangen (Germany)
E-mail: peter.gmeiner@medchem.uni-erlangen.de

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.201100499.

gated, and subtype-selective differences for the negative cooperativity on dopaminergic binding affinities were identified.

Results and Discussion

Modulation of ligand binding affinity

Because of a broad overlap of protein expression in brain tissue, dopamine D_2 and D_3 receptors and neurotensin receptor subtypes NTS_1 and NTS_2 were chosen to be studied for their cross-receptor interactions. Analogously to our previously described in vitro test system co-expressing D_{2L} and NTS_1, we established co-expressing systems of human dopamine D_{2L} or D_3 receptors in combination with the human NT receptor subtypes NTS_1 or NTS_2 by transient transfection in HEK293 cells and performed radioligand binding studies to identify cooperative effects on binding affinities.

To ensure approximately equimolar receptor expression levels, transient HEK293 cell transfections were performed with equal amounts of cDNA for co-expression of D_3–NTS_1 and D_3–NTS_2 and with a fourfold excess of NTS_2 cDNA for the D_{2L}–NTS_2 system.

Radioligand displacement studies were performed with membrane preparations of cells singly expressing D_{2L}, D_3, NTS_1 or NTS_2, or a co-expression of a dopamine receptor subtype with NTS_1 or NTS_2. Orthosteric binding sites of NTS_1 and NTS_2 were determined by the use of [^{3}H]NT. No influence on the binding affinity of NT onto co-expressed neurotensin receptors was observed in the presence of the dopamine receptor agonist 7-OH-DPAT (Supporting Information). Hence, the agonist-bound dopamine receptor conformations of D_{2L} and D_3 were unable to alter the neurotensin receptor affinity for NT. Saturation binding experiments were performed to investigate the influence of NT on dopamine receptor antagonist binding of [^{3}H]spiperone. For all newly investigated co-expressing cell lines (D_3–NTS_1, D_3–NTS_2, and D_{2L}–NTS_2), NT did not induce changes in the binding properties of the antagonist-bound dopamine receptor conformation.

To determine the modulatory effects of NT on dopamine receptor agonist binding of co-expressed D_{2L} or D_3 receptors, homologous competition experiments were carried out to determine the equilibrium dissociation constant (K_D) values for [^{3}H]7-OH-DPAT in the presence and absence of NT. In analogy to our previously described D_{2L}–NTS_1 system, agonist binding of D_{2L} in the presence of NTS_2 was negatively modulated by the addition of NT, leading to a threefold increase in K_D from 6.9 to 21 nM (Table 1). Singly expressed D_{2L} receptors or mixed homogenates with singly expressed NTS_2 receptors did not show significant changes in affinity induced by NT.

By using the test system expressing D_3 and NTS_1, radioligand binding revealed a fourfold decrease in dopamine agonist affinity to the high-affinity binding site of D_3 receptors in the presence of NT, whereas the K_D values for singly expressed D_3 receptors, as well as the mixture of singly expressed D_3 and

Table 1. Affinities of the co-expressing cell lines reveal various effects in the presence of neurotensin using [^{3}H]7-OH-DPAT as radioligand.[a]

		K_D [nM][b] 7-OH-DPAT	K_i [nM] Dopamine	5	6
Co-expression D_{2L}–NTS_1	−NT	7.2 ± 0.6[c]	8.8 ± 1.2[c]	3.3 ± 1.0	1.4 ± 0.33
	+NT	62 ± 11[c]	82 ± 3.3[c]	29 ± 8.0[d]	15 ± 3.1[d]
	K_{i+NT}/K_{i-NT}	**8**	**9**	**9**	**11**
Co-expression D_{2L}–NTS_2	−NT	6.9 ± 1.8	13 ± 1.5	2.5 ± 0.42	0.57 ± 0.08
	+NT	21 ± 6.3[d]	130 ± 18[d]	19 ± 1.9[d]	4.2 ± 0.22[d]
	K_{i+NT}/K_{i-NT}	**3**	**9**	**7**	**7**
D_{2L}	−NT	8.4 ± 0.6	–	–	–
	+NT	8.9 ± 11[d]	–	–	–
	K_{i+NT}/K_{i-NT}	**1**	**–**	**–**	**–**
Mixture of Singly Expressed D_{2L} and NTS_2	−NT	7.0 ± 1.3	–	–	–
	+NT	5.3 ± 1.6	–	–	–
	K_{i+NT}/K_{i-NT}	**1**	**–**	**–**	**–**
Co-expression D_3–NTS_1	−NT	0.8 ± 0.01	5.8 ± 0.9	0.78 ± 0.15	0.72 ± 0.10
	+NT	3.7 ± 1.1[d]	46 ± 18[d]	8.7 ± 1.1[d]	3.7 ± 0.5[d]
	K_{i+NT}/K_{i-NT}	**4**	**8**	**11**	**5**
Mixture of Singly Expressed D_3 and NTS_1	−NT	0.47 ± 0.016	–	–	–
	+NT	0.75 ± 0.14	–	–	–
	K_{i+NT}/K_{i-NT}	**1.6**	**–**	**–**	**–**
Co-expression D_3–NTS_2	−NT	0.54 ± 0.08	4.1 ± 0.93	0.15 ± 0.04	0.68 ± 0.20
	+NT	1.9 ± 0.21[d]	27 ± 6.1[d]	3.1 ± 0.83[d]	2.4 ± 0.30[d]
	K_{i+NT}/K_{i-NT}	**3**	**6**	**20**	**3**
D_3	−NT	1.6 ± 0.020	–	–	–
	+NT	1.1 ± 0.014	–	–	–
	K_{i+NT}/K_{i-NT}	**0.7**	**–**	**–**	**–**
Mixture of Singly Expressed D_3 and NTS_2	−NT	1.0 ± 0.0010	–	–	–
	+NT	1.0 ± 0.11	–	–	–
	K_{i+NT}/K_{i-NT}	**1**	**–**	**–**	**–**
Co-expression D_3–NTS_1W129A	−NT	0.30 ± 0.010	–	–	–
	+NT	0.52 ± 0.05	–	–	–
	K_{i+NT}/K_{i-NT}	**1.7**	**–**	**–**	**–**
Co-expression D_3–NTS_1 W134A	−NT	0.47 ± 0.01	–	–	–
	+NT	0.41 ± 0.03	–	–	–
	K_{i+NT}/K_{i-NT}	**0.8**	**–**	**–**	**–**

[a] The affinities of investigated substances were determined on membrane preparations of transiently transfected HEK293 cells co-expressing D_{2L} or D_3 and NTS_1 or NTS_2 using [^{3}H]7-OH-DPAT for displacement experiments; final NT concentration was 100 nM for all +NT rows. Data represent the mean ± SEM and are derived from three to ten individual experiments, each done in triplicate. Hill slopes were between −0.80 and −1.20. The ratio K_{i+NT}/K_{i-NT} indicates the change in affinity. [b] K_D values were derived from three to six individual saturation experiments for D_3–NTS_1 and for D_3–NTS_2, or homologous displacement experiments for D_2–NTS_2, each done in triplicate; they were determined on membrane preparations of transiently transfected HEK293 cells using the radioligand [^{3}H]7-OH-DPAT. [c] Binding data according to Koschatzky et al.[6] [d] Significance was calculated in an unpaired *t* test relative to control experiments in the absence of NT: $p < 0.023$.

NTS_1, remained unchanged in the presence of NT. Finally, saturation experiments with a D_3–NTS_2 co-expressing cell line also demonstrated a negative cooperative effect, as indicated by an increase in the K_D value of 7-OH-DPAT from 0.54 to 1.9 nM by NT, relative to wild-type D_3 receptors. Agonist-bound NTS_1 and NTS_2 also exerted substantial negative cooperativity on the D_2 and D_3 binding of the endogenous ligand dopamine, as indicated by the K_{i+NT}/K_{i-NT} ratios between 6 and 9.

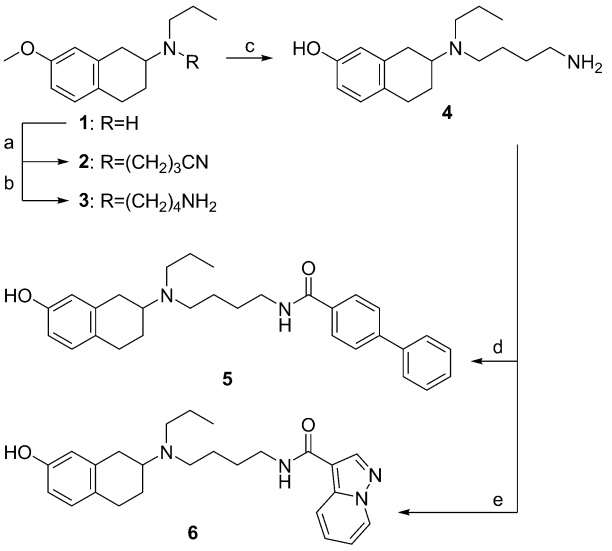
The unchanged binding affinities of the singly expressed D_3 receptors for NT-induced modulations underscore the requirement of co-expression and co-processing of both receptor subtypes in the cell membrane to facilitate an intramembrane receptor–receptor interaction. To confirm that the allosteric effect 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 NTS_1 (W129A and W134A), which show a complete loss in agonist binding of $[^3H]NT_{(8-13)}$, but which exhibit retention of antagonist binding of $[^3H]$SR4869.[8] We produced transiently co-expressing cell lines of D_3 and NTS_1W129A or NTS_1W134A. As expected, no specific binding of $[^3H]$NT was detected. On the other hand, the binding affinity of the dopamine receptor agonist 7-OH-DPAT remained unchanged in the presence of NT (100 nM). Thus, agonist binding of the neurotensin receptor and subsequent transition into the active receptor conformation seems necessary for the cooperative effects within the heterodimer.

Synthesis of 7-OH-DPAT analogues

Very recent SAR studies indicate that the replacement of a propyl substituent in the dopaminergic agent *N*,*N*-dipropylaminoindane with a biphenylcarboxamidobutyl group or a 7a-azaindole analogue thereof leads to highly potent dopamine D_2 and D_3 receptor agonists.[6,9] We planned to take advantage of this strategy to determine whether variously substituted 7-OH-DPAT analogues of type A show distinct susceptibility to the formation of heterodimers and, putatively, subtype-specific modulation profiles. Therefore, this required the preparation and investigation of molecular probes **5** and **6** for dimer-specific binding properties.[10] Synthesis of the new dopaminergics **5** and **6** was performed by starting from racemic 7-methoxy-2-(*N*-propylamino)tetralin (**1**).[11] Reduction of the carbonitrile function by lithium aluminum hydride afforded primary amine **3**. Subsequent ether cleavage yielded the hydroxytetralin derivative **4**, which served as a central intermediate. For the transformation of **4** into the final products **5** and **6**, N-acylation was carried out with 4-biphenylcarboxylic acid chloride and a TBTU-promoted coupling of pyrazolo[1,5-*a*]pyridine-3-carboxylic acid, respectively (Scheme 2).

Trans-modulatory effect on the binding affinities of the long-chain 7-OH-DPAT derivatives

To determine a correlation between structural modifications and the magnitude of the NT-induced cooperativity via cross-receptor interaction, test compounds **5** and **6** were evaluated



Scheme 2. *Reagents and conditions:* a) 4-bromobutyronitrile, K_2CO_3, NaI, CH_3CN, 24 h, reflux, (59%); b) $LiAlH_4$ in Et_2O, 6 h, RT, (92%); c) BBr_3 in CH_2Cl_2, 2 h, −78 °C, 16 h, RT; d) 4-biphenylcarboxylic acid chloride, NEt_3, CH_2Cl_2, 18 h, RT, (8%); e) pyrazolo[1,5-*a*]pyridine-3-carboxylic acid, TBTU, DIPEA, CH_2Cl_2, 4 h, RT, (15%).

for their binding behavior and heterodimer-selective susceptibilities toward NT and the co-expressing cell lines D_{2L}–NTS_1, D_{2L}–NTS_2, D_3–NTS_1, and D_3–NTS_2.

K_{i+NT}/K_{i-NT} ratios, which indicate the potency of a cooperative, NT-dependent effect for test compounds **5** and **6** relative to dopamine and our reference D_2/D_3 agonist 7-OH-DPAT, are depicted in Figure 1. For all four co-expressing cell lines, the endogenous ligand dopamine exhibits similar but marked decreases in dopamine binding affinities (between six- and ninefold) induced by NT bound to its receptor. The synthetic agonist 7-OH-DPAT displays a substantial (eightfold) decrease in affinity for the D_{2L}–NTS_1 system, whereas binding affinities for

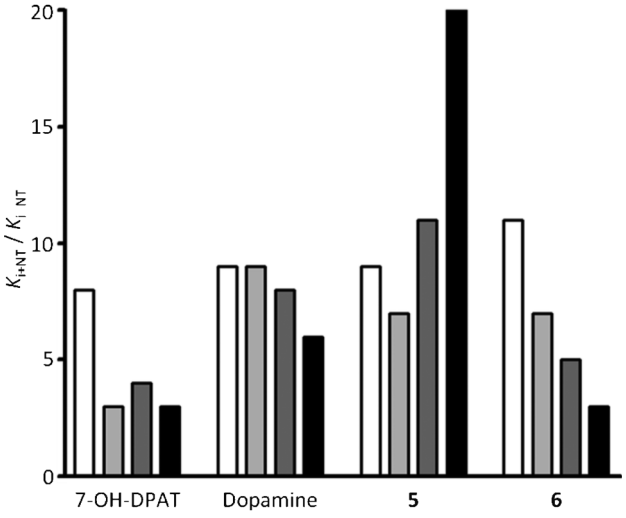


Figure 1. Subtype-dependent differences of the negative cooperativity on dopamine receptor agonist binding: □, D_{2L}–NTS_1; ■, D_{2L}–NTS_2; ■, D_3–NTS_1; ■, D_3–NTS_2.

D_3–NTS_1, D_3–NTS_2, and D_{2L}–NTS_2 were less influenced (three- to fourfold). The azaindole **6** induced stronger susceptibilities of binding affinities for the two D_{2L}-based heterodimers than to the D_3–$NTS_{1/2}$ systems. Interestingly, a significant decrease in affinity was observed for biphenylcarboxamide **5**, in the presence of NT, toward D_3–NTS_1 and D_3–NTS_2 co-expressing cell lines, with K_{i+NT}/K_{i-NT} values of 11 and 20, respectively.

Conclusions

Radioligand binding studies of the physical interaction between co-expressed dopamine D_{2L} or D_3 and neurotensin NTS_1 or NTS_2 receptors indicate substantial cross-inhibitory effects of both receptor subtypes NTS_1 and NTS_2 on the agonist binding of D_{2L} or D_3 in the presence of neurotensin. To identify ligand-specific modulation and subtype-dependent differences, the novel dopamine receptor agonists **5** and **6** bearing the 7-OH-DPAT pharmacophore were synthesized. Exceptional ligand specificity was observed for D_3–NTS_2 co-expression, which gave a 20-fold decrease in affinity for biphenylcarboxamide **5** in the presence of neurotensin. Dopamine receptor subtype-selective profiles of the cross-inhibitory effect of neurotensin were observed by comparing the binding properties of the dopaminergic agents in the presence of neurotensin. Subtype-selective differences of modulatory effects between dopamine and neurotensin receptor binding sites might support the development of tissue-selective dopaminergic drugs based on the concept of GPCR heteromers as biological targets of future drugs.

Experimental Section

Materials

All cell culture material was purchased from Invitrogen LifeTechnologies (Karlsruhe, Germany). The radioligands [^{3}H]spiperone (102–114 Ci mmol^{-1}) and [^{3}H]7-OH-DPAT (157–163 Ci mmol^{-1}) were purchased from GE Healthcare (Freiburg, Germany), and [^{3}H]neurotensin (100–112 Ci mmol^{-1}) was purchased from PerkinElmer (Rodgau, Germany). Dopamine (3,4-dihydroxyphenethylamine), spiperone, haloperidol, 7-OH-DPAT [(*R*)-(+)-7-hydroxy-2-(*N*,*N*-di-*n*-propylamino)tetralin hydrobromide], and other substances were purchased from Sigma (Steinheim, Germany), unless otherwise stated.

Biology

Expression vectors

Wild-type $hNTS_1$, $hNTS_2$, and hD_{2L} cDNA was purchased from the UMR cDNA Resource Center and subcloned into a pcDNA 3.1(+) eukaryotic expression vector (Invitrogen, Karlsruhe, Germany) using *EcoR1*/*Xba1* restriction sites.[8,12,13] The pcDNA3.1(+) of the hDRD3 receptor was used as described previously.[9a] Oligonucleotide primers were purchased from Biomers.net (http://www.biomers.net/index.html; Ulm, Germany). Restriction enzymes were purchased from New England Biolabs (Frankfurt am Main, Germany). The fidelity of PCR amplification and introduction of the fluorescence protein tags in the receptor cDNAs were confirmed by sequencing at LGC Genomics (Berlin, Germany).

Cell culture

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

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 or neurotensin receptor constructs, or a mixture of the cDNAs, by using TransIT-293 transfection reagent according to the protocol given by the manufacturer. Transfected cells were cultivated for 48 h. For the membrane preparations, the cell medium was removed, and cells were washed once with phosphate-buffered saline. The cell material was then abraded with a cell scraper and resuspended in 10 mL 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 pellet was resuspended in 5 mL 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 (20000 rpm, 5 times for 5 s each in an ice bath) and pelleted at 50000 *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^{-1} bacitracin, 5 μg mL^{-1} soybean trypsin inhibitor, pH 7.4) and homogenized with a Potter–Elvehjem homogenizer. Membrane preparations were stored at −80 °C in small aliquots. Protein concentration was determined by the method of Lowry et al. using bovine serum albumin as a standard.[14]

Saturation experiments with [^{3}H]7-OH-DPAT

Dopamine receptor agonist binding site saturation experiments with [^{3}H]7-OH-DPAT (specific activity 157–163 Ci mmol^{-1}) as radioligand were performed in 24-well plates with a total assay volume of 500 μL using six different concentrations of radioligand (0.1–5 nM). Total and unspecific binding were measured in the presence of buffer or 7-OH-DPAT (10 μM), respectively. To investigate the influence of neurotensin receptor agonist NT on dopaminergic binding, either substance or buffer was added to the reaction mixture. After the addition of the membrane homogenates (90 μg (mL protein)$^{-1}$), 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), and radioactivity counted in a MicroBeta Trilux instrument (PerkinElmer).

Homologous and non-homologous displacement experiments with [^{3}H]7-OH-DPAT

To determine binding at the high-affinity binding site of the dopamine receptors, the agonist [^{3}H]7-OH-DPAT (specific activity 157–163 Ci mmol^{-1}) was used as tritiated radioligand to determine the K_D and B_{max} values in homologous competition experiments, or K_i values of various test compounds in non-homologous competition experiments. Competition assays were performed in 96-well plates at a total volume of 200 μL. A solution of the [^{3}H]7-OH-DPAT radioligand (1.0 nM) was used for labeling of protein (90 μg mL^{-1} per well). Varying concentrations of unlabeled test compound (0.01–

100 000 nM) were added to the radioligand. To determine unspecific binding, 10 μM 7-OH-DPAT was used. Total binding was determined in the absence of test compound. After addition of the membrane homogenates, the mixture was incubated for 1 h at 37 °C. The assay was stopped by rapid filtration as described above.

Saturation experiments using [^{3}H]spiperone and [^{3}H]neurotensin

Membrane preparations of co-expressed or singly expressed dopamine or neurotensin receptors in HEK293 cells were incubated in 96-well plates with 10 different concentrations (0.005–2 nM) of the tritiated dopamine receptor antagonist [^{3}H]spiperone (specific activity 102–114 Ci mmol^{-1}) or [^{3}H]NT (specific activity 100–112 Ci mmol^{-1}). Nonspecific binding was defined in the presence of 10 μM haloperidol or NT, respectively, and total binding was measured in the absence of any competing drug. To investigate the influence of various NTS_1 or D_3 receptor ligands, either substance or buffer was added to the reaction mixture. After addition of membrane preparations with protein concentrations of 40 μg mL^{-1}, the assay mixture was incubated for 30–60 min at 37 °C and stopped by rapid filtration and further processed as described above for [^{3}H]7-OH-DPAT.

Data analysis

Analyses of the saturation experiments were performed by nonlinear regression data analysis for the determination of K_D and B_{max} values using Prism (GraphPad Software, San Diego, CA, USA). The resulting competition curves were analyzed by nonlinear regression using the algorithms in Prism. The data were initially fitted using a sigmoid model and an IC_{50} value, representing the concentration corresponding to 50% maximal inhibition. Data were then calculated for a one-site model using Prism. IC_{50} values were transformed into K_i values according to the equation for the calculation of competition curves as described by Cheng and Prusoff.[15] Data are presented as mean ± SEM. Significances were calculated in an unpaired *t* test relative to control experiments in the absence of NT.

Chemistry

General

Reagents and dry solvents were of commercial quality and were used as purchased. All reactions were carried out under nitrogen atmosphere. MS was performed on a JEOL JMS-GC Mate II spectrometer by EI (70 eV) with solid inlet or a Bruker Esquire 2000 by APC or ionization. HR-EIMS analyses were run on a JEOL JMS-GC Mate II using Peak-Matching ($M/\Delta M > 5000$). NMR spectra were collected on a Bruker Avance 360 or a Bruker Avance 600 spectrometer relative to TMS in the solvents indicated (*J* values in Hz). IR spectra were run on a Jasco FT/IR 410 spectrometer. Melting points were determined with a MEL-TEMP II melting-point apparatus (Laboratory Devices, USA) in open capillaries and are uncorrected. Preparative RP-HPLC (Agilent 1100 preparative series) was performed under the following conditions: column: Zorbax Eclipse XDB-C_8, 21.2×250 mm, 5 μm particle size; eluent: CH_3OH (A) and 0.1% TFA in H_2O (B); flow rate: 20 mL min^{-1}; UV detection at λ 254 nm.

TLC was performed with Merck 60 F_{254} aluminum sheets, and analysis was by UV light (λ 254 nm). Analytical HPLC was performed on Agilent 1100 HPLC systems equipped with a VWL detector and a Zorbax Eclipse XDB-C_8 column (4.6 mm×150 mm, 5 μm). Purity was determined by using the aforementioned binary solvent system: A/B (10:90 *v*/*v*) to 100% A over 21 min, isocratic 100% A for 3 min; flow rate: 1.0 mL min^{-1}; $\lambda = 254$ nm.

4-[(7-methoxy-1,2,3,4-tetrahydronaphthalen-2-yl)-(propyl)amino]butanenitrile, 2: 4-Bromobutyronitrile (0.857 mL, 8.54 mmol) was added dropwise to a suspension of compound **1** (781 mg, 3.56 mmol), KI (533 mg, 3.2 mmol), and K_2CO_3 (2.76 g, 8.9 mmol) in CH_3CN (60 mL). After being held at reflux for 24 h, the mixture was allowed to cool to room temperature, and the solvent was evaporated. The residue was dissolved in H_2O, acidified with an excess of 2 N HCl, and extracted with Et_2O. The aqueous solution was basified with an excess of 2 N NaOH and extracted with Et_2O. The combined organic layers were dried ($MgSO_4$) and evaporated to give **2** as a yellow liquid (591 mg, 59%). ^{1}H NMR (360 MHz, $CDCl_3$): $\delta = 0.92$ (t, $J = 7.4$ Hz, 3H), 1.44–1.55 (m, 2H), 1.57–1.70 (m, 1H), 1.75–1.85 (m, 2H), 1.96–2.04 (m, 1H), 2.43–2.52 (m, 4H), 2.66 (t, $J = 6.44$ Hz, 2H), 2.71–3.00 (m, 5H), 3.80 (s, 3H), 6.65 (d, $J = 2.6$, 1H), 6.71 (dd, $J = 8.3$, 2.7 Hz, 1H), 7.01 ppm (d, $J = 8.3$ Hz, 1H); ^{13}C NMR (90 MHz, $CDCl_3$): $\delta = 11.85$, 14.57, 22.25, 24.91, 26.04, 29.04, 32.31, 48.38, 52.35, 55.30, 56.38, 112.22, 113.94, 120.17, 128.49, 129.47, 137.55, 157.63 ppm; IR $\tilde{\nu} = 2956$s, 2931s, 2244w, 1503s, 1262m cm^{-1}; MS (EI) *m*/*z* 286; purity 99% (HPLC).

***N*-(4-aminobutyl)-7-methoxy-*N*-propyl-1,2,3,4-tetrahydronaphthalen-2-amine, 3**: A solution of $LiAlH_4$ (1 M) in Et_2O (6.3 mL, 6.3 mmol) was added dropwise to a cooled solution of **2** (360 mg, 1.26 mmol) in THF (15 mL). After stirring at room temperature for 1 h, the mixture was cooled to 0 °C, quenched with aqueous $NaHCO_3$, filtered over Celite/Mg_2SO_4/Celite, and washed several times with CH_2Cl_2 and EtOAc. The solvent was evaporated to give **3** as a yellow liquid (336.6 mg, 92% yield). ^{1}H NMR (360 MHz, $CDCl_3$): $\delta = 0.92$ (t, $J = 7.3$ Hz, 3H), 1.47–1.74 (m, 7H), 2.00–2.07 (m, 1H), 2.11–2.39 (m, 2H), 2.47–2.65 (m, 4H) 2.71–2.92 (m, 5H), 2.95–3.08 (m, 1H), 3.80 (s, 3H), 6.66 (d, $J = 2.7$, 1H), 6.71 (dd, $J = 8.3$, 2.7 Hz, 1H), 7.01 ppm (d, $J = 8.3$ Hz, 1H); ^{13}C NMR (90 MHz, $CDCl_3$): $\delta = 11.93$, 22.15, 26.09, 26.46, 28.97, 29.06, 32.37, 42.06, 50.49, 52.60, 55.29, 56.73, 112.10, 113.96, 128.71, 129.46, 137.81, 157.53 ppm; IR $\tilde{\nu} = 2930$s, 2868m, 2361m, 1503s, 1262m cm^{-1}; MS (EI) *m*/*z* 290; purity 95% (HPLC).

7-[(4-aminobutyl)(propyl)amino]-5,6,7,8-tetrahydronaphthalen-2-ol, 4: Compound **3** (720 mg, 2.5 mmol) was added to a solution of BBr_3 (1 M; 11.3 mL, 11.3 mmol) in CH_2Cl_2, which was pre-cooled at −78 °C, and stirring was continued at −78 °C for 2 h. The mixture was then stirred for 23 h at room temperature. After quenching with an excess of aqueous $NaHCO_3$ for 30 min, the aqueous layer was adjusted to mild basic, and was extracted three times with CH_2Cl_2. The combined organic layers were dried ($MgSO_4$) and evaporated. The crude product of **4** was not further purified (101.5 mg). LC–MS (APCI) *m*/*z* 276.

***N*-{4-[(7-hydroxy-1,2,3,4-tetrahydronaphthalen-2-yl)-(propyl)amino]butyl}-4-phenylbenzamide, 5**: Compound **4** (53.3 mg, 0.19 mmol) was dissolved in 6 mL dry CH_2Cl_2, and 0.074 mL Et_3N (0.57 mmol) was added. The mixture was cooled to 0 °C. A solution of 4-biphenylcarboxylic acid chloride (39.7 mg, 0.18 mmol) in dry CH_2Cl_2 (6 mL) was then added dropwise. The mixture was stirred for 18 h at room temperature before aqueous $NaHCO_3$ was added. The aqueous layer was extracted with CH_2Cl_2, and the combined organic layers were dried ($MgSO_4$) and evapo-

rated. The residue was purified by preparative RP-HPLC (gradient: 10→90% A over 16 min, then 90% A for 2 min, 90→10% A over 4 min, t_R: 16.0 min) to give **5** as a gray–white solid (7 mg, 8% referenced to crude product **5**); mp: 106 °C; ^{1}H NMR (360 MHz, $CDCl_3$): δ = 0.89 (t, J = 7.3 Hz, 3H), 1.50 (tq, J = 7.5, 7.5 Hz, 2H), 1.55–1.73 (m, 5H), 1.98 −2.04 (m, 1H), 2.51 (t, J = 7.6 Hz, 2H), 2.60 (t, J = 7.0, 2H), 2.68–2.84 (m, 4H), 2.97–3.04 (m, 1H), 3.51 (dt, J = 6.4, 6.5 Hz, 2H), 6.55 (d, J = 2.5 Hz, 1H), 6.62 (dd, J = 8.2, 2.6 Hz, 1H), 6.92 (d, J = 8.2 Hz, 1H), 7.40 (tt, J = 7.4, 1.1 Hz, 1H), 7.47 (tt, J = 7.5, 1.5 Hz, 2H), 7.60–7.62 (m, 2H), 7.65 (tt, J = 8.5, 1.5 Hz, 2H), 7.85 ppm (tt, J = 8.2, 1.0 Hz, 2H); ^{13}C (90 MHz, $CDCl_3$): δ = 11.93, 21.68, 25.80, 26.14, 27.52, 28.94, 31.98, 40.06, 50.01, 52.58, 56.83, 113.36, 115.65, 127.19, 127.46, 127.90, 128.90, 129.54, 133.46, 137.53, 140.04, 153.83, 167.51 ppm; IR $\tilde{\nu}$ = 3299s, 2930s, 1635s, 1541s, 1506s, 1485s, 1263m cm^{-1}; HRMS (EI) *m/z* calcd for $C_{30}H_{36}N_2O_2$: 456.2777, found 456.2777; purity 98% (HPLC).

***N*-{4-[(7-hydroxy-1,2,3,4-tetrahydronaphthalen-2-yl)-(propyl)amino]butyl}pyrazolo[1,5-*a*]pyridine-3-carboxamide, 6**: DIPEA (109 μL, 0.66 mmol) was added to a solution of pyrazolo[1,5-*a*]pyridine-3-carboxylic acid (34.5 mg, 0.21 mmol) in CH_2Cl_2 (6 mL). The mixture was cooled to 0 °C before a solution of TBTU (95.3 mg, 0.297 mmol) in DMF (1 mL) was added. A solution of **4** (76.2 mg, 0.27 mmol) in CH_2Cl_2 (6 mL) was added dropwise. The mixture was stirred at room temperature for 3 h, before aqueous $NaHCO_3$ was added. The aqueous layer was extracted with CH_2Cl_2, and the combined organic layers were dried ($MgSO_4$) and evaporated. The residue was purified by preparative RP-HPLC (gradient: 10→80% A over 15 min, then 80% A for 2 min, 80→10% A over 3 min, t_R: 12.4 min) to give **6** as a gray–white solid (17 mg, 15% referenced to crude product **5**); mp: 156 °C; ^{1}H NMR (360 MHz, $CDCl_3$): δ = 0.88 (t, J = 7.4 Hz, 3H), 1.59 (tq, J = 7.6, 7.6 Hz, 2H), 1.52–1.70 (m, 5H), 1.99–2.04 (m, 1H), 2.54 (t, J = 7.7 Hz, 2H), 2.63 (t, J = 6.9 Hz, 2H), 2.65–2.81 (m, 4H), 3.00–3.01 (m, 1H), 3.49 (dq, J = 6.5, 6.5 Hz, 2H), 6.54 (d, J = 2.5 Hz, 1H), 6.55–6.59 (m, 1H), 6.63 (dd, J = 8.2, 2.64 Hz, 1H), 6.88 (d, J = 8.2 Hz, 1H), 6.91 (ddd, J = 6.9, 6.9, 1.4 Hz, 1H), 7.33 (ddd, J = 8.9, 6.8, 1.1 Hz, 1H), 8.23 (s, 1H), 8.31 (dt, J = 9.0, 1.2 Hz, 1H), 8.47 ppm (ddd, J = 7.0, 1.0, 1.0 Hz, 1H); ^{13}C (90 MHz, $CDCl_3$): δ = 11.85, 21.31, 25.63, 25.71, 27.62, 28.77, 31.86, 39.27, 50.09, 52.59, 57.13, 106.90, 113.60, 113.65, 115.70, 119.62, 126.49, 127.69, 128.78, 129.51, 140.52, 140.66, 154.27, 163.68 ppm; IR $\tilde{\nu}$ = 3307s, 2932s, 1637s, 1557s, 1503s, 1273m cm^{-1}; HRMS (EI) *m/z* calcd for $C_{25}H_{32}N_4O_2$: 420.2525, found 420.2526; purity 99% (HPLC).

Acknowledgements

We gratefully acknowledge the Deutsche Forschungsgemeinschaft for financial support (DFG 13/8). Manuel Plomer is acknowledged for providing the cDNA of hNTS$_2$, and Dr. Harald Hübner, for reference binding data.

Keywords: dopamine · GPCRs · heterodimers · neurotensin · receptors

[1] a) S. R. George, B. F. O'Dowd, S. P. Lee, *Nat. Rev. Drug Discovery* **2002**, *1*, 808–820; b) G. Milligan, *Mol. Pharmacol.* **2004**, *66*, 1–7; c) G. Milligan, *Biochim. Biophys. Acta Biomembr.* **2007**, *1768*, 825–835.

[2] a) N. J. Birdsall, *Trends Pharmacol. Sci.* **2010**, *31*, 499–508; b) J. P. Vilardaga, V. O. Nikolaev, K. Lorenz, S. Ferrandon, Z. Zhuang, M. J. Lohse, *Nat. Chem. Biol.* **2008**, *4*, 126–131; c) D. J. Daniels, N. R. Lenard, C. L. Etienne, P.-Y. Law, S. C. Roerig, P. S. Portoghese, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 19208–19213; d) J. Hillion, M. Canals, M. Torvinen, V. Casadó, R. Scott, A. Terasmaa, A. Hansson, S. Watson, M. E. Olah, J. Mallol, E. I. Canela, M. Zoli, L. F. Agnati, C. F. Ibánez, C. Lluis, R. Franco, S. Ferré, K. Fuxe, *J. Biol. Chem.* **2002**, *277*, 18091–18097; e) R. Rozenfeld, L. A. Devi, *Trends Pharmacol. Sci.* **2010**, *31*, 124–130; f) J.-P. Pin, R. Neubig, M. Bouvier, L. Devi, M. Filizola, J. A. Javitch, M. J. Lohse, G. Milligan, K. Palczewski, M. Parmentier, M. Spedding, *Pharmacol. Rev.* **2007**, *59*, 5–13; g) A. J. Rashid, B. F. O'Dowd, S. R. George, *Endocrinology* **2004**, *145*, 2645–2652; h) J. Kühhorn, H. Hübner, P. Gmeiner, *J. Med. Chem.* **2011**, *54*, 4896–4903; i) W. Guo, E. Urizar, M. Kralikova, J. C. Mobarec, L. Shi, M. Filizola, J. A. Javitch, *EMBO J.* **2008**, *27*, 2293–2304; j) C. Jomphe, P.-L. Lemelin, H. Okano, K. Kobayashi, L.-E. Trudeau, *Eur. J. Neurosci.* **2006**, *24*, 2789–2800.

[3] a) E. B. Binder, B. Kinkead, M. J. Owens, C. B. Nemeroff, *Pharmacol. Rev.* **2001**, *53*, 453–486; b) M. Boules, A. Shaw, P. Fredrickson, E. Richelson, *CNS Drugs* **2007**, *21*, 13–23; c) K. Fuxe, D. Marcellino, A. S. Woods, L. Giuseppina, T. Antonelli, L. Ferraro, S. Tanganelli, L. F. Agnai, *J. Neural Transm.* **2009**, *116*, 923–939.

[4] a) G. von Euler, P. Mailleux, J.-J. Vanderhaeghen, K. Fuxe, *Neurosci. Lett.* **1990**, *109*, 325–330; b) S. Tanganelli, X.-M. Li, L. Ferraro, G. von Euler, W. T. O'Connor, C. Bianchi, L. Beani, K. Fuxe, *Eur. J. Pharmacol.* **1993**, *230*, 159–166; c) X.-M. Li, L. Ferraro, S. Tanganelli, W. T. O'Connor, U. Hasselrot, U. Ungerstedt, K. Fuxe, *J. Neural Transm.* **1995**, *102*, 125–137; d) L. F. Agnati, K. Fuxe, F. Benfenati, N. Basttistini, *Acta Physiol. Scand.* **1983**, *119*, 459–461; e) G. von Euler, K. Fuxe, *Acta Physiol. Scand.* **1987**, *131*, 625–626.

[5] Y. Liu, M. Hillefors-Berglund, G. von Euler, *Brain Res.* **1994**, *643*, 343–348.

[6] S. Koschatzky, N. Tschammer, P. Gmeiner, *ACS Chem. Neurosci.* **2011**, *2*, 308–316.

[7] a) I. Boyfield, M. C. Coldwell, M. S. Hadley, C. N. Johnson, G. J. Riley, E. E. Scott, R. Stacey, G. Stemp, K. M. Thewlis, *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1995–1998; b) N. Tschammer, M. Dörfler, H. Hübner, P. Gmeiner, *ACS Chem. Neurosci.* **2010**, *1*, 25–35.

[8] S. Härterich, S. Koschatzky, J. Einsiedel, P. Gmeiner, *Bioorg. Med. Chem.* **2008**, *16*, 9359–9368.

[9] a) M. Dörfler, N. Tschammer, K. Hamperl, H. Hübner, P. Gmeiner, *J. Med. Chem.* **2008**, *51*, 6829–6838; b) N. Tschammer, J. Elsner, A. Goetz, K. Ehrlich, S. Schuster, M. Ruberg, J. Kühhorn, D. Thompson, J. Whistler, H. Hübner, P. Gmeiner, *J. Med. Chem.* **2011**, *54*, 2477–2491.

[10] G. Stemp, C. N. Johnson, Patent, **1998**, EP0817767.

[11] L. A. van Vliet, P. G. Tepper, D. Dijkstra, G. Damsma, H. Wikstroem, T. A. Pugsley, H. C. Akunne, T. G. Heffner, S. A. Glase, L. D. Wise, *J. Med. Chem.* **1996**, *39*, 4233–4237.

[12] K. Ehrlich, A. Götz, S. Bollinger, N. Tschammer, L. Bettinetti, S. Härterich, H. Hübner, H. Lanig, P. Gmeiner, *J. Med. Chem.* **2009**, *52*, 4923–4935.

[13] J. Einsiedel, C. Held, M. Hervet, M. Plomer, N. Tschammer, H. Hübner, P. Gmeiner, *J. Med. Chem.* **2011**, *54*, 2915–2923.

[14] O. H. Lowry, N. R. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **1951**, *193*, 265–275.

[15] Y.-C. Cheng, W. H. Prusoff, *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.

Received: October 27, 2011
Revised: December 5, 2011
Published online on December 23, 2011

