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Bivalent Dopamine D_2 Receptor Ligands: Synthesis and Binding Properties

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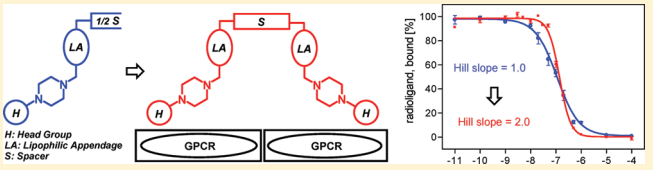
Supporting Information

ABSTRACT: Dopamine D_2 receptor homodimers might be of particular importance in the pathophysiology of schizophrenia and, thus, serve as promising target proteins for the discovery of atypical antipsychotics. A highly attractive approach to investigate and control GPCR dimerization may be provided by the exploration and characterization of bivalent ligands, which can act as molecular probes simultaneously binding two adjacent binding sites of a dimer. The synthesis of bivalent dopamine D_2 receptor ligands of type **1** is presented, incorporating the privileged structure of 1,4-disubstituted aromatic piperidines/piperazines (1,4-DAPs) and triazolyl-linked spacer elements. Radioligand binding studies provided diagnostic insights when Hill slopes close to two for bivalent ligands with particular spacer lengths and a comparative analysis with respective monovalent control ligands and unsymmetrically substituted analogues indicated a bivalent binding mode with a simultaneous occupancy of two neighboring binding sites.

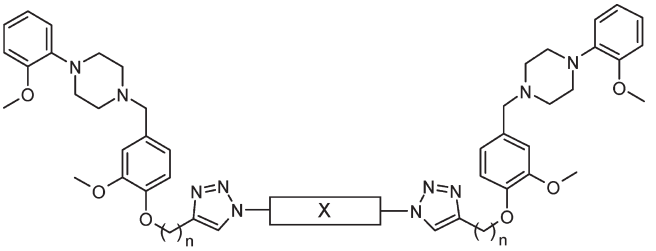


INTRODUCTION

G-protein-coupled receptors (GPCRs) have been traditionally considered to exist as monomers. However, in recent years, evidence has accumulated to suggest that GPCRs can cross-react forming homo- or heterodimers or higher-order oligomers.[1,2] Moreover, receptor dimerization is often essential for receptor function and can also modulate ligand pharmacology, signal transduction, and cellular trafficking.[3] Previous investigations indicate that dopamine D_2 receptors, which are associated with many central nervous system diseases including schizophrenia, Parkinson's disease, drug addiction, and erectile dysfunction, exist as homomeric[4−8] or heteromeric[9,10] complexes. Using functional complementation assays, communication between protomers of the D_2 receptors could be observed leading to a significant modulation of GPCR activation in transfected cell lines.[11] According to very recent findings,[12] homodimers of dopamine D_2 receptors might be of particular importance in the pathophysiology of schizophrenia and, thus, serve as promising targets for the discovery of atypical antipsychotics. Investigating postmortem striatal sections from schizophrenia patients and striatal tissue of animal models of schizophrenia, the expression of D_2 dimers proved to be significantly enhanced, while expression of D_2 monomers was decreased. Further studies in this exciting field will request the invention of new methodologies and selective pharmacological tools.

A highly attractive approach to investigate and control GPCR dimerization may be provided by the exploration and characterization of bivalent ligands, which can act as molecular probes simultaneously binding two adjacent binding sites of a dimer. Containing two identical or distinct pharmacophores linked by a spacer, bivalent ligands can either address homomers[13−18] or heteromers.[19−22] Employing bivalent ligands as pharmacological tools, a differential analysis of homo- and heteromers, tissue-selective modulation, and a reversible control of monomer/dimer equilibriums may be facilitated. In this paper, we describe the synthesis of homodimeric dopamine D_2 receptor ligands of type **1** (Chart 1) incorporating the privileged structure of 1,4-disubstituted aromatic piperidines/piperazines (1,4-DAPs) and triazolyl-linked spacer elements. Radioligand binding studies are also reported when Hill slopes close to two for homodimeric ligands with particular spacer lengths and a comparative analysis with respective monomers and unsymmetrically substituted analogues indicated a bivalent binding mode with a simultaneous occupancy of two adjacent binding sites.

Chart 1. General Structure of the Dopamine D_2 Receptor Bivalent Ligands



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RESULTS AND DISCUSSION

Design. The biological properties of 1,4-DAPs are encoded by an aromatic headgroup, which controls intrinsic activity, and

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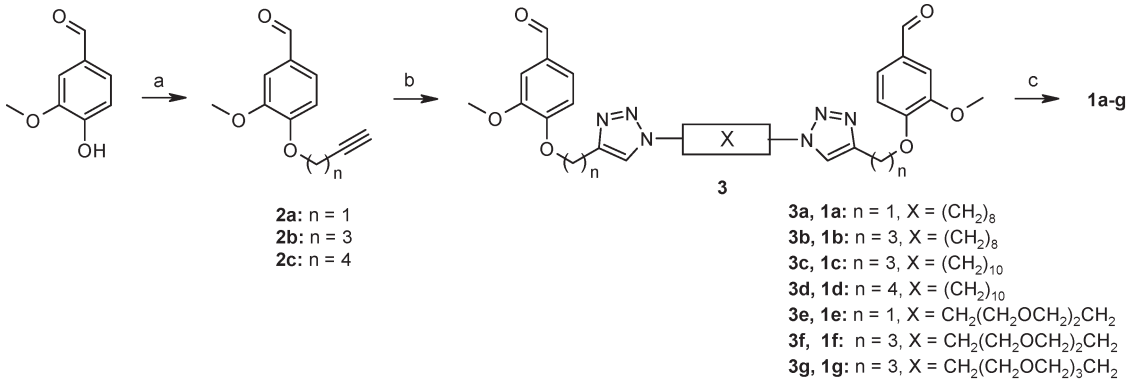
Scheme 1[a]



[a] Reagents and conditions: (a) 3-bromo-1-propyne, 5-chloro-1-pentyne, or 6-chloro-1-hexyne, K_2CO_3, KI, CH_3CN, reflux, 16−24 h (47−85%); (b) 1,8-diazidooctane, 1,10-diazidododecane, 1,2-bis(2-azidoethoxy)ethane, or 1,1′-oxybis[2-(2-azidoethoxy)ethane], sodium ascorbate, $CuSO_4$, H_2O/^{t}BuOH/CH_2Cl_2 1:2:2, RT, 20 h (65−97%); (c) 2-methoxyphenylpiperazine, $Na(OAc)_3BH$, CH_2Cl_2, RT, 16 h (38−73%).

an amine moiety, which is responsible for the formation of a reinforced hydrogen bond to the crucial residue Asp$^{3.32}$ in the transmembrane helix 3 (TM3). Thus, the phenylpiperidine/phenylpiperazine scaffold simulates the endogenous biogenic amine.[23] Besides this, a lipophilic appendage is necessary enhancing ligand affinity. According to recent mutagenesis and molecular docking studies, the lipophilic appendage, which is usually represented by a heterocyclic system, directs to a hydrophobic microdomain that is formed by the extracellular portion of TM2, TM3, and TM7 and specific residues of the extracellular loop 2 (EL2).[24−26] Because a formal elongation of this appendage is expected to lead to the "entrance region" of the receptor and from there to the binding pocket of a neighboring protomer, the *para*-position of an aromatic moiety terminating the lipophilic appendage was considered to be perfectly applicable as a point of attachment for a linker unit. Instead of heterocyclic systems that we used for our originally described monomeric molecular probes, we chose a vanilline-derived carbocyclic moiety that could be easily reacted in the *para*-position with a part of the linker sequence by a Williamson ether synthesis and attached to the phenylpiperazine moiety by reductive amination. Dimerization and construction of a symmetric linker by click chemistry should result in spacer units of approximately 25 Å, which is in agreement with structure−activity relationships of recently described bivalent GPCR ligands.[21,27]

Chemical Synthesis. A collection of homodimeric dopamine receptor ligands with spacers built from 18−26 atoms was synthesized. Because conformational flexibility and physicochemical properties of the spacer seem to be essential for high specific binding,[18,28] flexible hydrophobic and hydrophilic spacer elements were implemented. The chemical composition of the bridging moiety was varied using hydrophobic linkers composed of methylene units as well as hydrophilic spacers incorporating oligo(ethylene glycol) units. As a dopamine surrogate, we employed *ortho*-methoxyphenylpiperazine that was attached to a benzylic CH_2 position of a carbocyclic arene moiety.[29] For comparative purposes, monovalent fragments containing one-half of the linker and asymmetric "dummy ligands" incorporating one original pharmacophore while the second pharmacophore is replaced by a structurally similar nonbinding motif were synthesized.

In detail, our central building block vanilline bearing a free hydroxyl group in the *para*-position of the carbaldehyde was converted to the corresponding alkynylethers **2a**−**c** by alkylation with 3-bromo-1-propyne, 5-chloro-1-pentyne, or 6-chloro-1-hexyne in the presence of K_2CO_3 and KI.[30] Employing copper(I)-catalyzed azide−alkyne cycloaddition (CuAAC),[31] the terminal alkynes **2a**−**c** were reacted with 1,8-diazidooctane,[32] 1,10-diazidododecane,[32] 1,2-bis(2-azidoethoxy)ethane,[33] or 1,1′-oxybis-[2-(2-azidoethoxy)ethane][33] to afford the synthetic intermediates **3a**−**g** in good to excellent yields (65−97%). Reductive amination of **3a**−**g** with *ortho*-methoxyphenylpiperazine furnished the final products **1a**−**g** in 38−73% yield (Scheme 1).

For the preparation of the monovalent ligands **4a**−**c** containing capped spacers, the central building blocks **2a**−**c** were reacted with butylazide[34] and reductively aminated by *ortho*-methoxyphenylpiperazine (Scheme 2). To efficiently synthesize the oxa-analogues **6a**,**b**, the reaction sequence was inverted when the final triazole formation was done using an excess of 1-azido-2-ethoxyethane. The synthesis of the asymmetric molecular probes of type **8** and **9** started from the terminal alkyne **5b**, which was subjected to CuAAC using a 5-fold excess of 1,8-diazidooctane to afford the intermediate **7** in 52% yield. The synthetic intermediates **5c**,**d** were obtained by reductive amination of the vanilline derivative **2b** with either methyl- or cyclohexylpiperazine. Cycloaddition of the azide **7** and the alkynes **5c**,**d** and pentyne afforded the asymmetric ligands **9a**,**b** and **8**, respectively.

Receptor Binding. In vitro binding affinity of the molecular probes **1a**−**g**, along with their monovalent control agents **4a**−**c**, **6a**,**b**, **8**, **9a**,**b**, and the reference drug haloperidol was measured by displacement of the radioligand [^{3}H]spiperone from human D_{2long}, D_{2short}, D_3, and $D_{4.4}$ receptors stably expressed in Chinese hamster ovary cells (CHO). D_1 receptor binding affinities were determined utilizing striatal membranes and the D_1 selective radioligand [^{3}H]SCH 23390. Our initial investigations were directed to a careful comparison of the dimeric ligands **1a** and **1b** displaying oxymethylene and oxypropylene units, respectively, between the triazole and the benzene rings with their monovalent analogues **4a**,**b**. The test compounds gave significant specific binding for D_{2long}, D_{2short}, D_3, and D_4 (Table 1) and clearly reduced D_1 affinity (Table 1). For both variants of the D_2 subtype, K_i values in the double-digit nanomolar range were observed with slightly higher affinities for the dimeric test compounds. D_3 affinity was substantially lower with K_i values of 31−84 nM for the dimers and 320−610 nM for the monomeric analogs. K_i values of 5.4−15 nM and 3.3−7.2 nM for **1a**,**b** and **4a**,**b**, respectively, indicated that D_4 binding was high for both dimeric and monomeric ligands. Whereas **1a**,**b** displayed only a 5-fold preference for D_4

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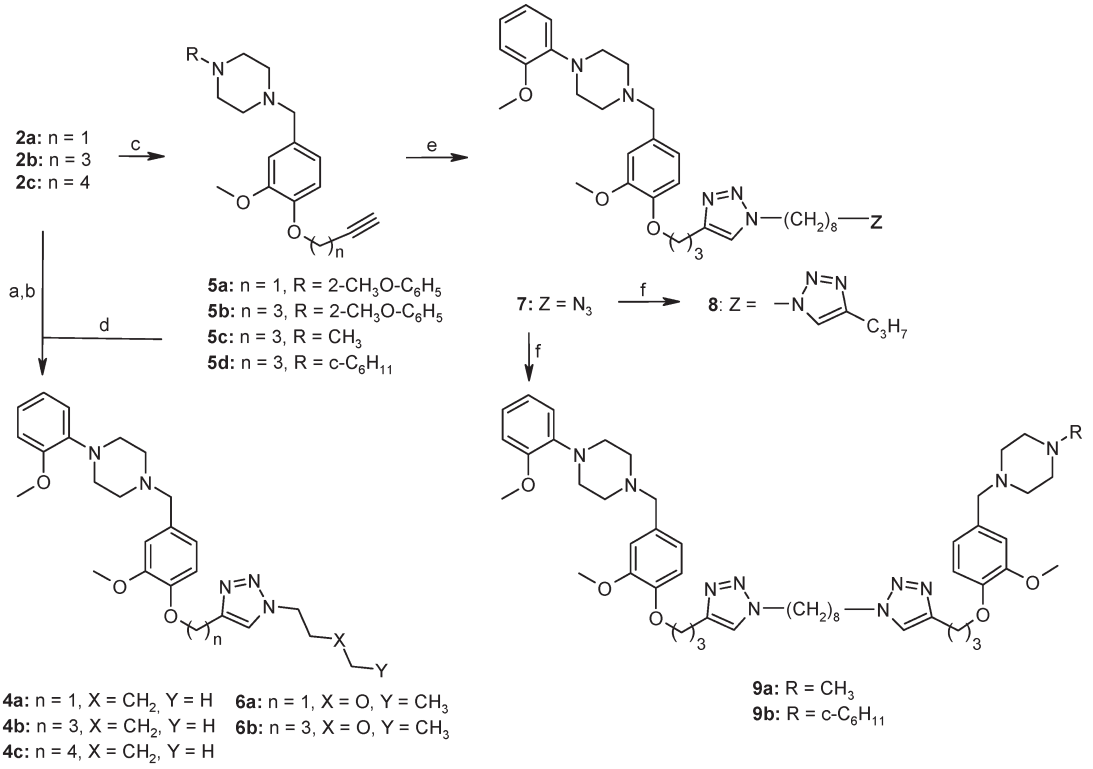
Scheme 2[a]



[a] Reagents and conditions: (a) butyl azide, sodium ascorbate, $CuSO_4$, H_2O:^{t}BuOH:CH_2Cl_2 1:2:2, RT, 16 h (60−94%); (b) 2-methoxyphenylpiperazine, $Na(OAc)_3BH$, CH_2Cl_2, RT, 14 h (68−79%); (c) 2-methoxyphenylpiperazine or methylpiperazine or cyclohexylpiperazine, $Na(OAc)_3BH$, CH_2Cl_2, RT, 16 h (69−98%); (d) **5a** or **5b**, 1-azido-2-ethoxyethane, sodium ascorbate, $CuSO_4$, CH_2Cl_2:MeOH 4:1 (73−83%); (e) **5b**, 1,8-diazidooctane, sodium ascorbate, $CuSO_4$, CH_2Cl_2:MeOH 4:1, RT, 42 h (52%); (f) pentyne or **5c** or **5d**, sodium ascorbate, $CuSO_4$, CH_2Cl_2:MeOH 4:1, RT, 16 h (58−81%).

Table 1. Receptor Binding Data for the Bivalent Compounds and Their Monomeric Counterparts in Comparison to the Reference Haloperidol Employing Porcine Dopamine D_1 Receptor and the Human Subtypes D_{2long}, D_{2short}, D_3, and $D_{4.4}$

				K_i values[a] (nM) ± SEM and Hill slopes[b] [absolute value of n_H ± SEM]				
				[^{3}H]SCH 23390		[^{3}H]spiperone		
compd	n	spacer atoms	X	pD_1	hD_{2long}	hD_{2short}	hD_3	$hD_{4.4}$
1a	1	18	$(CH_2)_8$	210 ± 28	16 ± 2.1 [1.6 ± 0.1]	25 ± 8.4 [1.7 ± 0.1]	31 ± 3.5 [1.5 ± 0.1]	5.4 ± 0.78 [1.5 ± 0.1]
1b	3	22	$(CH_2)_8$	220 ± 61	22 ± 2.4 [2.0 ± 0.1]	41 ± 8.2 [2.0 ± 0.1]	84 ± 11 [1.6 ± 0.1]	15 ± 1.3 [1.6 ± 0.1]
1c	3	24	$(CH_2)_{10}$	250 ± 77	23 ± 4.0 [1.6 ± 0.1]	17 ± 4.0 [1.7 ± 0.2]	96 ± 15 [1.6 ± 0.2]	19 ± 4.6 [1.7 ± 0.2]
1d	4	26	$(CH_2)_{10}$	300 ± 100	17 ± 5.2 [1.0 ± 0.1]	8.2 ± 2.5 [1.1 ± 0.1]	74 ± 33 [1.2 ± 0.2]	11 ± 2.6 [1.2 ± 0.1]
1e	1	18	$CH_2(CH_2OCH_2)_2CH_2$	390 ± 80	3.3 ± 0.92 [0.9 ± 0.0]	3.4 ± 0.55 [0.9 ± 0.1]	36 ± 9.4 [0.9 ± 0.1]	6.8 ± 1.5 [0.8 ± 0.1]
1f	3	22	$CH_2(CH_2OCH_2)_2CH_2$	220 ± 38	2.8 ± 0.26 [1.0 ± 0.1]	3.7 ± 1.1 [0.9 ± 0.1]	61 ± 7.6 [1.1 ± 0.1]	12 ± 0.95 [1.1 ± 0.1]
1g	3	25	$CH_2(CH_2OCH_2)_3CH_2$	270 ± 29	4.8 ± 0.68 [1.0 ± 0.0]	6.2 ± 1.4 [1.1 ± 0.1]	72 ± 5.7 [1.1 ± 0.1]	19 ± 2.5 [0.9 ± 0.1]
4a	1			2300 ± 410	63 ± 6.8 [1.0 ± 0.1]	70 ± 15 [1.0 ± 0.0]	320 ± 89 [0.9 ± 0.0]	3.3 ± 0.34 [0.9 ± 0.0]
4b	3			1500 ± 140	67 ± 12 [1.1 ± 0.0]	53 ± 11 [1.0 ± 0.1]	610 ± 190 [1.2 ± 0.2]	7.2 ± 1.3 [1.0 ± 0.1]
4c	4			2500 ± 540	52 ± 2.5 [0.9 ± 0.1]	67 ± 21 [0.9 ± 0.1]	590 ± 130 [1.0 ± 0.1]	13 ± 1.3 [0.9 ± 0.0]
6a	1			3800 ± 510	68 ± 13 [1.0 ± 0.0]	100 ± 17 [1.1 ± 0.1]	440 ± 94 [1.0 ± 0.1]	8.4 ± 1.6 [0.9 ± 0.1]
6b	3			870 ± 90	18 ± 2.6 [1.1 ± 0.1]	16 ± 2.5 [1.1 ± 0.1]	250 ± 64 [1.0 ± 0.2]	6.4 ± 0.25 [1.2 ± 0.2]
8	3	21	$(CH_2)_8$	400 ± 45	36 ± 6.6 [1.1 ± 0.1]	32 ± 6.7 [1.1 ± 0.0]	230 ± 25 [1.1 ± 0.1]	14 ± 2.0 [1.2 ± 0.1]
9a	3	22	$(CH_2)_8$	980 ± 120	9.8 ± 4.0 [0.9 ± 0.1]	7.3 ± 0.55 [0.90 ± 0.0]	220 ± 34 [0.9 ± 0.1]	12 ± 2.9 [0.9 ± 0.1]
9b	3	22	$(CH_2)_8$	480 ± 130	19 ± 2.9 [1.2 ± 0.1]	17 ± 2.3 [1.0 ± 0.1]	100 ± 12 [1.1 ± 0.1]	19 ± 1.8 [1.3 ± 0.1]
haloperidol				93 ± 11	1.5 ± 0.44 [1.0 ± 0.1]	1.1 ± 0.16 [1.1 ± 0.1]	6.3 ± 1.6 [1.1 ± 0.1]	6.3 ± 1.3 [1.2 ± 0.1]

[a] K_i values ± SEM derived from 4−10 experiments each done in triplicate. [b] Hill slopes ± SEM are derived from the same binding curves recorded for the determination of K_i values; the original n_H was negative but is displayed as absolute value.

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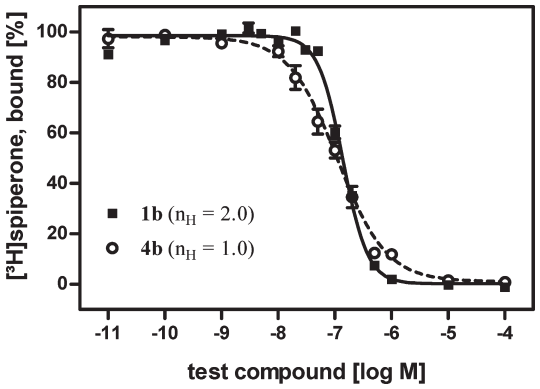

Figure 1. Representative binding curves of **1b** and **4b** at the human D_{2short} receptor displaying different curve shapes resulting in Hill slopes (absolute value of n_H) of 2.0 (for **1b**) and 1.0 (for **4b**) indicating a bivalent and a monovalent binding mode, respectively.

over D_3, approximately 80−100-fold selectivity was observed for **4a**,**b**. To gain diagnostic insights into the binding modes of our bivalent test compounds, we analyzed the profiles of the competition binding curves in detail. Competitive binding curves that follow the law of mass action have a slope of one. A Hill slope (n_H) less than one is considered a reflection of negative cooperativity or the ability of a ligand to bind to G-protein precoupled receptors with a higher affinity than uncoupled receptors. Thus, agonists often show Hill slopes between 0.5 and 0.7. Antagonists tend to have Hill slope values close to one since they fail to differentiate between the precoupled and uncoupled receptor populations, thereby following the general law of mass action for single site competition.[35] Examination of the binding curves of the monomeric ligands **4a**,**b** revealed Hill slopes close to one (n_H = 0.9−1.2) indicating antagonist properties and a binding mode that one receptor protomer binds one ligand. Interestingly, careful analysis of the heterologous competition experiments that we conducted with the dimers **1a**,**b** revealed a steepening of the competition curves leading to Hill slopes between 1.5 and 2.0. This is indicative of a positive cooperative binding. Positive cooperativity is usually observed as a consequence of an allosteric effect inducing conformational crosstalk within one receptor protomer or modulation of the interaction between two protomers of a receptor dimer.[36,37] Bivalent ligands addressing two adjacent binding sites of receptor dimers will also induce such cooperativity because binding of the second pharmacophore is significantly accelerated due to the vicinity of ligand and the thus facilitated enrichment of local concentration. Thus, bivalent binding leads to the liberation of two equivalents of radioligand and substantial steepening of the competition curve. The highest Hill slope of 2.0 was calculated for the interaction of D_{2long} and D_{2short} with the dimeric probe **1b** incorporating a 22-atom spacer (Figure 1). To learn whether this effect depends on the length of the spacer arm, the dimeric homologues **1c** and **1d** with 24- and 26-atom spacers, respectively, were investigated. The competitive binding studies gave Hill coefficients between 1.6 and 1.7 for **1c** and slopes close to one for the homologue **1d** (1.0−1.2) indicating that lengthening of the distance beyond 22 atoms leads to a reduction of a bridging binding mode by increasing the confinement volume. The monovalent control ligand **4c** showed Hill slopes close to one (0.9−1.0). Subsequently, the more hydrophilic oligo(ethylene glycol) derivatives **1e**−**g** were investigated. Although significantly higher D_2 affinity was observed (K_i = 2.8−6.2 nM), these probes gave Hill slopes close to one (n_H = 0.9−1.1). Thus, length and physicochemical properties of the spacer clearly affect the binding mode when a hydrophobic molecular bridge and a linker length of approximately 25 Å appear to be best for a bivalent binding of two interacting receptor protomers, which is in accordance with previous studies on bivalent GPCR ligands.[21,27] To corroborate a receptor-chelating binding mode, further control experiments were performed using the asymmetric ligands **8** and **9a**,**b** that are composed of a pharmacophore with the complete spacer arm or one pharmacophore, a spacer of 22 atom length, and a structurally distorted second pharmacophore with a cyclohexyl or a methyl unit displacing the methoxyphenyl headgroup. In fact, Hill coefficients close to one (1.0−1.2) were observed, indicating that the two D_2 binding pharmacophores appear to be essential for the cooperative binding mode. Ligand affinity of **8** and **9a**,**b** for D_{2long} and D_{2short} (K_i = 7.3−36 nM) was similar to the monovalent and bivalent parent compounds **1b** and **4b**.

To better characterize the biological properties and the determinants for the unusual Hill slopes, the bivalent ligand **1b** was further investigated in comparison to the control ligand **4b**. Since the concentration of sodium ions was shown to significantly influence D_2 affinity, maximal binding capacity, and ligand efficacy,[38−40] the influence of Na^+ on the ligand binding mode was studied. While the monovalent ligand **4b** showed very similar binding properties in the absence (K_i = 53 ± 11 nM; n_H = 1.0 ± 0.1) and in the presence of 100 mM sodium chloride (K_i = 35 ± 6.0 nM; n_H = 0.8 ± 0.0), flattening of the binding curve could be observed for the bivalent ligand **1b** revealing a Hill slope close to one (n_H = 1.1 ± 0.1 [+Na^+]; n_H = 2.0 ± 0.1 [−Na^+]) and K_i values of 12 ± 1.5 nM [+Na^+] and 41 ± 8.2 nM [−Na^+]). Dopamine D_2 receptor dimerization is described to be dependent on pH values and temperature.[41] According to our data, the ionic strength of the system might also control the dimerization state. Thus, sodium ions might perturb and destabilize receptor dimers, thus precluding a receptor-bridging binding mode of **1a**.

To exclude that the unusual binding behavior of the bilvalent ligand **1b** is due to an allosteric modulation,[42−45] we examined the influence of **1b** and **4b** on the dissociation of the radioligand [^{3}H]spiperone from the D_2 receptor. Accelerating dissociation of [^{3}H]spiperone, negative allosteric modulation of the dopamine D_2 receptor was observed for amiloride and for zinc ions.[46,47] Positive allosteric cooperation should markedly decrease the dissociation rate. After preincubation of the receptor with the radioligand to reach equilibrium, dissociation was observed in a time-dependent way after adding an excess of haloperidol as a competitor, which resulted in a k_{off} = 0.0054 min^{-1} and a corresponding dissociation half-life of $t_{1/2}$ = 130 min. In fact, these parameters were identical in presence of **1b** (k_{off} = 0.0054 min^{-1}, $t_{1/2}$ = 130 min) and highly similar in presence of **4b** (k_{off} = 0.0057 min^{-1}, $t_{1/2}$ = 120 min) indicating that the atypical binding mode of the bivalent ligand **1b** may not be due to an allosteric modulation.

Agonist behavior of GPCR ligands is a consequence of a binding preference for the precoupled receptor state leading to Hill coefficients smaller than one. The bivalent ligand **1b** and the control agent **4b** were tested for their functional behavior in a mitogenesis assay measuring an agonist stimulated incorporation of [^{3}H]thymidine into cells expressing the human D_{2long} receptor. As we expected from the structure of the pharmacophore, both ligands **1b** and **4b** displayed neutral antagonist properties in the functional experiments revealing that the different binding profiles are not a result of varying functional properties.

CONCLUSION

When bivalent ligands are employed as pharmacological tools, a differential analysis of homo- and heteromers, tissue-selective modulation, and a reversible control of monomer/dimer equiliriums may be facilitated. We herein present bivalent ligands of type **1**, differing in length and structure of the spacer unit that links two identical pharmacophores. Radioligand binding assays revealed that the bivalent ligands exhibited a distinct binding profile compared with monovalent analogues containing capped spacer and asymmetric "dummy ligands" incorporating one original pharmacophore while the second pharmacophore is replaced by a structurally similar nonbinding motif. Some of the bivalent ligands revealed a steepening of the competition curve for the D_2 receptors. The profiles of the test compounds were dependent on the structure and on the length of the spacer arm when the bivalent ligand **1b** containing a 22-atom spacer gave the steepest curve and a Hill slope of 2. Since homodimerization of dopamine D_2 receptors is assumed to be of particular importance in the pathophysiology of schizophrenia, molecular probes of type **1** will serve as promising tools for the discovery of atypical antipsychotics.

EXPERIMENTAL SECTION

Chemistry. Dry solvents and reagents were of commercial quality and were used as purchased. MS were run 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 were run on a JEOL JMS-GC Mate II using Peak-Matching ($M/\Delta M > 5000$). NMR spectra were obtained on a Bruker Avance 360 or a Bruker Avance 600 spectrometer relative to TMS in the solvents indicated (J value in hertz). Melting points were determined with a MEL-TEMP II melting point apparatus (Laboratory Devices, USA) in open capillaries and are given uncorrected. IR spectra were performed on a Jasco FT/IR 410 spectrometer. Purification by flash chromatography was performed using silica gel 60; TLC analyses were performed using Merck 60 F254 aluminum sheets and analyzed by UV light (254 nm). Analytical HPLC was performed on Agilent 1100 HPLC systems employing a VWL detector. As column, a ZORBAX ECLIPSE XDB-C18 (4.6 mm × 150 mm, 5 μm) was used. HPLC purity was measured using following binary solvent systems: system A, eluent CH_3OH in 0.1% aqueous trifluoroacetic acid, 10% to 100% CH_3OH in 15 min, 100% for 3 min, flow rate 1.0 mL/min, λ 254 nm; system B, eluent CH_3CN in 0.1% aqueous trifluoroacetic acid, 10% CH_3CN for 2 min to 95% CH_3CN in 18 min, 95% for 1 min, flow rate 1.0 mL/min, λ 254 nm. The purity of all test compounds and key intermediates was determined to be >95%. CHN elementary analyses were performed at the chair of Organic Chemistry of the Friedrich-Alexander University Erlangen-Nürnberg.

*3-Methoxy-4-(pent-4-yn-1-yloxy)benzaldehyde (**2b**).* A suspension of 4-hydroxy-3-methoxybenzaldehyde (2.3 g, 15 mmol), 5-chloropent-1-yne (3.2 g, 30 mmol), K_2CO_3 (2.1 g, 15 mmol), and KI (0.5 g, 3.0 mmol) in anhydrous CH_3CN (50 mL) was stirred at reflux temperature for 16 h. Then the reaction mixture was allowed to cool to room temperature, and the solvent was evaporated. The residue was dissolved in H_2O and extracted with CH_2Cl_2. The combined organic layers were dried ($MgSO_4$) and evaporated. The residue was purified by flash chromatography (hexane−EtOAc 4:1) to give **2b** as a white solid (1.55 g, 47% yield); mp 81 °C. IR 3747, 2360, 2337, 1676, 1587, 1279, 1134, 995, 658 cm^{-1}. ^1H NMR (360 MHz, $CDCl_3$) δ 1.98 (t, J = 2.6 Hz, 1H), 2.10 (m, 2H), 2.44 (dt, J = 6.9, 2.6 Hz, 2H), 3.92 (s, 3H), 4.22 (t, J = 6.3 Hz, 2H), 7.00 (d, J = 8.2 Hz, 1H), 7.41 (d, J = 2.0 Hz, 1H), 7.44 (dd, J = 8.2, 1.8 Hz, 1H), 9.85 (s, 1H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 15.1, 27.8, 56.0, 67.4, 69.2, 83.1, 109.3, 111.6, 126.6, 130.0, 149.8, 153.9, 190.8. HPLC system A (254 nm) purity >99%. (t_R = 11.9 min); system B (254 nm) purity >99% (t_R = 13.7 min). HR-EIMS calcd m/z for $C_{13}H_{14}O_3$ 218.0943, found 218.0942.

*4,4′-[Octane-1,8-diylbis(1H-1,2,3-triazole-1,4-diylpropane-3,1-diyloxy)]-bis(3-methoxybenzaldehyde) (**3b**).* A suspension of **2b** (334 mg, 1.5 mmol), 1,8-diazidooctane (100 mg, 0.5 mmol), sodium ascorbate (20 mg, 0.1 mmol), and copper sulfate pentahydrate (6.4 mg, 25 μmol) in H_2O (2.5 mL), ^{t}BuOH (5 mL), and CH_2Cl_2 (5 mL) was stirred at room temperature for 16 h. The reaction was quenched using saturated $NaHCO_3$ and 0.1 N EDTA, extracted with CH_2Cl_2, dried (Na_2SO_4), and evaporated. The residue was purified by flash chromatography (hexane/EtOAc 1:1−2:98) to give **3b** as a white solid (290 mg, 90% yield); mp 96 °C. IR 2360, 1681, 1587, 1510, 1269, 1136 cm^{-1}. ^1H NMR (360 MHz, $CDCl_3$) δ 1.24−1.32 (m, 8H), 1.85 (m, 4H), 2.28 (m, 4H), 2.94 (t, J = 7.2 Hz, 4H), 3.93 (s, 6H), 4.17 (t, J = 6.5 Hz, 4H), 4.29 (t, J = 7.2 Hz, 4H), 6.96 (d, J = 8.2 Hz, 2H), 7.26 (s, 2H), 7.41 (brs, 2H), 7.43 (d, J = 1.8 Hz, 2H), 9.84 (s, 2H). ^{13}C NMR (90 MHz, $CDCl_3$) δ 21.9, 26.1, 28.3, 28.5, 30.1, 49.9, 55.9, 68.1, 109.2, 111.5, 120.7, 126.7, 129.9, 146.6, 149.8, 153.9, 190.6. APCI-MS calcd m/z for $C_{34}H_{44}N_6O_6$ 632.7, found 634 $(M + H)^+$. Anal. ($C_{34}H_{44}N_6O_6$) C, H, N.

*1,1′-[Octane-1,8-diylbis[1H-1,2,3-triazole-1,4-diylpropane-3,1-diyloxy-(3-methoxy-4,1-phenylene)methylene]]bis[4-(2-methoxyphenyl)-piperazine] (**1b**).* A solution of **3b** (50 mg, 0.08 mmol), 1-(2-methoxyphenyl)piperazine (45.6 mg, 0.24 mmol), and $Na(OAc)_3BH$ (53 mg, 0.24 mmol) in anhydrous CH_2Cl_2 (10 mL) was stirred under nitrogen atmosphere at room temperature for 20 h. The reaction mixture was quenched with saturated aqueous $NaHCO_3$ and extracted with CH_2Cl_2. The combined organic layers were dried (Na_2SO_4) and evaporated. The residue was purified by flash chromatography (EtOAc/MeOH 99:1−98:2 + 1% TEA) to give **1b** as a white solid (54.7 mg, 70% yield); mp 103 °C. IR 2933, 2813, 1590, 1502, 1452, 1238 1140, 1030, 748 cm^{-1}. ^1H NMR (360 MHz, $CDCl_3$) δ 1.23−1.37 (m, 8H), 1.85 (m, 4H), 2.21 (m, 4H), 2.64 (m, 8H), 2.93 (t, J = 7.6 Hz, 4H), 3.09 (m, 8H), 3.51 (s, 4H), 3.85 (s, 6H), 3.87 (s, 6H), 4.06 (t, J = 6.3 Hz, 4H), 4.28 (t, J = 7.2 Hz, 4H), 6.79−7.00 (m, 14H), 7.30 (s, 2H). ^{13}C NMR (90 MHz, $CDCl_3$) δ 22.2, 26.3, 28.7, 28.9, 30.2, 50.1, 50.7, 53.3, 56.1, 62.9, 68.2, 111.2, 113.0, 118.2, 120.8, 121.0, 121.5, 122.9, 131.0, 141.5, 147.3, 147.5, 149.4, 152.3. HPLC system A (254 nm) purity 97% (t_R = 11.9 min); system B (254 nm) purity 97% (t_R = 12.7 min). APCI-MS calcd m/z for $C_{56}H_{76}N_{10}O_6$ 985.3, found 986 $(M + H)^+$.

*1-[[4-[3-(1-Butyl-1H-1,2,3-triazol-4-yl)propoxy]-3-methoxyphenyl]-methyl]-4-(2-methoxyphenyl)piperazine (**4b**).* Sodium azide (5.93 g, 91 mmol) was added to a solution of 1-bromobutane (3.94 mL, 36 mmol) in anhydrous CH_3CN (36 mL) and H_2O (4 mL) and heated at reflux temperature overnight. The reaction was quenched with saturated aqueous $NaHCO_3$, extracted with n-hexane, dried ($MgSO_4$), and evaporated to 3.83 mL to obtain a concentration of 1-azidobutane of 4.13 mmol/mL.

A suspension of **2b** (344 mg, 1.58 mmol), a 4.13 M solution of 1-azidobutane in n-hexane (1.15 mL, 4.74 mmol), sodium ascorbate (62.5 mg, 0.32 mmol), and copper sulfate pentahydrate (19.7 mg, 0.08 mmol) in H_2O (5 mL), ^{t}BuOH (10 mL), and CH_2Cl_2 (10 mL) was stirred at room temperature overnight. The reaction was quenched with saturated $NaHCO_3$ and 0.1 N EDTA, extracted with CH_2Cl_2, dried (Na_2SO_4), and evaporated. The residue was purified by flash chromatography (hexane/EtOAc 1:3) to give 4-[1-butyl-1*H*-1,2,3-triazol-4-yl)propoxy]-3-methoxybenzaldehyde as a white solid (354 mg, 71% yield); mp 36 °C. IR 2958, 2935, 2873, 1682, 1589, 1512, 1466, 1423, 1269, 1134, 1030, 806 cm^{-1}. ^1H NMR (360 MHz, $CDCl_3$) δ 0.95 (t, J = 7.4 Hz, 3H), 1.34 (m, 2H), 1.86 (m, 2H), 2.29 (m, 2H), 2.94 (t, J = 7.5 Hz, 2H), 3.93 (s, 3H), 4.18 (t, J = 6.5 Hz, 2H), 4.32 (t, J = 7.3 Hz, 2H), 6.97 (d, J = 8.4 Hz, 1H), 7.31 (s, 1H), 7.40−7.45 (m, 2H), 9.85 (s, 1H). ^{13}C NMR (90 MHz, $CDCl_3$) δ 13.4, 19.7, 22.0, 28.5, 32.3, 49.9, 56.0, 68.1, 109.3, 111.6, 120.8, 126.8, 130.1, 146.8, 149.9, 154.0, 190.8. HPLC system A (254 nm) purity 97% (t_R = 12.0 min); system B (254 nm)

purity 98% (t_R = 12.9 min). HR-EIMS calcd m/z for $C_{17}H_{23}N_3O_3$ 317.1739, found 317.1739.

A solution of 4-[1-butyl-1*H*-1,2,3-triazol-4-yl)propoxy]-3-methoxybenzaldehyde (70 mg, 0.22 mmol), 1-(2-methoxyphenyl)piperazine (63.6 mg, 0.33 mmol), and $Na(OAc)_3BH$ (98.0 mg, 0.44 mmol) in anhydrous CH_2Cl_2 (10 mL) was stirred under nitrogen atmosphere at room temperature for 14 h. The reaction mixture was quenched with saturated aqueous $NaHCO_3$ and extracted with CH_2Cl_2. The combined organic layers were dried (Na_2SO_4) and evaporated. The residue was purified by flash chromatography (hexane/EtOAc 1:1 + 1% TEA) to give **4b** as a colorless oil (86 mg, 79% yield). IR 2935, 2873, 2816, 1592, 1501, 1452, 1260, 1240, 1141, 1031, 1011, 931 cm^{-1}. ^1H NMR (600 MHz, $CDCl_3$) δ 0.94 (t, J = 7.4 Hz, 3H), 1.34 (m, 2H), 1.86 (m, 2H), 2.22 (m, 2H), 2.64 (m, 4H), 2.93 (t, J = 7.4 Hz, 2H), 3.09 (m, 4H); 3.52 (s, 2H), 3.85 (s, 3H), 3.88 (s, 3H), 4.06 (t, J = 6.4 Hz, 2H), 4.31 (t, J = 7.2 Hz, 2H), 6.81 (d, J = 8.3 Hz, 1H), 6.83−6.87 (m, 2H), 6.89−6.96 (m, 3H), 6.99 (ddd, J = 7.7, 7.4, 1.7 Hz, 1H), 7.31 (s, 1H). ^{13}C NMR (90 MHz, $CDCl_3$) δ 13.4, 19.7, 22.2, 28.9, 32.3, 49.9, 50.7, 53.3, 55.3, 56.1, 62.9, 68.2, 111.2, 113.0, 118.2, 120.8, 121.0, 121.5, 122.8, 131.1, 141.5, 147.3, 147.5, 149.4, 152.3. HPLC system A (254 nm) purity >99% (t_R = 11.5 min); system B (254 nm) purity >99% (t_R = 11.8 min). HR-EIMS calcd m/z for $C_{28}H_{39}N_5O_3$ 493.3051, found 493.3053.

Radioligand Binding Studies. Receptor binding studies were carried out as described previously.[48] In brief, competition binding experiments with the human D_{2long}, D_{2short}, D_3, and $D_{4.4}$ receptors were run on the membrane preparations from CHO cells stably expressing the corresponding receptor and [^{3}H]spiperone (specific activity = 84 Ci/mmol, PerkinElmer, Rodgau, Germany) at final concentrations of 0.1−0.5 nM according to the individual K_D values in binding buffer (50 mM Tris, 1.0 mM EDTA, 5.0 mM $MgCl_2$, 100 μg/mL bacitracin, and 5 μg/mL soybean trypsin inhibitor at ph 7.4). The K_D values were 0.051−0.12, 0.067−0.21, 0.095−0.19, and 0.16−0.29 nM for the D_{2long}, D_{2short}, D_3, and D_4 receptor, respectively. The corresponding B_{max} values were in the range of 870−1700 fmol/mg for D_{2long}, 1200−2600 fmol/mg for D_{2short}, 1600−4700 fmol/mg for D_3, and 630−2200 fmol/mg for D_4 receptor, respectively. Nonspecific binding was determined in the presence of 10 μM haloperidol. Specific binding represented about 85% of the total binding. Experiments in the presence of sodium were performed in binding buffer supplemented with NaCl at a final concentration of 100 mM. The D_1 receptor binding experiments were performed with homogenates prepared from porcine cerebral cortex as described.[48] Assays were run with membranes at protein concentrations per well of 40−60 μg/mL and radioligand concentrations of 0.5 nM [^{3}H]SCH 23390 with K_D values of 0.56−0.95 nM. Protein concentration was established by the method of Lowry using bovine serum albumin as a standard.[49]

Data Analysis. The resulting competition curves of the receptor binding experiments were analyzed by nonlinear regression using the algorithms in PRISM 5.0 (GraphPad Software, San Diego, CA). Competition curves were fitted to a sigmoid curve by nonlinear regression analysis in which the log EC_{50} value and the Hill coefficient were free parameters. EC_{50} values were transformed to K_i values according to the equation of Cheng and Prusoff.[50]

■ ASSOCIATED CONTENT

Supporting Information. Experimental and spectroscopic data of the non-key target compounds **1a**, **1c**−**g**, **4a**, **4c**, **8**, **9a**, and **9b** and the respective precursors thereof, receptor binding data employing porcine 5-HT_1, 5-HT_2, and α_1 receptors, experimental details and results of receptor binding experiments in the presence of sodium, dissociation experiments, and mitogenesis assays for functional activity. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

GPCR, G-protein coupled receptor; 1,4-DAP, 1,4-disubstituted aromatic piperidines/piperazines; TM, transmembrane domain; EL, extracellular loop; CuAAC, copper(I)-catalyzed azide−alkyne cycloaddition; CHO, Chinese hamster ovary; K_i, inhibition constant; SEM, standard error of the mean; n_H, Hill slope; HR-EIMS, high-resolution electron ionization mass spectrometry; APCI-MS, atmospheric-pressure chemical ionization mass spectrometry; CHN, combustion/elemental analysis

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