Full Paper

Ring Expansions of Tetrahydroprotoberberines and Related Dibenzo[*c,g*]azecines Modulate the Dopamine Receptor Subtype Affinity and Selectivity

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The affinities of tetrahydroprotoberberines for dopamine receptors dramatically decrease after cleaving the central C-N bond to the analogous ten-membered dibenzo[*c*,*g*]azecines [1]. In the present work, we also synthesized eleven-membered homologues of these heterocycles and measured the affinities of the resulting dibenzazaundecenes and their underlying homoberberines for human dopamine receptors as well as the cytotoxic effects of all target compounds on human glia cells. The tetracyclic iso-C-homoberberine-derivatives revealed to be D_4-selective antagonists, while all other active compounds showed a significant D_1/D_5 selectivity. Distances in energy-minimized conformations were measured in order to explain our findings.

Keywords: Azecines / Cytotoxicity / Dopamine receptor / SAR / Scaffold

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Introduction

Dopamine receptors play a key role in many psychiatric, motoric, or endocrinologic disorders. Even though there are many compounds targeting the dopamine system, it is not clarified yet if a certain selectivity profile at the dopamine receptor subtypes might be advantageous for clinical use. Antipsychotic drugs used in therapy exert different selectivity profiles (*e. g.* haloperidol and clozapine). While haloperidol has the highest affinities for D_2 and D_3 receptors, clozapine preferably binds to D_1 and D_4 receptors and causes less extrapyramidal side effects [2]. Thus, it is still attractive to look for new scaffolds with novel selectivity profiles to investigate the therapeutic value of subtype-selective compounds and to perform

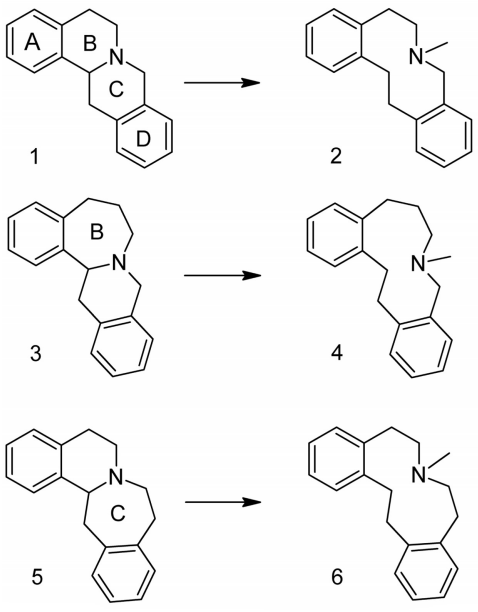

Figure 1. Scaffolds of the test compounds: the underlying tetracyclic structures and ring-opened azecine- and azacycloundecene scaffolds.

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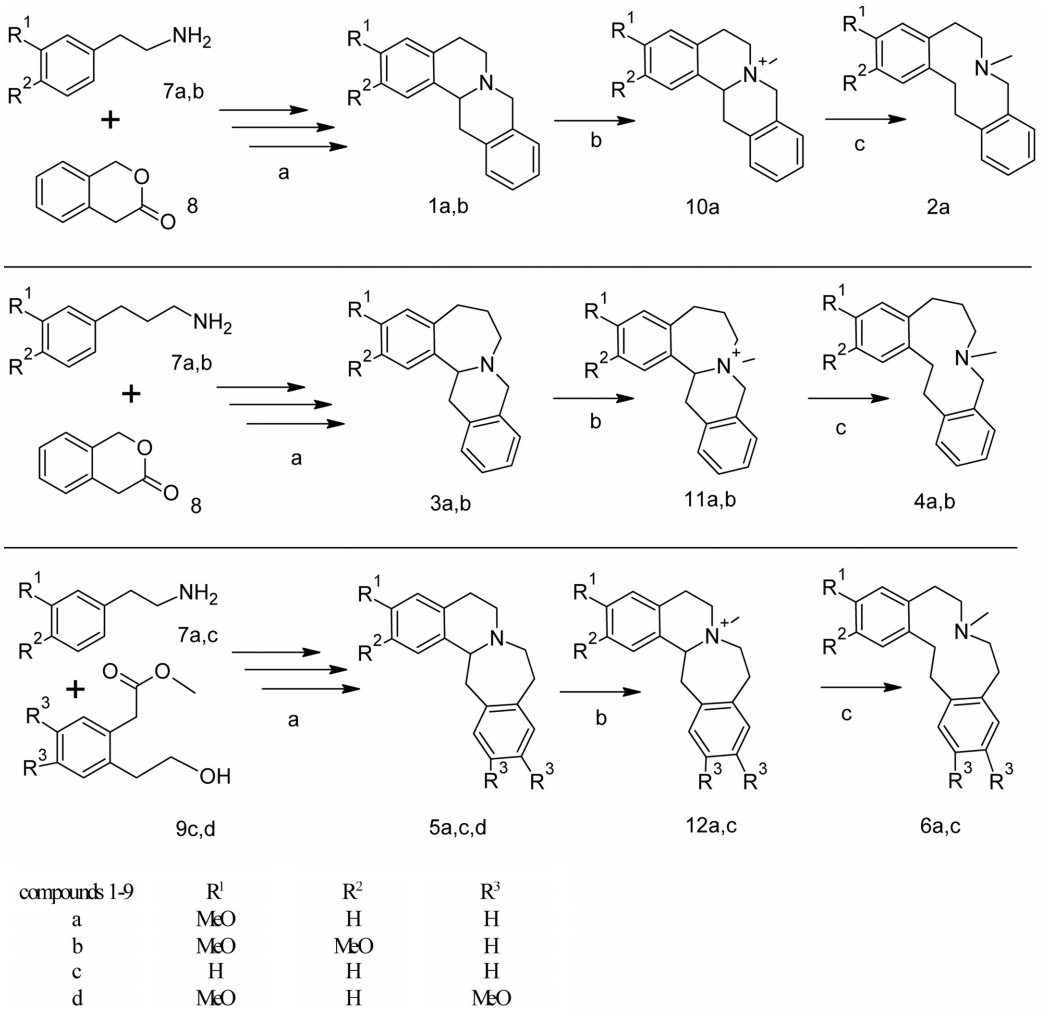
Abbreviation: tetrahydroprotoberberines (THPBs)



compounds 1-9	R^1	R^2	R^3
a	MeO	H	H
b	MeO	MeO	H
c	H	H	H
d	MeO	H	MeO

Reagents and Conditions: (a) i) toluene reflux; ii) $POCl_3$, MeCN; iii) $NaBH_4$/MeOH; (b) methyl iodide, MeCN; (c) Na/NH_3^{liq}.

Scheme 1. Synthesis of compounds **1–6**.

cross-target SAR-studies within the dopamine receptors. In former studies, we investigated tetrahydroprotoberberines (THPBs) **1** along with their ring-opened derivatives **2** and found that ring opening of **1a** to **2a** decreases dopamine receptor affinities dramatically [1]. Furthermore, ring expansion goes along with a shift of selectivity from similar antagonist activities at all receptors for **1a**, toward D_1/D_5 selectivity for **2a**.

In the present study, we applied this ring-opening approach on ring-enlarged THPBs to obtain B- (**3**) and C-homologues **5**, which were synthesized by Meise *et al*. [3, 4]. Compounds **3** and **5** were quaternized with methyl iodide, followed by cleavage of the central C-N bond with Na in liq. NH_3. The resulting eleven-membered homologues **4** and **6** represent derivatives of novel heterocyclic ring systems (Fig. 1). They were investigated together with the THPB-homologues **3** and **5** with respect to their affinity and selectivity profiles for all dopamine receptor subtypes (radioligand binding and functional calcium assay).

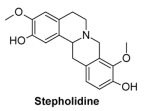
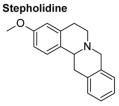
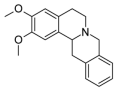
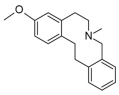
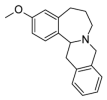
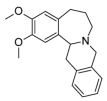
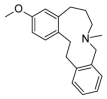
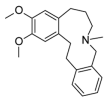
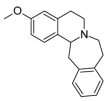
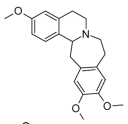
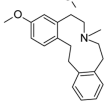
Low general cytotoxicity of candidate compounds is a basic requirement for drug development. Accordingly, we measured the cytotoxicity of our target compounds up to 250 μM by an *in-vitro* cell-culture-based MTT assay.

Results and discussion

Chemistry

The synthesis of compounds **1–6** is outlined in Scheme 1. Ring expanded THPB homologues **3** and **5** were synthesized according to previously described procedures [3, 4], starting with substituted phenylethyl- or phenylpropylamines **7** and a suitable lactone **8**, or its corresponding

Table 1. Affinities (K_i) for dopamine D_1–D_5 receptor subtypes determined by radioligand-binding experiments.

Compound		(Radioligand binding studies) K_i (nM)				
		D_1	D_{2L}	D_3	$D_{4.4}$	D_5
 Stepholidine		5.9 {	974 {	30 (rat) {	3748 {	4.4 {
	**1a**	183.5 ± 27.5 #	320 ± 159 §	251 ± 56 #	2565 ± 1673 §	185 ± 97 #
	**1b**	474 ± 156 §	1093 ± 61.5 #	1122 ± 30.5 #	>10000	398 ± 150.5 #
	**2a**	1727 ± 238 §	>10000	>10000	>10000	1508 ± 136 #
	**3a**	>10000	>10000	>10000	>10000	>10000
	**3b**	>10000	>10000	>10000	>10000	>10000
	**4a**	>10000	>10000	>10000	>10000	>10000
	**4b**	2343 ± 43 #	>10000	>10000	>10000	>10000
	**5a**	662 ± 172 #	>10000	>10000	224 ± 117 #	1174 ± 221 #
	**5c**	1446 ± 176 #	4475 ± 14.5 #	>10000	427 ± 230 #	626 ± 230 §
	**5d**	1482 ± 329 #	>10000	>10000	282 ± 115 §	843 ± 243 #
	**6a**	119 ± 74 §	1502 ± 443 #	3120 ± 1085 #	726 ± 131 #	492 ± 293 §
	**6c**	118 ± 57 §	917 ± 151 #	3045 ± 22 #	475 ± 254 #	97.5 ± 30 #

§ Experiments were performed in triplicate, values are given as mean ± SD; # experiments were performed twice, values are given as mean ± SEM; { values are from the PDSP database [5].

hydroxy ester **9**. Ring opening was performed after quaternization with methyl iodide under Birch conditions (liq. NH_3/Na; Scheme 1).

Pharmacology

The affinities of the tetracyclic and tricyclic compounds were determined by radioligand-binding experiments at all human cloned dopamine receptors according to a previously described method [1]. The K_i values of the binding assay are given in nM units. Their functionalities were defined by an intracellular calcium assay [1] (Table 1).

The investigated tetracyclic THPBs **1a**, **b** showed affinities for all dopamine receptors, but preferable for D_1/D_5. The lowest affinities were detected for the D_4 receptor. Stepholidine, a well studied representative of the THPBs is a ligand for dopamine receptors, which has agonistic activities at D_1 and antagonist properties at D_2 receptors [6]. Yet, all our compounds turned out to be antagonists for D_1 and D_2 receptors. This may be explained by the different substitution pattern compared to stepholidine, whose phenolic group in position 2 is similar to the intrinsic agonist dopamine. At all our molecules, this OH-group is missing. It can be speculated that this particular phenolic group is crucial for the functionality at dopamine receptors. Ring opening of **1a** to dibenzo[*c*,*g*]-azecine **2a** lead to a nearly complete loss of affinities. Only at the D_1 and D_5 receptor micromolar affinities could be detected. Neither ring expansion of ring B of the THPBs **1a**, **b** to isochino[3,2-*a*][2]benzazepines **3a**, **b** nor their ring opening to the respective dibenzo[*c*,*g*]azacycloundecenes **4a**, **b** led to ligands with noteworthy affinities for dopamine receptors. But on the contrary, when THPBs **1a**, **b** were expanded at the central C-ring, we obtained antagonists with K_i values comparable with those of the parent THPBs. In contrast to THPBs, the homo-C congeners **5a**, **c**, **d** exhibited a distinct D_4 selectivity and almost no affinity for D_3 receptors. This might be interesting, since Seeman *et al*. observed that D_4 receptors were elevated in the brains (*post mortem*) of schizophrenic patients [8]. Furthermore, the atypical antipsychotic drug clozapine was found to bind more tightly to D_4 receptors than to the other dopamine receptor subtypes [2]. Clinical evidence for the influence of D_4 receptors in schizophrenia was proved by D_4 selective antagonists [9]. Interestingly, ring opening of homo-C THPB derivatives **5a**, **c** to compounds **6a**, **c** increased the affinities for D_1, D_2, D_3, and D_5 receptors. Only at D_4 receptors, affinities revealed to be the same or were even decreased. Thus, compounds **6a**, **c** are D_1- or in case of **6c** D_1/D_5-selective, with the weakest affinity for D_3. The increase of affinities by providing higher conformational flexibility was discussed in a previous paper [1]. Conclusively, we can demonstrate that ring opening, going along with higher conformational flexibility, maintains or even increases the affinity of the already active homo-C THPBs but does not turn the inactive homo-B derivatives into compounds with distinct affinity.

Table 2. MTT test results measured on human glia cells.

Compound	Viability at 100 μM (%)	cc_{50} (μM)
1a	97.8 ± 5.2	>250
2a	77.3 ± 22.6	158.7 ± 14.7
3a	94.2 ± 3.3	>250
3b	91.8 ± 4.2	>250
4a	115.8 ± 9.9	165.4 ± 9.8
4b	78.9 §	poor solubility
5a	63.7 ± 1.4	poor solubility
5c	99.3 ± 11.0	>250
5d	93.2 ± 8.0	>250
6a	71.2 ± 8.4	125.1 ± 15.7
6c	91.7 ± 17.3	177.5 ± 18.0

§ Due to poor solubility, all values except one were discarded.

It is an essential feature for all CNS drugs not to be toxic. During screening investigations, we observed for the dibenz[*c*,*g*]azecine **2a** cytotoxic effects on glia cells. Therefore, we screened all target compounds using an *in-vitro* MTT-assay on human U87-MG glia cells at 100 μM and determined cc_{50} values up to 250 μM (see Table 2). Solubility of all compounds in all concentrations was monitored by microscopy. Values of incompletely dissolved compounds were neglected.

Ring expansion of THPBs **1** to C- and B-homologues, **3** and **5**, respectively, and their ring-opened derivatives, **4** and **6**, respectively, did not decrease cytotoxicity in general. The results are rather inconsistent. Neither a specific scaffold nor one of the substitution patterns could be identified as responsible for toxicity. In general, ring opening led to higher toxicity. Almost all ring-closed compounds showed no toxicity up to 250 μM. At 100 μM, the methoxylated dibenzo[*c*,*g*]azecine **2a**, the C-ring-expanded monomethoxylated compounds **5a** and **6a** and the dimethoxy-substituted B-homologue **4b** displayed cytotoxic effects (viability of cells compared to control <90%). Unfortunately, the poor solubility of compounds **4b** and **5a** precluded us from determining their exact cc_{50} values. Focusing on the monomethoxy series, C-ring expansion led to higher toxicity, while the B-homologues showed no (**3a**) or less (**4a**) toxic effects. The unsubstituted homo-C-derivatives **5c** and **6c** showed significant lower cytotoxicity than their aromatic-substituted congeners.

In-silico studies

Structure-based modeling is difficult for the dopamine receptors, since the X-ray structure or a reliable homol-

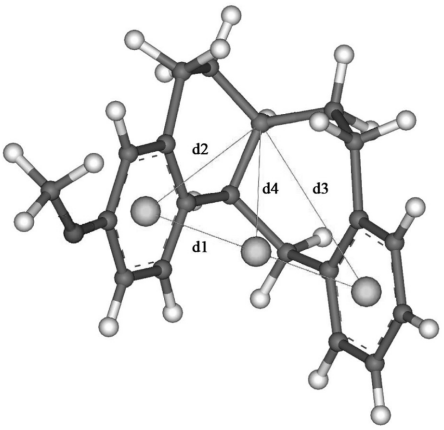


Figure 2. Measured distances of energy-minimized conformation of compound **5a**.

ogy model is not available so far. Nevertheless, we wanted to compare the conformational space of the pharmacophors in order to correlate distances with the affinities for dopamine receptors.

Therefore, we measured the following distances: Between the centroids of the two aromatic moieties (d1), from the nitrogen to the substituted aromatic moiety (d2) and the unsubstituted one (d3), respectively. Moreover, we determined the distance between the nitrogen and the center between the two aromatic moieties, which is a parameter for the angulations of the whole molecule (d4). In Fig. 2, as an example, these distances are demonstrated for compound **5a**.

In order to select a reasonable conformation for the minimization, we relied on the observations reported for (*l*)-stepholidine before [10]. Herein it is assumed that (*l*)-stepholidine binds in its *cis*-protonated conformation to the binding pocket. Hence, we used the *cis*-protonated *(l)*-stepholidine as template for our tetracyclic compounds (**1**, **3**, and **5**) prior to energy minimization. To complement our investigations, we also measured the respective *trans*-isomers (**1a**, **3a**, **5a**) (Table 3). Due to their high flexibility, it is difficult to include the ring-opened derivatives (**2**, **4**, and **6**) to this investigation. Computational examinations on this special topic are ongoing.

The active compounds within our investigated set are **1a-cis** and **5a-cis**. It is obvious that the distances between the two aromatics (d1) of these two compounds are significantly shorter compared to all other molecules we examined. The other way around can be observed for the distance between the nitrogen and the center between the aromatic rings (d4) where the distances are significantly longer. These two parameters reflect the strongly angulated shape of compounds **1** and **5**. Since the homo-B derivative **3a**, which lacks affinity, does not show such a picture, the flexed structure of **1** and **5** might be responsible for their affinity.

Table 3. Distances in energy-minimized conformations of the tetracycles **1**, **3**, **5** between the centroids of the two aromatic moieties (d1), from the nitrogen to the substituted aromatic moiety (d2) and to the unsubstituted one (d3), and between the nitrogen and the center between the two aromatic moieties (d4).

Compound	Measured distances (Å)			
	d1	d2	d3	d4
1a *cis*	4.678	3.844	3.774	3.007
1a *trans*	6.612	3.806	3.755	1.834
3a *cis*	6.535	3.944	3.697	1.984
3a *trans*	6.592	3.940	3.773	2.005
5a *cis*	4.504	3.821	4.478	3.501
5a *trans*	6.311	3.842	3.747	2.108

Conclusion

Though our affinities gains were, in general, only moderate, we could observe some interesting changes in the selectivity at the dopamine receptor subtypes by ring enlargement of tetrahydroprotoberberines (THPBs) and their corresponding dibenz[*c*,*g*]azecines. Ring expansion of the C-ring in the THPBs to isochino[1,2-*b*][3]benzazepines (**5a**, **c**, **d**) yielded dopamine antagonists with distinct D_4 selectivity, while the expansion of the other central ring (B) led to compounds with much lower affinities for all dopamine receptor subtypes. We assume that the highly flexed conformation might be responsible for the affinities of the homo-C derivatives **5** and the missing affinities of homo-B derivatives **3**. By ring opening of the enlarged, but still active tetracycles **5** to compounds **6**, the D_4 selectivity of **5** changed into a D_1/D_5 preference, going along with increased affinities. Cytotoxic effects were found preferable for the ring-opened compounds. Substitution at the aromatic ring systems seems to be disadvantageous regarding cytotoxicity. The unsubstituted compound **5c** displays the lowest cytotoxicity of all active compounds. Furthermore, **5c** shows a new selectivity profile and, hence, might be used for the further development of atypical antipsychotics.

Experimental

Chemistry

Syntheses were performed under nitrogen with solvents and reagents of commercial availability with no further purification. Melting points are uncorrected and were measured in open capillary tubes, using a Gallenkamp melting point apparatus (Weiss-Gallemkamp, London, UK). ^{1}H- and ^{13}C-NMR spectral data

were obtained from a Bruker Advance 250 spectrometer (250 MHz) and Advance 400 spectrometer (400 MHz), (Bruker, Rheinstatten, Germany) respectively. Elemental analyses were performed on a Heraeus Vario EL apparatus (Heraeus, Hanau, Germany) for all test compounds. MS data were determined by GC/MS, using a Hewlett–Packard GCD-Plus (G1800C) apparatus (HP-5MS column; J&W Scientific, Folsom, CA, USA). Synthesis and analytical data of compounds **1** and **2** were described previously [1].

*3-Methoxy-5,6,7,9,14,14a-hexahydroisoquino[3,2-a][2]benzazepine **3a** and 2,3-dimethoxy-5,6,7,9,14,14a-hexahydroisoquino[3,2-a][2]benzazepine **3b***

Isochroman-3-one (25 mmol, 3.7 g) and a suitable phenethylamine/phenpropylamine, respectively (25 mmol) were refluxed in toluene (100 mL) for 6 h. After cooling to room temperature, the amide precipitated as a white solid, which could be filtered off. The solid was washed twice with toluene and dried *in vacuo* to remove the starting material. The product could be identified by the typical two singlets in ^{1}H-NMR spectrum at 4.65 and 3.59 ppm. The amide was refluxed in 50 mL of a mixture of phosphoryl chloride in acetonitrile (1:8) for 18 h. After evaporation of the solvents, the resulting brown oil was washed twice with petroleum ether and the residue was solved in methanol. Cooled by an ice bath, a ten-fold excess of sodium boron hydride was added in small portions. The suspension was refluxed for half an hour. After evaporation of the solvent, the residue was dissolved in water and extracted with ethyl acetate. The organic layer was dried over $MgSO_4$ and evaporated *in vacuo*. The resulting yellow oil was crystallized from methanol to yield the respective tetracycle. Analytical data is given in reference [4]. Yields and melting points: for compound **3a**: yield 29%, white crystals, m. p. 129°C; for compound **3b**: yield 31%, white crystals, m. p. 91–93°C.

*3-Methoxy-5,6,8,9,14,14a-hexahydroisoquino[1,2-b][3]benzazepine **5a** and 5,6,8,9,14,14a-hexahydroisoquino[1,2-b][3]benzazepine **5c***

The synthesis was performed according to a procedures described before [3], starting with a phenethylamine (2.4 mmol) and methyl[2-(2-hydroxyethyl)phenyl]acetate (2 mmol). The crude products were crystallized from toluene. Analytical data is given in reference [3]. Yields and melting points: for compound **5a**: yield 32%, white crystals, m. p. 80–82°C; for compound **5c**: yield 22%, white crystals, m. p. 61–62°C.

*3-Methoxy-8-methyl-5,6,7,9,14,14a-hexahydroisoquino[3,2-a][2]benzazepinium iodide **11a**, 2,3-dimethoxy-8-methyl-5,6,7,9,14,14a-hexahydroisoquino[3,2-a][2]benzazepinium iodide **11b**, 3-methoxy-7-methyl-5,6,8,9,14,14a-hexahydroisoquino[1,2-b][3]benzazepinium iodide **12a**, and 7-methyl-5,6,8,9,14,14a-hexahydroisoquino[1,2-b][3]benzazepinium iodide **12c***

10 mmol of the tetracyclic compounds (**3**, **5**) were solved in acetonitrile (20 mL). A 20-fold excess of methyl iodide was added and the mixture stirred overnight. The quaternary ammonium salt precipitated as white to light yellow powder and could be isolated by filtration. Washing with small amounts of acetonitrile and drying *in vacuo* resulted in the pure products with almost 100% yield.

*Compound **11a***

Yield: 98.2%, white powder, m.p.: 215°C; ^{1}H-NMR 250 MHz ($CDCl_3$) δ: 1.97–2.13 (m, 2H), 2.76–2.91 (m, 1H), 3.11–3.29 (m, 2H), 3.39 (s, 3H, NMe), 3.53–3.82 (m, 2H), 3.78 (s, 3H, OMe), 3.91–3.96 (d, 1H, *J* = 13.9 Hz), 5.18–5.24 (d, 1H, *J* = 15.2 Hz), 5.59–5.69 (m, 2H), 6.72–6.78 (m, 2H), 7.13–7.29 (m, 4H), 7.74–7.77 (d, 1H, *J* = 8.3 Hz); ^{13}C-NMR, *dept* 250 MHz ($CDCl_3$) δ: 23.1, 30.5, 34.0, 48.6 (NMe), 55.5 (MeO), 59.8, 67.5, 73.6 (14a), 112.0, 117.6, 123.5, 126.6, 127.4, 127.8, 128.4, 128.8, 129.6, 134.4, 141.4; 160.6. Anal. calcd. for $C_{20}H_{24}INO \cdot 0.25\ H_2O$: C 56.41%, H 5.80%, N 3.29%; found: C 56.21%, H 5.75%, N 3.11%.

*Compound **11b***

Yield: 96.5%, white powder, m.p.: 220°C; ^{1}H-NMR 250 MHz (DMSO-d_6) δ: 1.92–2.22 (m, 2H), 2.71–2.81 (m, 2H), 3.05 (s, 3H, NMe), 3.24–3.52 (m, 2H), 3.75–3.84 (m, 8H), 4.73–5.05 (dd, 2H, *J* = 64.6; 15.5 Hz), 5.10–5.17 (dd, 1H, *J* = 13.2; 4.8 Hz); 6.92 (s, 1H); 7.04 (s, 1H), 7.24–7.32 (m, 4H); ^{13}C-NMR 250 MHz (DMSO-d_6) δ: 23.1, 29.9, 32.5, 48.9 (NMe), 56.1, 56.7, 59.2, 67.8, 73.7, 115.6, 116.4, 124.3, 127.2, 127.5, 127.7, 128.6, 129.2, 131.2, 133.8, 147.3, 149.4. Anal. calcd. for $C_{21}H_{26}INO \cdot 0.10\ H_2O$: C 55.30%, H 6.03%, N 2.79%.

*Compound **12a***

Yield: 94.0%, white solid, m.p.: 255°C; ^{1}H-NMR 250 MHz (methanol-d_4) δ: 3.27 (s, 3H, NMe), 3.13–3.35 (m, 4H), 3.41–3.57 (m, 2H), 3.78 (s, 3H, MeO), 3.65–3.97 (m, 3H), 4.15–4.32 (m, 1H), 4.94–4.98 (d, 1H, *J* = 9.8 Hz), 6.85 (s, 1H), 6.90–6.94 (d, 1H, *J* = 8.7 Hz), 7.22–7.39 (m, 5H). Anal. calcd. for $C_{20}H_{24}INO \cdot 0.25\ H_2O$: C 56.13, H 6.01, N 2.98.

*Compound **12c***

Yield: 91.8%, white solid, m.p: 257°C; ^{1}H-NMR 250 MHz (methanol-d_4) δ: 3.27 (s, 3H, NMe), 3.30–3.49 (m, 4H), 3.55–4.01 (m, 5H), 4.20–4.41 (m, 1H), 5.01–5.05 (d, 1H, *J* = 9.9 Hz), 7.24–7.39 (m, 8H). Anal. calcd, for $C_{19}H_{22}IN$: C 58.32%, H 5.67%, N 3.58%.

*11-Methoxy-6-methyl-6,7,8,9,14,15-hexahydro-5H-dibenzo[c,g]azacycloundecene hydrochloride **4a**, 11,12-dimethoxy-6-methyl-6,7,8,9,14,15-hexahydro-5H-dibenzo[c,g]azacycloundecene hydrochloride **4b**, 3-methoxy-7-methyl-6,7,8,9,14,15-hexahydro-5H-dibenzo[d,h]azacycloundecene hydrochloride **6a**, and 7-methyl-6,7,8,9,14,15-hexahydro-5H-dibenzo[d,h]azacycloundecene hydrochloride **6c***

In a 100-mL three-neck flask equipped with a balloon as an overflow tank and cooled in a liquid nitrogen bath, ammonia was condensed until it was 3/4 filled. The cooling bath was removed and the ammonia was allowed to liquefy. After suspending 1 mmol of the quaternary ammonium salts (**11**, **12**) in the liquid ammonia, small pieces of sodium were added to the stirred mixture until the developing blue colour remained for 10–15 min. The mixture was quenched with 1–2 drops of saturated aqueous NH_4Cl. The ammonia was evaporated under nitrogen and the residue portioned between ether and water. The aqueous phase was extracted with ether (3–15 mL) and the pooled organic

phases were dried over $MgSO_4$ and evaporated. Products were purified by column chromatography ($CH_3OH/CHCl_3$; 1:3). The product fractions were merged and the solvent removed *in vacuo*. The resulting oil was dissolved in little methanol, followed by the addition of a few drops of etheric HCl and crystallization from methanol/ether.

*Compound **4a***

Yield: 32%, white powder, m.p.: 231°C; ^{1}H-NMR 400 MHz (methanol-d_4) δ: 2.05–2.47 (m, 4H), 2.76–2.90 (m, 2H), 3.01–3.15 (m, 5H), 3.41 (s, 1H, 5), 3.34–3.49 (m, 2H), 3.77 (s, 3H, OMe), 4.16 (s, 1H, 5), 6.75–6.82 (m, 2H), 7.23–7.25 (d, J = 8.3 Hz, 1H), 7.33–7.38 (t, J = 7.5 Hz, 1H), 7.48–7.55 (m, 3H); ^{13}C-NMR 400 MHz (methanol-d_4) δ: 23.9, 24.8, 28.3, 33.3, 33.6, 42.2, 54.4, 55.5, 111.9, 115.2, 126.7, 130.2, 130.5, 130.5, 130.8, 131.4, 132.5, 140.4, 142.8, 158.4. Anal. calcd. for $C_{20}H_{26}ClNO \cdot 0.20\ H_2O$: C 71.61%, H 8.21%, 3.96%.

*Compound **4b***

Yield: 43%, white crystals, m.p.: 218°C; ^{1}H-NMR 250 MHz (methanol-d_4) base δ: 1.58–1.90 (m, 4H), 2.16 (s, 3H, NMe), 2.37–2.51 (m, 2H), 2.59–2.90 (m, 4H), 2.92–3.50 (m, 2H), 3.86 (s, 3H, OMe), 3.92 (s, 3H, OMe), 6.70 (s, 1H, 1), 6.80 (s, 1H, 4), 7.15–7.34 (m, 4H); ^{13}C-NMR 250 MHz (methanol-d_4) δ: 23.8, 31.7, 32.6, 32.9, 47.6, 55.1, 55.1, 55.2, 114.7, 116.4, 125.7, 126.2, 126.8, 126.9, 129.1, 134.0, 136.5, 139.9, 146.9, 149.5; GC-MS (*m/z*): 265 (4%), 250 (2%), 234 (4%), 219 (1%), 207 (2%), 193 (5%), 178 (8%), 160 (14%), 146 (29%), 130 (13%), 115 (66%), 104 (100%), 91 (53%), 78 (87%), 65 (28%). Anal. calcd. for $C_{21}H_{28}ClNO_2 \cdot 2\ H_2O$: C 63.53%, H 7.85%, N 3.26%.

*Compound **6a***

Yield: 73%, white powder, m.p.: 96°C; ^{1}H-NMR 250 MHz (methanol-d_4) δ: 2.87–2.91 (m, 7H), 3.02–3.10 (m, 4H), 3.47–3.51 (m, 4H), 3.76 (s, 3H, OMe), 6.79–6.82 (m, 2H), 7.12–7.38 (m, 5H); ^{13}C-NMR, *dept* 250 MHz (Methanol-d_4) δ: 26.4, 26.6, 33.4, 34. 3, 40.7 (N-Me), 53.7, 54.4 (O-Me), 112.8, 115.4, 126.3, 127.4, 129.9, 130.1, 131.0, 131.9, 134.9, 136.1, 140.1, 158.3; GC-MS (*m/z*): 325 (2%), 292 (2%), 283 (2%), 265 (8%), 253 (4%), 239 (5%), 220 (12%), 206 (4%), 189 (1%), 178 (15%), 165 (7%), 154 (11%), 146 (24%), 131 (20%), 117 (61%), 104 (100%), 91 (67%), 78 (82%), 65 (32%). Anal. calcd. for $C_{20}H_{26}ClNO \cdot H_2O$: C 69.39%, H 8.19%, N 3.73%.

*Compound **6c***

Yield: 62%, white powder, m.p.: 145°C; ^{1}H-NMR 450 MHz (methanol-d_4) δ: 2.91–2.98 (m, 7H), 3.09–3.13 (t, J = 7.4 Hz, 4H), 3.47–3.51 (t, J = 7.5 Hz, 4H), 7.16–7.21 (m, 2H), 7.24–7.28 (m, 4H), 7.39–7.41 (m, 2H); ^{13}C-NMR, *dept* 400 MHz (methanol-d_4) δ: 26.3, 34.1, 40.6 (N-Me), 53.8, 126.4, 127.4, 130.0, 130.2, 134.8, 140.0; GC-MS (*m/z*): 295 (1%), 280 (2%), 264 (3%), 249 (1%), 235 (1%), 223 (3%), 204 (1%), 190 (4%), 178 (5%), 160 (5%), 147 (24%), 132 (14%), 117 (47%), 104 (100%), 91 (72%), 78 (79%), 65 (32%). Anal. calcd. for $C_{19}H_{24}ClN \cdot 0.30\ H_2O$: C 72.83%, H 8.33%, N 4.24%.

Pharmacology

Radioligand binding experiments and the functional calcium assay were performed as described in detail in a former publication [1]. Human D_1, D_{2L}, D_3, and D_5 receptors were expressed in HEK cells and $D_{4.4}$ receptors were expressed in CHO cells, respectively.

Radioligand binding experiments

Cells were grown at 37°C under a humidified atmosphere of 5% CO_2:95% air in HAM/F12-medium (Sigma-Aldrich, Germany) for CHO cells and Dulbecco's modified Eagles Medium Nutrient mixture F-12 Ham for HEK293 cells, each supplemented with 10% fetal bovine serum, 1 mM L-glutamine and 0.2 µg/mL of G 418 (all by Sigma-Aldrich).

Radioligand binding assays with a whole-cell-suspension [1] were carried out in triplicate in a volume of 550 µL in 96-well plates (Greiner bio-one, Frickenhausen, Germany), containing: TRIS-Mg^{2+}-buffer (345 µL), [^{3}H]-ligand (50 µL), whole-cell-suspension (100 µL), and appropriate drug dilutions (55 µL). [^{3}H] SCH23390 was used as radioligand for D_1 and D_5, and [^{3}H] spiperone for D_2-D_3. Non-specific binding was determined using fluphenazine (100 µM) for D_1 and D_5 tests and haloperidol (10 µM) for D_2, D_3, and D_4 tests. For determining the K_i values at least two independent experiments, each in triplicate, were performed. The competition binding data were analyzed with Graph Pad Prism™ 3.0. K_i values were calculated from IC_{50} values applying the equation of Cheng and Prusoff [11].

Functional calcium assay

Screening for agonistic and antagonist activity was performed in an intracellular calcium assay using a NOVOstar microplate reader™ (BMG LabTechnologies, Offenburg, Germany) with a pipettor system. Agonistic activities were tested by injecting 20 µL buffer alone as negative control, standard agonist in buffer as positive control (final concentration: 1 µM), and test compounds in buffer in rising concentrations, respectively, each into separate wells. Screening for antagonist activities was performed by pre-incubating the cells with 20 µL of the test compound dilutions (final concentrations: 100 µM, 50 µM, 10 µM, 5 µM, 1 µM, 500 nM, 100 nM, 50 nM, 10 nM, 1 nM, 0.1 nM) at 37°C 30 min prior to injection of 20 µL standard agonist per well. Fluorescence measurement started simultaneously to the automatic injection. SKF 38393 was used as standard agonist for D_1 receptors and quinpirole for D_2 receptors. At least two independent experiments each in four or six replications were performed. Fluorescence intensity was measured at 520 nm (bandwidth 25 nm) for 30 s at 0.4-s intervals. Excitation wavelength was 485 nm (bandwidth 20 nm). Agonistic or antagonist activities were assessed by a dose-response curve obtained by determination of the maximum fluorescence intensity of each data set and nonlinear regression with sigmoidal dose-response equation using Graph Pad Prism™ 3.0.

MTT-test

U87-MG glia cells (ATCC HTB-14) were cultured at 37°C, and 5% CO_2 in DMEM (PAA, E15-883) + 10% FCS (Thermo Scientific HyClone. Logan UT, USA). In 96-well plates, 15 000 cells were dispersed in 200 µL into each well. After 24 h, the medium was replaced by DMEM + FCS containing the test compound solved in a final concentration of 0.25% DMSO. To minimize edge-effects as mentioned by Rasmussen [12], each concentration of each compound was measured in six wells distributed across the 96-well plate and only the inner 6 6 10 wells of the plate were analyzed. The outer wells were filled with 200 µL PBS-buffer. As positive control, six wells with cells and medium containing 0.25% DMSO were analyzed. After 24 h of incubation, the medium was removed and 100 µL MTT (Fluka), dissolved in phenol-red-free DMEM (without FCS) at 0.5 mg/mL, were added to

each well. The plates were incubated for 4 h. Cells were killed and formazan crystals were solubilized by addition of 100 μL of 20% sodium dodecylsulfate (SDS) in H_2O followed by incubation overnight at 37°C. Optical density was measured at 544 nm using a microplate reader (Galaxy FluoStar, BMG Labtechnologies) with background substraction (compounds in the same preparation without cells). Cytotoxicity values were calculated as percentage of positive control (= 100%). Cc_{50} values were calculated with Graph Pad Prism™ 3.0. All experiments were repeated at least three times.

In-silico studies

Energy minimization was performed with ChemAxon Marvin software and measuring of the distances was done with Accelrys® Discovery Studio Visualizer 2.0.

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