N-*n*-Propyl-Substituted 3-(Dimethylphenyl)piperidines Display Novel Discriminative Properties between Dopamine Receptor Subtypes: Synthesis and Receptor Binding Studies[1]

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3-Phenylpiperidines (PPEs) have been thoroughly investigated in view of their interesting dopaminergic activity, and the *N*-*n*-propyl substitution has been suggested as the most effective among several PPEs differently substituted on the phenyl ring. In previous studies, we found that the dimethyl substitution on the phenyl ring of N-unsubstituted PPEs provided compounds active toward α_2-adrenergic receptors (α_2-ARs), which proved to possess interesting selectivity properties. The high degree of homology between the binding domains of α_2-ARs and D_4-dopaminergic receptors (D_4-DARs) prompted us to verify whether this kind of substitution on the aromatic ring might prove to be active against retinal DARs of the D_4 subtype. On the basis of these premises, we synthesized the dimethylphenyl-substituted PPEs **4a**−**f**, in which an *n*-propyl chain is present on the aminic nitrogen. Radioligand binding assays on bovine retina and striatum membranes for D_1-like and D_2-like DARs indicated that PPEs **4a, 4b**, and **4f** possess a high affinity and selectivity for the D_4-DAR subtype of bovine retina.

Introduction

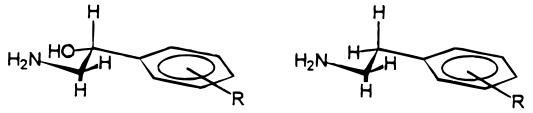
Dopamine (DA), like other biogenic amines, is found in the central nervous system in pathways essential for controlling sensory and motor performance, as well as higher functions. Although dopaminergic neurons form a relatively small class of cells clustered in discrete regions of the brain, they control, with their connections, virtually all areas of the brain. DA triggers its physiological and pathological actions upon binding to receptors located on the surface membrane of target cells. Dopamine receptors (DARs) have been classified as either D_1 or D_2, based on their ability to activate or inhibit the enzyme adenylyl cyclase, respectively.[3]

Since 1988 several DAR subtypes[4] sharing a common structural motif with other members of the G-protein-coupled receptor superfamily[5] have been cloned and assigned to the D_1-like or D_2-like subfamilies. The D_2-like receptor superfamily includes two isoforms of the D_2 subtype generated by alternative splicing:[6−9] the D_3[10] and D_4 subtypes.[11]

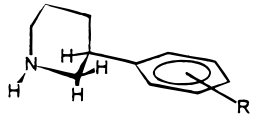
D_2-like DAR subtypes are not uniformly expressed in either rat or human brain. The heterogeneous distribution of D_2-like receptors opens the way to the targeting of therapeutic interventions in critical brain areas, by designing selective pharmacological agents to exploit differences in receptor subtypes. In this respect, the observation that the atypical neuroleptic clozapine has a 10-fold higher affinity for D_4-DAR than for D_2/D_3 receptors[11] has aroused considerable interest in the synthesis of D_4-selective drugs.

3-Phenylpiperidines (PPEs) have been thoroughly investigated since the early 1980s, in view of their interesting dopaminergic activity.[12,13] Several PPEs have consequently been studied and substituted on both the aromatic ring and the aminic nitrogen. The most interesting of these is 3-(3-hydroxyphenyl)-*N*-(*n*-propyl)-piperidine (3-PPP, preclamol),[14−16] reported to be the first selective D_2-like DA autoreceptor agonist; its *S*-(−)-enantiomer has been reported to be more active than the *R*-one. The *N*-*n*-propyl substitution has been suggested to be the most effective also for other PPEs differently substituted on the aromatic ring.[12,14,17−19]

In a previous paper[20] we described certain N-unsubstituted PPEs with the general formula **3** which display good activity and selectivity on α_2-adrenergic receptors (α_2-ARs). PPEs **3** were studied as conformationally restricted analogues of adrenergic drugs with a phenylaminoethanolic structure (**1**), in which the C(1)−C(2) side chain of amino alcohols **1** is incorporated into a piperidine ring.



1 **2**



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However, PPEs **3** lack the benzyl hydroxyl group of **1** and are, therefore, congeners of drugs with a phenylethylamine structure such as DA differently substituted

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Table 1. Binding Affinities of PPEs for D_1-like and D_2-like Dopamine Receptors

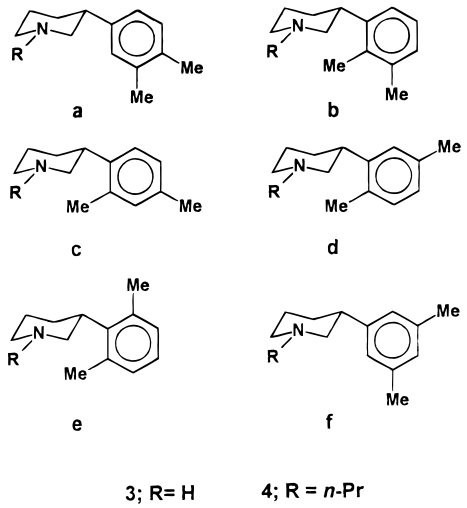
	K_i (nM)[a]			
compd	D_1 (retina)[b]	D_2 (retina)[c,d] high affinity	D_2 (retina)[c] low-affinity	D_2 (striatum)[c]
4a	(37 000, 102 000)	19.1 ± 8.5	8 200 ± 5 700	4 990 ± 290[e]
4b	(33 000, 60 000)	75.3 ± 12.9[f]	7 800 ± 2 300[f]	(4 600, 4 900)
4c	(86 000, 100 000)	(240, 720)	(13 000, 17 000)	nd[g]
4d	112 000 ± 21 000	(360, 540)	(21 000, 25 000)	nd
4e	(98 000)	(156)	(23 000)	nd
4f	60 000 ± 6 000	(16, 52)[h]	(6 400, 9 300)[h]	(1 000, 1 300)
3a	nd	(78, 140)	(6 800, 19 000)	nd
3b	nd	(33, 43)	(4 000, 16 000)	nd
3f	nd	(90, 240)	(19 000, 24 000)	nd
dopamine	4 300 ± 1 200	14.9 ± 5.9[i]	1 520 ± 740[i]	617 ± 75
quinpirole	nd	59.4 ± 11.4[i]	2 580 ± 570[i]	(1 300, 1 500)
S-(−)-3-PPP	nd	(530, 530)	(7 600, 12 000)	(1 500, 2 100)

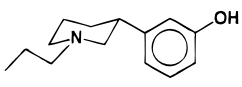
[a] K_i values are expressed as the mean ± standard error of the mean (SEM), for compounds which were tested three or more times. When less than three assays were performed, K_i values estimated from each assay are reported in parentheses. [b] D_1-like receptors labeled with [^{3}H]SCH23390. [c] D_2-like receptors labeled with [^{3}H]YM-09-151-2. [d] The high-affinity site represents 28%, 17%, 19%, 17%, 16%, and 27% of total binding for compounds **4a**−**f**, respectively, and 11%, 19%, and 20% of total binding for compounds **3a**, **3b**, and **3f**, respectively. The high-affinity site represented 51%, 53%, and 45% of total binding for dopamine, quinpirole, and *S*-(−)-3-PPP, respectively. [e] K_i was 4300 nM when tested against striatal receptors labeled with [^{3}H]raclopride. [f] In the presence of 200 μM GTP-γ-S the competition curve became monophasic, with a Hill coefficient of 0.98. [g] Not determined. [h] In the presence of 200 nM raclopride, K_i's were 44 and 9300 for the high- and low-affinity sites, respectively, and the high-affinity site was 46% of total binding. [i] For the purposes of comparison with striatal DARs, dopamine and quinpirole K_i's in the retina were 134 ± 27 and 356 ± 134 nM, respectively.

on the phenyl ring (**2**). The most intriguing of the PPEs proved to be the 3-(3,4-dimethylphenyl)-substituted one, **3a**. Its interesting biopharmacological properties were subsequently revealed in the study carried out on its five dimethylphenyl-substituted isomers **3b**−**f**.[2]

The high degree of homology between the binding domains of α_2-ARs and D_4-DARs[21] prompted us to verify whether this kind of substitution on the aromatic ring might prove to be active against retinal DARs of the D_4 subtype.

In light of these considerations, we synthesized the dimethylphenyl-substituted PPEs **4a**−**f** in which an *n*-propyl chain is present on the aminic nitrogen. Compounds **4a**−**f** were screened for their affinity for D_1-like and D_2-like retinal receptors by radioligand binding assays.





Furthermore, in consideration of the known differences in the binding domains between D_4-DARs and D_2/D_3-DARs,[21] compounds **4a**, **4b**, and **4f**, which have a higher affinity for retinal receptors than the remaining compounds, were also screened in membrane preparations from striatum, containing D_2 and D_3 receptors. For the sake of comparison, the full agonist quinpirole and the partial agonist *S*-(−)-3-PPP were also screened in both retinal and striatal membrane preparations. Furthermore, the N-unsubstituted analogues **3a**, **3b,** and **3f**,[2] corresponding to the most active compounds **4a**, **4b**, and **4f**, were tested for their affinity for D_4-DARs. PPEs **4a**, **4b**, and **4f** were also tested for their affinity for α_2-ARs.

Chemistry

The *N*-*n*-propyl-substituted 3-(dimethylphenyl)piperidines **4a**−**f** were obtained directly by reductive alkylation of the corresponding N-unsubstituted 3-(dimethylphenyl)piperidines **3a**−**f**[2] with propionaldehyde and sodium cyanoborohydride.

The arylpiperidines **3a**−**f** were in turn prepared by hydrogenation in the presence of PtO_2 of the corresponding 3-arylpyridines obtained through a cross-coupling reaction of the appropriate arylmagnesium bromide with 3-bromopyridine catalyzed by dichlorobis-(triphenylphosphine)nickel(II).

Conformational analysis was carried out on compounds **4a**−**f** by means of the Discover[22] molecular mechanics program, following the same procedure already used for the corresponding N-unsubstituted compounds **3a**−**f**.[2] The results of calculations indicate that compounds **4a**−**f** exist in the expected conformation,[2] in which the piperidine ring is in a chair conformation and the aromatic ring is in the equatorial position.

Radioligand Binding Assays

The affinities of the PPEs studied **(3a**, **3b**, and **3f**, and **4a**−**f)** for DARs were estimated by means of radioligand competition assays carried out on bovine retinal and striatal membrane preparations (Table 1). The antago-

nists [^{3}H]SCH23390 and [^{3}H]YM-09-151-2 were used as specific radioligands for D_1-like and D_2-like DARs, respectively. [^{3}H]Raclopride was also used as a selective antagonist radioligand for D_2- and D_3-DARs.

The affinities of PPEs **4a**, **4b**, and **4f** for α_2-ARs were determined by radioligand binding assays carried out in rat brain membrane preparations. The antagonist [^{3}H]rauwolscine was used as a specific radioligand.

Bovine Retina D_1-like Dopamine Receptors. PPEs **4a**−**f** had monophasic inhibition curves when tested against D_1-like DARs labeled with [^{3}H]SCH23390. Their mean inhibition constants were 10−20 times higher than the DA inhibition constant.

Bovine Retina D_2-like Dopamine Receptors. The PPEs had biphasic inhibition curves when tested against DARs labeled with [^{3}H]YM-09-151-2, as shown in Figure 1A for **4a**−**c** and **4f**. The high-affinity inhibition constants of PPEs **4a**, **4b**, and **4f** were similar to those of DA and quinpirole and lower than that of *S*-(−)-3-PPP. The remaining compounds (**4c**−**e**) had high-affinity inhibition constants higher than those of DA and quinpirole. As regards the *N*-*n*-propyl-unsubstituted PPEs **3a**, **3b**, and **3f**, while **3a** and **3f** had high-affinity inhibition constants higher than those of the corresponding PPEs **4a** and **4f**, **3b** displayed an inhibition constant value smaller than that of **4b**.

For all PPEs **(3a, 3b, 3f**, and **4a**−**f**), the high-affinity site was less than 30% of total binding, while for DA and quinpirole, the high-affinity site was more than 50%. The high-affinity sites of PPE **4f** and DA were 46% and 65%, respectively, when tested in the presence of 200 nM of the selective D_2 and D_3 receptor blocker raclopride, with no major changes in affinity.

Bovine Striatum D_2-like Dopamine Receptors. Compounds **4a**, **4b**, and **4f** were also tested against striatal D_2-like DARs labeled with [^{3}H]YM-09-151-2. Their inhibition curves were monophasic, as shown in Figure 1B for **4a**, with average inhibition constants in the micromolar range. A similar inhibition constant was found in striatal membranes for the parent compound *S*-(−)-3-PPP. PPE **4a** was also tested against striatal receptors labeled with [^{3}H]raclopride, and the inhibition constant was in the micromolar range.

Rat Brain α_2-Adrenergic Receptors. PPEs **4a**, **4b**, and **4f** showed K_i values of 630, 670, and 500 nM, respectively, which were about 2 orders of magnitude higher than that of NE (4.8 nM). *S*-(−)-3-PPP showed a higher affinity compared with the other PPEs tested, with a K_i value of 163 nM.

Discussion

The results of the binding assays indicate that PPEs **4a**−**f** were virtually inactive on retinal D_1-like receptors labeled with [^{3}H]SCH23390, with monophasic inhibition curves and inhibition constants higher than 10 μM.

On the other hand, compounds **4a**−**f** had inhibition constants in the nanomolar range for the high-affinity site of retinal DARs labeled with [^{3}H]YM-09-151-2, indicating an approximately 1000-fold selectivity between D_1-like and D_2-like retinal DARs.

The distinctive feature of PPEs **4a**−**f** competition curves against retinal D2-like receptors labeled with [^{3}H]YM-09-151-2 was the presence of two binding components. The observation that GTP-γ-S, a nonhydrolyzable GTP analogue, converts the high-affinity state to a low-affinity one indicates that compounds **4a**−**f** are akin to agonists. The relative percentage of the high-affinity component for compounds **4a**−**f** (15−30%) is lower than that of DA and quinpirole (around 50%). Since raclopride competition experiments suggest the presence in the retina of D_2/D_3 receptors (20%) along with D_4 (80%) receptors, differences in the percentage of the high-affinity component may therefore be attributed to the failure of **4a**−**f** to interact with a high affinity with D_2 and D_3 receptors. In agreement with this idea, PPEs **4a**, **4b**, and **4f** had monophasic inhibi-

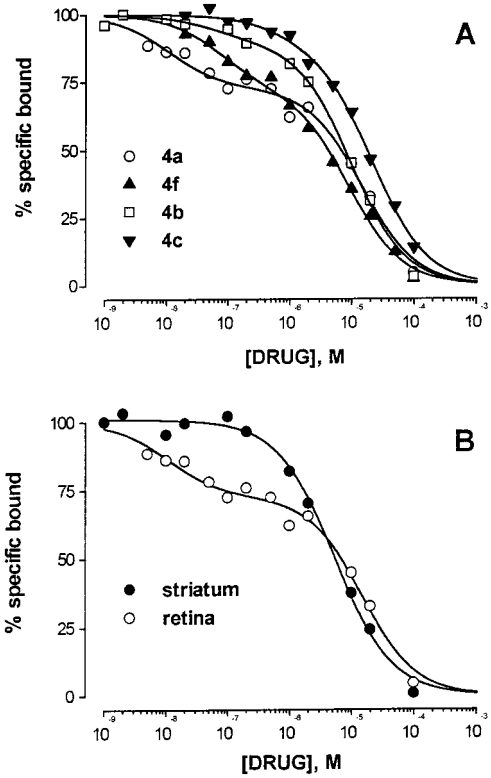


Figure 1. (A) Examples of typical biphasic competition experiments between PPEs and [^{3}H]YM-09-151-2 for retinal DARs. The ordinate is the [^{3}H]YM-09-151-2 specific bound at each drug concentration, expressed as a percent of the quantity bound in the absence of the competitor. High-affinity site K_i's from the single experiments plotted in panel A are 7.9, 87, 720, and 52 nM for **4a** (open circles), **4b** (open squares), **4c** (solid upward triangles), and **4f** (solid downward triangles), respectively. The continuous curves through the data points were drawn in accordance with to a two-site model (see eq 2 in Experimental Section), using parameters that minimize the sum of squares (*SS*) between experimental data and the theoretical curve. The *F*-test (see Experimental Section), suggests that the two-site model provides a significantly better fit to data than the one-site model, with $P < 0.005$ for **4a** and **4f**, and $P < 0.01$ for **4b**. For compound **4c** $P > 0.05$, but if the parameter n in eq 1 was fixed to unity, then $P < 0.01$. This result indicates that the competition curve for **4c** is not truly monophasic, and we have therefore accepted a two-site model also for compound **4c**. (B) Examples of representative displacement experiments between **4a** and [^{3}H]YM-09-151-2 for striatal (solid circles) and retinal (open circles) DARs, respectively. A two-site model (see eq 1 in Experimental Section) does not provide a better fit than a one-site model for displacement curves in striatal membranes ($F = 88.6$ for the two-site equation and $F = 83.9$ for the one-site equation). The continuous curve has been drawn with a Hill coefficient (n) of 0.95 and a K_i of 4400 nM. Data for retinal membranes (open circles) are replotted from panel A for comparison.

tion curves, with inhibition constants in the low-micromolar range, when tested against striatal (mostly D_2 and D_3) DARs labeled with [^{3}H]YM-09-151-2. This indicates a 20−100-fold selectivity of PPEs **4a**, **4b**, and **4f** between retinal and striatal DARs. However, competition experiments in the presence of 200 nM raclopride indicated that the high-affinity component is 48% for PPE **4f** and 65% for DA. This result does not support the idea that differences in the percentage of the high-affinity component between **4a**−**f** and DA and/or quinpirole may be explained by the heterogeneity of retinal D_2-like DARs. The possibility that PPEs **4a**−**f** are agonists with an intrinsic activity lower than that of full agonists will require specific behavioral and functional tests.

Differences in the affinities for retinal and striatal DARs labeled with [^{3}H]YM09-151-2 may be a consequence of the different properties of DAR subtypes expressed in the retina and the striatum. In general agreement with this notion, the affinity of PPE **4f** for retinal DARs is not affected by 200 nM raclopride; furthermore, PPE **4a** has similar affinities for striatal DARs labeled with [^{3}H]YM-09-151-2 or with [^{3}H]raclopride, confirming the low affinity of compound **4a** for the D_2- and D_3-DAR subtypes. For the purpose of reference, the affinity of clozapine, which has been reported to have a moderate selectivity for D_4- over D_2-DARs, was 38 nM in the bovine retina, similar to the values reported for cloned human D_4-DARs[23] (21−30 nM).

We therefore suggest that compounds **4a**, **4b**, and **4f** have a high selectivity for the high-affinity site of the D_4 receptor.

Compounds **4a**, **4b**, and **4f** show an increase approximately up to 20-fold in the K_i for the high-affinity site of retinal D_4-like DARs with respect to compounds **4c**−**e**. A common characteristic of **4a**, **4b**, and **4f** is the presence of a methyl group in the meta position which could be important for eliciting a high affinity. By contrast, the presence of the methyl in the ortho position seems to have a negative effect, as can be seen from the regularly lower affinity of the *o*-methyl-substituted compounds **4b**−**e** compared with the non-*o*-methyl-substituted **4a** and **4f**.

Conclusions

A major drawback of schizophrenia treatment with D_2- and D_3-selective antagonists is the appearance of a severe motor syndrome, such as tardive dyskinesia.[24] Furthermore, neuroleptic drugs, which are effective against the so-called "positive" symptoms (delusion and hallucinations), may have a low efficacy against the so-called "negative" symptoms of schizophrenia (social withdrawal and flattened effect).[25]

The observation that clozapine, an atypical neuroleptic drug with a low propensity to induce motor problems, effective against the negative symptoms, has an approximately 10-fold higher affinity for D_4-DARs than for D_2- or D_3-DARs[11] has encouraged the development of D_4-selective compounds for use as antipsychotic agents. However, as the therapeutic effectiveness of clozapine may result from its activity at muscarinic[26] and serotonergic[27] receptors, in addition to its antagonism of D_4 receptors, D_4-selective compounds are needed in order to assess the role of D_4-DARs in the treatment of schizophrenia.

PPEs have been thoroughly investigated in view of their interesting dopaminergic activity, especially if *N*-*n*-propyl-substituted.[12−18] The dimethyl substitution on the phenyl ring of N-unsubstituted PPEs has been found to provide compounds active as α_2-adrenergic agents, which have proved to possess interesting selectivity properties.[2] In this study a substitution of this kind in *N*-*n*-propyl-substituted PPEs was found to provide agents possessing a high selectivity toward retinal D_2-like (mostly D_4) DARs. In particular, the PPEs which are dimethyl-substituted on the phenyl ring, **4a**, **4b**, and **4f**, were found to have a higher affinity and selectivity for D_4-DARs than preclamol, the most widely investigated PPE. Furthermore, PPEs **4a**, **4b**, and **4f** did not display any higher α-adrenergic affinity.

However, the *N*-*n*-propyl substitution is associated with an increase in affinity only for compounds **4a** and **4f** and not for **4b**. The lack of improvement in the affinity of compound **3b** with the *N*-*n*-propyl substitution is in general agreement with the above-mentioned notion of the negative effect of the *o*-methyl substitution.

In PPEs dimethyl-substituted on the phenyl ring, the meta position appears to be critical in order to provide both a high affinity for D_4-DARs and a good selectivity between D_4- and D_2/D_3-DARs. The importance of a methyl substituent on a phenyl ring for increasing the selectivity toward the D_4-DARs versus D_2/D_3-DARs is in general agreement with the results of recent reports.[28,29]

Compounds **4a**, **4b**, and **4f**, which have a good D_4 selectivity, may thus be useful for addressing the controversial role of D_4-DARs in schizophrenia.

Experimental Section

Chemistry. Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. IR spectra for comparisons between compounds were taken with a FT-IR Mattson 1000 Unicam spectrometer, as Nujol mulls in the case of solid substances or as liquid film in the case of liquids. ^{1}H NMR spectra were routinely detected with a Varian CFT-20 spectrometer operating at 80 MHz in ca. 5% D_2O, using $Me_3Si(CH_2)_3SO_3Na$ as the internal standard. The electron impact mass spectra were recorded on a Hewlett Packard 5988A spectrometer by direct introduction at a nominal electron energy of 70 eV and a source temperature of 350 °C. Evaporations were performed in vacuo (rotating evaporator). $MgSO_4$ was always used as the drying agent.

Elemental analyses were performed by our analytical laboratory and agreed with theoretical values to within ±0.4%.

General Procedure for the Synthesis of 3-(3,4-Dimethylphenyl)- (4a·HCl), 3-(2,3-Dimethylphenyl)- (4b·HCl), 3-(2,4-Dimethylphenyl)- (4c·HCl), 3-(2,5-Dimethylphenyl)- (4d·HCl), 3-(2,6-Dimethylphenyl)- (4e·HCl), and 3-(3,5-Dimethylphenyl)-*N*-*n*-propylpiperidine Hydrochloride (4f·HCl). Sodium cyanoborohydride (1.06 mmol) was added portionwise to a mixture of the appropriate piperidine hydrochloride **3a**−**f**[2] (0.44 mmol) and propionaldehyde (1.06 mmol) in anhydrous MeOH (5 mL); the reaction mixture was stirred at room temperature for 24 h and then poured into H_2O (30 mL) and extracted with AcOEt. Evaporation of washed (H_2O) and dried extracts yielded the appropriate crude **4a**−**f** as an oil, which was dissolved in anhydrous Et_2O and then treated with an excess of Et_2O·HCl. The resulting solid precipitate was filtered and crystallized from MeOH−Et_2O to yield pure **4a**−**f** as hydrochloride salts. **4a·HCl** (45%): mp 188−190 °C; ^{1}H NMR δ 0.97 (t, 3H, J = 7.2 Hz), 1.45−2.1 (m, 6H), 2.25 (s, 6H), 2.6−3.75 (m, 7H), 7.09 (m, 3H); MS m/z 231

$(M - HCl)^+$. Anal. ($C_{16}H_{26}NCl$) C, H, N. **4b·HCl** (50%): mp 178−180 °C; ^{1}H NMR δ 0.97 (t, 3H, J = 7.2 Hz), 1.45−2.15 (m, 6H), 2.26, 2.30 (2s, 6H), 2.70−3.80 (m, 7H), 7.16 (m, 3H); MS m/z 231 $(M - HCl)^+$. Anal. ($C_{16}H_{26}NCl$) C, H, N. **4c·HCl** (60%): mp 166−168 °C; ^{1}H NMR δ 1.0 (t, 3H, J = 7.2 Hz), 1.60−2.12 (m, 6H), 2.30, 2.34 (2s, 6H), 2.80−3.70 (m, 7H), 7.13 (m, 3H); MS m/z 231 $(M - HCl)^+$. Anal. ($C_{16}H_{26}NCl$) C, H, N. **4d·HCl** (50%): mp 219−221 °C; ^{1}H NMR δ 0.96 (t, 3H, J = 7.2 Hz), 1.42−2.10 (m, 6H), 2.29 (s, 6H), 2.80−3.75 (m, 7H), 7.12 (m, 3H); MS m/z 231 $(M - HCl)^+$. Anal. ($C_{16}H_{26}NCl$) C, H, N. **4e·HCl** (20%): mp 217−219 °C; ^{1}H NMR δ 0.95 (t, 3H, J = 7.2 Hz), 1.50−2.10 (m, 6H), 2.40 (s, 6H), 2.75−3.75 (m, 7H), 7.10 (m, 3H); MS m/z 231 $(M - HCl)^+$. Anal. ($C_{16}H_{26}NCl$) C, H, N. **4f·HCl** (60%): mp 206−208 °C; ^{1}H NMR δ 0.97 (t, 3H, J = 7.2 Hz), 1.30−2.14 (m, 6H), 2.30 (s, 6H), 2.70−3.75 (m, 7H), 6.96 (m, 3H); MS m/z 231 $(M - HCl)^+$. Anal. ($C_{16}H_{26}NCl$) C, H, N.

Radioligand Binding Methods. Radioreceptor binding studies with [^{3}H]SCH23390 (a D_1-like receptor antagonist, 80 Ci/mmol; New England Nuclear, Boston, MA) and [^{3}H]YM-09-151-2 (a D_2-like receptor antagonist, 81-87 Ci/mmol; New England Nuclear, Boston, MA) were performed in membrane preparations from bovine retina and striatum. Striatum was homogenized in a saline solution (1:20 w/v) containing in mM: Tris, 50; EDTA, 1; $CaCl_2$, 1.5; $MgCl_2$, 4; KCl, 5; NaCl, 120; pH 7.4. The homogenate was centrifuged at 48000*g* for 20 min at 4 °C, resuspended 1:20, recentrifuged, and resuspended at a final dilution of about 1.25 mg of original wet tissue/mL of saline (1:800 w/v) for use in the binding assay. Retina were treated similarly to striata except that the last resuspension for use in the binding assay was at about 4 mg of original wet tissue/mL of saline (1:250 w/v). For competition experiments membranes were incubated at 30 °C in the presence of 0.1 nM [^{3}H]YM-09-151-2 or 0.5 nM [^{3}H]SCH23390 for 60 or 20 min, respectively. For [^{3}H]SCH23390 binding, duplicate test tubes contained a final volume of 1 mL: 0.5 mL of membranes, 0.1 mL of drug, and 0.1 mL of tracer. For [^{3}H]-YM-09-151-2 binding, duplicate test tubes contained a final volume of 2 and 1.5 mL, for striatum and retina, respectively: 0.5 mL of membranes, either 0.15 or 0.2 mL of tracer, and drug for retina or striatum, respectively. The affinity of [^{3}H]-SCH23390 and [^{3}H]YM-09-151-2 was measured by saturation experiments in similar conditions, and the values were 2 and 0.4 nM, respectively. Nonspecific binding was assessed by 2 mM DA.

At the end of the incubation period, the radioactivity bound to the receptor was separated from the free ligand by rapid filtration under a vacuum, using a 30-well filtration apparatus (Brandel). Filters were counted by liquid scintillation spectroscopy (LS-1600, Packard, Canberra Co.) and converted from cpm to dpm.

Specific binding was obtained by subtracting nonspecific binding from totals and normalized to specific binding in the absence of drugs. Normalized data from 10−12 concentrations were fitted by either a single-site model (eq 1) or a two-site model, (eq 2) by nonparametric fitting using a modified Lowenberg−Marquardt algorithm implemented in the data analysis program Microcal Origin, version 3.5 (Microcal Software, Inc., Northampton, MA 01060).

$$B(L) = 100 - \frac{100 \cdot L^n}{L^n + IC_{50}^n} \quad (1)$$

$$B(L) = 100 - \frac{B_H \cdot L}{L + K_H} - \frac{(100 - B_H) \cdot L}{L + K_L} \quad (2)$$

The acceptance of a two-site model versus a single-site model was performed in accordance with the criteria described by Munson and Rodbard.[30] Briefly, the statistical significance of a one-site versus a two-site model was assessed by the F-test, in accordance with the relation:

$$F = \frac{\frac{(SS_1 - SS_2)}{SS_2}}{\frac{(df_1 - df_2)}{df_2}}$$

where SS_1 and SS_2 are the sum of squares for the vertical distance between experimental data and the computed curve for the one-site and two-site models, respectively; for the 12-point competition curves, the degrees of freedom (df_1 and df_2) are 9 and 8 for the one-site and two-site models, respectively. The probability of finding an F value equal or higher by chance was taken from the tabulated values of the F distribution with ($df_1 - df_2$) and df_2 degrees of freedom. K_i values were obtained from IC_{50} by the Cheng−Prusoff equation:[31] $K_i = IC_{50}/(1 + [L]/K_d)$, where IC_{50} is the drug concentration inhibiting 50% of specific binding. K_d values were 2, 0.4, and 1 nM for [^{3}H]-SCH23390, [^{3}H]YM-09-151-2, and [^{3}H]raclopride, respectively.

α_2-Receptor binding assays were performed in rat cerebral cortex membranes, as elsewhere reported.[32,33] The values of inhibition constants (K_i, nM) were calculated from the respective IC_{50} values by applying the Cheng−Prusoff equation.[31] [^{3}H]Rauwolscine was used as the radioligand at a concentration of 2.0 nM.

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