New *N*-*n*-Propyl-Substituted 3-Aryl- and 3-Cyclohexylpiperidines as Partial Agonists at the D_4 Dopamine Receptor

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Received August 6, 2002

We have previously reported that compounds dimethyl-substituted on the phenyl ring of *N*-*n*-propyl-3-phenylpiperidines (PPEs) have a high (nM) affinity and selectivity toward the D_4 dopamine receptor (D_4 DAR) with *m*,*p*-dimethyl PPE (**1**) having the highest affinity and selectivity. In the present paper we have investigated the role of the methyl substitution by the synthesis of monomethylated (**2a**−**c**) and nonmethylated (**2d**) PPEs followed by the characterization of their biological properties using receptor binding assays. Our findings reveal that the methyl substitution of the phenyl ring is not necessary for a high and selective binding affinity to the D_4 DAR. Moreover, we have also synthesized cyclohexylpiperidines (CHPEs, **3a**−**d**), which all showed higher binding affinities for the D_4 DAR than their aromatic counterparts. These results indicate that a $\pi-\pi$ type interaction of the phenyl ring of PPEs with the D_4 DAR might not be essential, whereas a simple hydrophobic attraction between the cyclohexyl substituent of CHPEs and a hypothesized lipophilic pocket of the receptor might be crucial. Furthermore, functional assays indicate that **3d**, as well as **1**, are partial agonist at the D_4 DAR and therefore might represent new pharmacological tools to investigate the role of D_4 DAR activation in the control of cognitive functions and emotional states in health and disease.

Introduction

Dopaminergic neurons projecting to the prefrontal cortex and limbic areas play a key role in the control of cognitive functions[1] and emotional states,[2] respectively. Upon release, dopamine interacts with several receptors (DARs) that belong to the superfamily of G-protein-coupled receptors (GPCR).[3] DARs have been classified as D_1- or D_2-like according to their ability to activate or inhibit adenylyl cyclase, respectively.[4] The D_2-like subfamily of DARs includes D_2,[5] D_3,[6] and D_4[7] subtypes. D_2 and D_3 receptors are the target of classic and some atypical neuroleptics, but no conclusive evidence is presently available from biological and genetic studies for D_4 receptors as the target for atypical neuroleptics.[8]

Genetic studies have indicated a certain association between a specific polymorphic variant of the D_4 gene, which translated into a receptor with a blunted response to dopamine, and an attention deficit with hyperactivity disorder (ADHD), a disturbance affecting from 3 to 5% of children.[9] Although the association between the D_4 polymorphism and ADHD has been controversial, a recent meta-analysis[10] has provided convincing evidence for the association between several allelic variations of the D_4 DAR and ADHD. In addition, allelic variations of the D_4 DAR have been associated with specific personality traits such as novelty seeking or impulsive, compulsive, and addictive behaviors, like susceptibility to drug abuse and compulsory gambling.[11] It is relevant to note that recent investigations at the cellular level indicate that mice lacking the D_4 gene are supersensitive to methamphetamine, cocaine, and alcohol[12] and display cortex hyperexcitability.[13] Intriguingly, the psychostimulants amphetamine and methylphenidate, which block the reuptake of dopamine, thus increasing synaptic dopamine levels,[14] are an effective treatment for 70−80% of subjects diagnosed with ADHD and HD. This observation opens up the possibility that agonists selective for D_4 receptors may have therapeutic value in ADHD and HD.

A general concern is that the altered behavior may result from a functional imbalance between the mesocortical and mesolimbic pathways, and compensation with stimulants or full agonists of a hypoactive pathway may lead to excessive stimulation of the other pathway. A therapeutic approach with partial agonists may solve the problem of imbalance between different dopaminergic pathways.[15,16] By acting as a stimulant in those brain areas where the dopaminergic projection is hypofunctional, while preventing full activation of other brain regions that receive the hyperactive or normal projection, a partial agonist would adjust the balance between different anatomically and functionally distinct

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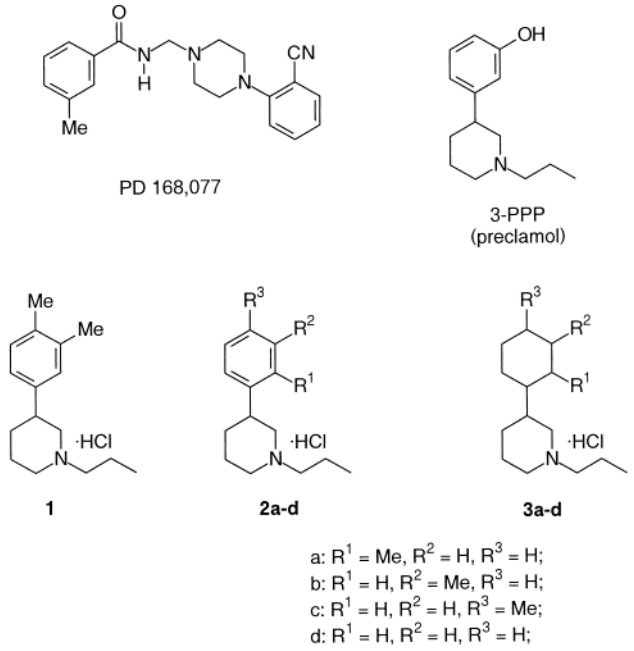
10.1021/jm021019a CCC: $25.00
Published on Web 11/28/2002

Chart 1



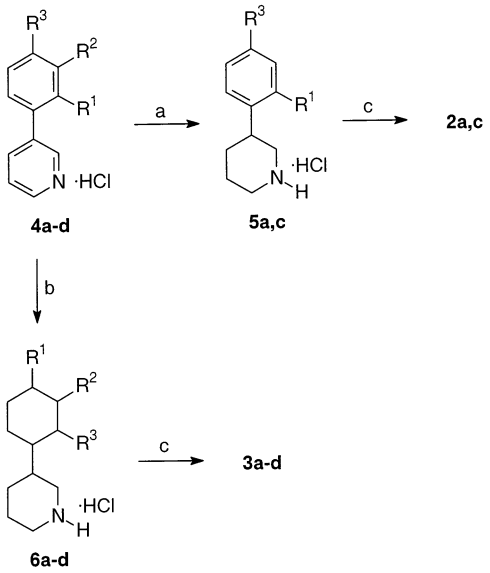
dopaminergic pathways. In this regard, the partial dopamine agonist aripiprazole has recently been proposed as the first member of a new class of antipsychotic drugs.[17]

We have previously reported that a dimethyl substitution on the phenyl ring of 3-phenylpiperidines (PPEs), studied for several years in view of their dopaminergic properties,[18−25] leads to compounds possessing a high (nM) affinity toward the D_4 DAR.[26,27] In particular, the *m*,*p*-dimethyl-substituted derivative **1** proved to be the most potent and selective compound. Furthermore, some of these compounds have been reported to activate the G proteins coupled to these receptors, which is an indication of their agonist type of activity on D_4 DAR.[26]

It is possible to hypothesize that the aromatic moiety of these derivatives may interact with a lipophilic pocket at the level of the active site of the D_4 DAR, in line with the fact that the presence of polar substituents on the aryl leads to a drop in D_4 DAR affinity as for preclamol (3-PPP),[26,27] a 3-(3-hydroxyphenyl)-1-propylpiperidine, reported as the first autoreceptor-selective agonist.[20]

Considering that (see above) compounds with an agonist activity on the D_4 DAR may represent a useful tool for the study and the treatment of the ADHD, we thought it conceivable to further develop these studies through the synthesis of new PPE derivatives. In particular, in this paper, we report the biological evaluation of mono-methyl-substituted (**2a**−**c**) and unsubstituted (**2d**) derivatives, to verify the importance of the presence of the two methyl groups on the phenyl ring of compound **1** for the affinity of PPE's on the D_4 DAR. We also prepared completely saturated analogues of compounds **2a**−**d**, namely cyclohexylpiperidines (CHPEs, **3a**−**d**), with the aim of determining whether the nature of the interaction of these compounds with the hypothesized lipophilic pocket is of $\pi-\pi$ type with an aromatic receptor moiety or it is simple hydrophobic.

Scheme 1[a]



[a] Reagents and conditions: (a) H_2 (1 atm), PtO_2 (10 mol %), concentrated HCl, MeOH, rt, 4 h; (b) H_2 (1 atm), PtO_2 (50 mol %), concentrated HCl, MeOH, rt, 24 h; (c) CH_3CH_2CHO, $NaBH_3CN$, MeOH, rt, 24 h, then HCl.

Chemistry

Compounds **2a**,**c** and **3a**−**d** were synthesized as shown in Scheme 1, whereas compounds **2b** and **2d** were prepared as previously reported.[18]

Mild hydrogenation of 3-arylpyridines **4a**[28] and **4c**[29] under 1 atm of molecular hydrogen in the presence of 10 mol % of platinum oxide gave aryl piperidines **5a** and **5c**. Subsequent treatment with proprionic aldehyde in the presence of $NaBH_3CN$ and final salification with gaseous HCl in Et_2O gave *N*-*n*-propylated 3-arylpiperidines **2a**,**c**. The synthesis of cyclohexylpiperidines **3a**−**d** started with a more vigorous hydrogenation of 3-arylpyridines **4a**,[28] **4b**,[18] **4c**,[29] and **4d**,[30] using a higher amount of catalyst (50 mol % of platinum oxide), which afforded completely aliphatic intermediates **6a**−**d**. The same propylation conditions (proprionic aldehyde, $NaBH_3$-CN) reported above, followed by salification with hydrochloric acid, afforded *N*-*n*-propylated cyclohexylpiperidines **3a**−**d**. It is important to note that the non-methylated compound **3d** is made up of a single diastereoisomer, whereas in the case of **3a** and **3b**, up to four diastereoisomers could be obtained; moreover, also **3c** can exist as two different diastereoisomeric forms. Due to the difficult separation of these mixtures, compounds **3a**−**c** were submitted to biological tests as unresolved diastereoisomeric mixtures.

Results

Radioligand Binding Assays. The affinities of the PPEs (**2a**−**d**) and CHPEs (**3a**−**d**) for DARs were estimated by means of radioligand competition assays carried out on bovine retinal and striatal membrane preparations (Table 1). The antagonists [^{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.

Bovine Retina D_1-like Dopamine Receptors. PPEs and CHPEs had monophasic inhibition curves when tested against D_1-like DARs labeled with [^{3}H]-SCH23390.

Table 1. Binding Affinities of PPE's and CHPE's for D_1-like and D_2-like Dopamine Receptors

	K_i (nM)[a]			
compound	D_1 (retina)[b]	D_2 (retina)[c,d] high-affinity	D_2 (retina)[c] low-affinity	D_2 (striatum)[c]
2a	>10 000 (2)	449 ± 99 (3)	26 700 ± 4800 (3)	1510; 2270 (2)
2b	>10 000 (2)	42; 124 (2)	15 900−11 000 (2)	nd[e]
2c	>10 000 (2)	44 ± 28 (3)	35 400 ± 1800 (3)	13 800; 8400 (2)
2d	>10 000 (2)	39 ± 16 (3)	43 700 ± 2300 (3)	10 800; 3600 (2)
3a[f]	>10 000 (2)	26 ± 9 (3)	43 200 ± 8700 (3)	420; 1620 (2)
3b[f]	>10 000 (1)	5 (1)	27 000 (1)	10 000 (1)
3c[f]	>10 000 (2)	4 (1)	64 000 (1)	20 000 (1)
3d	>10 000 (2)	4 ± 1 (3)	24 900 ± 7200 (3)	14 400; 46 300 (2)
1	>10 000 (2)	27 ± 6 (6)	7 600 ± 1100 (6)	8100 ± 1700 (3)
PD 168 077	>10 000 (1)	25 ± 8 (3)	6 300 ± 880 (3)	18 300 (1)

[a] K_i's for retinal D_1 DARs and striatal D_2 DARs were obtained from IC_{50} in eq 1. K_i's for D_2-DARs were obtained from K_i^H and K_i^L in eq 2. K_d's were 2 nM and 0.4 nM for [^{3}H]-SCH23390 and [^{3}H]-YM-09-151-2, respectively; the number of independent experiments is reported in parentheses. K_i values are expressed as the mean ± standard error of the mean (SEM), for compounds which were tested three or more times. When two assays were performed, K_i values estimated from each assay are reported separated by a semicolon. [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 16%, 18%, 16%, 13%, 16%, 15%, 20%, 20%, 16% of total binding for compounds **1**, **2a**, **2b**, **2c**, **2d**, **3a**, **3b**, **3c**, **3d**. The high affinity site represents 64% of total binding for PD 168,077 and the difference with compounds **1**, **2a**−**d** and **3a**−**d** was statistically significant ($P < 0.01$, 1-way ANOVA). [e] Not determined. [f] Tested as a mixture of diastereomers.

Their mean inhibition constants were higher than 10 μM (Table 1).

Bovine Retina D_2-like Dopamine Receptors. The PPEs and CHPEs had biphasic inhibition curves when tested against DARs labeled with [^{3}H-]YM-09-151-2, as shown in Figure 1A for **2a** and **2d**, and in Figure 1B for **3a** and **3d** as representative compounds.

Among the PPEs, compound **2a**, characterized by the presence of a methyl group in the *ortho* position of the phenyl ring possesses an affinity 10-fold lower (higher K_i^H) than those of *m*- and *p*-monomethyl-substituted derivatives (**2b**,**c**) and of the unsubstituted derivative **2d**, whose values are similar to that of *m*,*p*-dimethyl-substituted derivative **1**.

All CHPEs **3a**−**d** proved to possess a significant affinity. The 2′-monomethyl substituted derivative **3a** showed a K_i^H about 20-fold lower than its aromatic counterpart **2a**. Also in this series, the best affinities were found with the unsubstituted derivative **3d** and 3′- and 4′-monomethyl-substituted derivatives (**3b**,**c**).

Table 1 reports the high- and low-affinity inhibition constants (K_i^H and K_i^L), respectively, for PPE and CHPEs.

Bovine Striatum D_2-like Dopamine Receptors. All the PPEs and CHPEs had monophasic inhibition curves with K_i in the micromolar range when tested against the D_2 striatal receptors labeled with [^{3}H-]YM-09-151-2.

Functional Assays for Agonist Activity. To assess the agonist properties of PPEs and CHPEs, two different functional assays were carried out on the *m*,*p*-dimethyl-substituted derivative **1**, the completely aliphatic unsubstituted derivative **3d**, and the reference compound PD 168,077.

Effects of Guanine Nucleotide Analogues on the Binding Affinity for D_4 DARs. The presence of a high affinity component in the displacement curves of PPEs and CHPEs indicates functional heterogeneity of D_4 DARs in retinal membrane preparations. To test the hypothesis that PPEs and CHPEs recognize with a high affinity D_4 DARs coupled to a GTP binding protein, we carried out competition binding assays in the presence or absence of guanosine 5′-[β,γ-imido]triphosphate (GMP-PNP), a non-hydrolyzable analogue of GTP. As shown in Figure 2 for **1**, **3d**, and PD 168,077, GMP-PNP abolished the high affinity state of D4 DARs, and the competition assays are best described by monophasic

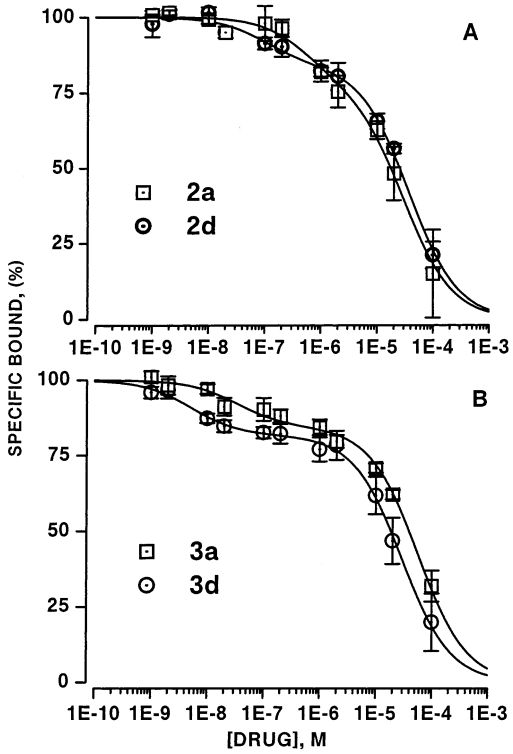


Figure 1. Competition curves of PPE and CHPE for retinal D_4 DARs are biphasic. Examples of typical biphasic competition experiments between [^{3}H]-YM-09-151-2 and *o*-methyl substituted (open squares, compounds **2a** and **3a**) or nonsubstituted (open circles, compounds **2d** and **3d**) derivatives of PPE and CHPE for retinal DARs are illustrated in panels A and B, respectively. The ordinate is the [^{3}H]-YM-09-151-2 specifically bound at each drug concentration, expressed as a percentage of the quantity bound in the absence of the competitor. Average high-affinity constants are reported in in Table 1 as K_i^H. The continuous curves through the data points were drawn in accordance with 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 (eq 3). For all the compounds, 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 $P < 0.01$.

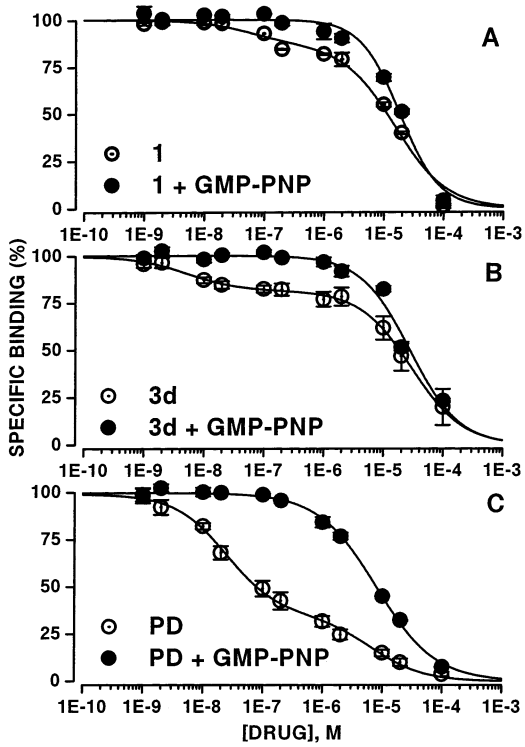


Figure 2. Effects of GTP analogue GMP-PNP on high-affinity interaction of **1**, **3d**, and PD 168 077 with D_4-DARs. (A) Datapoints are average values, with their standard error of the mean, from two independent competition assays for **1** in the absence (open circles) and in the presence (filled circles) of 200 μM GMP-PNP. (B) Datapoints are average values, with their standard error of the mean, from 3 independent competition assays for **3d** in the absence (open circles) and in the presence (filled circles) of 200 μM GMP-PNP. (C) Datapoints are average values, with their standard error of the mean, from two independent competition assays for PD 168 077 (PD)in the absence (open circles) and in the presence (filled circles) of 200 μM GMP-PNP. For **1**, **3d**, and PD, in the absence of GMP-PNP the two-site model provides a better fit than the one-site model ($P < 0.01$). In the presence of GMP-PNP, the two-site model is not significantly better than the one-site model ($P < 0.1$).

curves with K_i values close to K_i^L of control experiments.

Melatonin Synthesis Assays. Compounds **1**, **3d**, and PD 168,077 were evaluated for their agonist properties at the D_4 DARs by a functional test that measures the suppression of melatonin synthesis by retinal photoreceptors.

As shown in Figure 3 Forskolin (FRSK) increased the synthesis of melatonin and the selective D_4 DAR agonist PD 168,077, at the concentration of 0.2 μM, completely blocked the FRSK-induced increase of melatonin synthesis. Compounds **1** and **3d**, at the same concentration of 0.2 μM, suppressed FRSK-induced increase of melatonin synthesis, but to a smaller extent than PD 168 077. Note that a 5000-fold increase in concentration did not enhance the extent of suppression. These results may indicate that compounds **1** and **3d** are partial agonists at the D_4 DARs.

To assess the possibility that **1** and **3d** are partial agonists at the D_4 DAR, we measured the effect of 0.2 μM **1** or **3d** on the suppression of FRSK-induced melatonin release by 0.2 μM PD 168,077. As shown in Figure 3, in the presence of either **1** or **3d** the efficacy

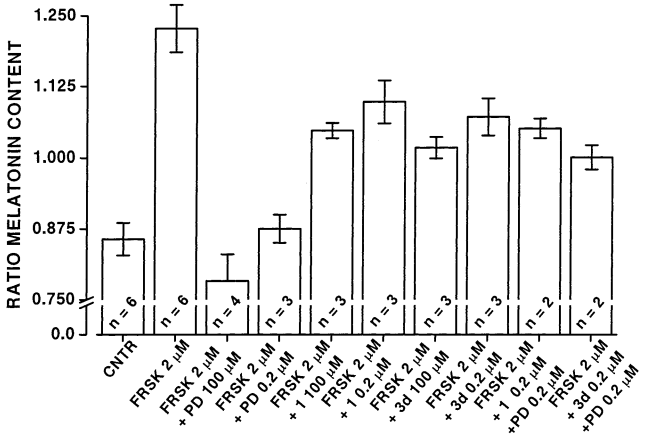


Figure 3. Effects of **1**, **3d**, and PD 168 077 on melatonin synthesis and release in the retina. Data plot of the average value and the sem of the ratio between melatonin measured in the extracellular medium upon synthesis and release in response to treatment compared with basal levels (see methods). CNTR, FRSK, and PD indicate control, forskolin, and PD 168 077, respectively. Note the ordinate axis break between 0.2 and 0.7. The number of independent replicates (*n*) is reported inside each column.

of PD 168,077 was reduced and became similar to that of **1** and **3d** alone.

Discussion and Conclusions

We have previously reported[26] that among the dimethyl-substituted PPEs, compound **1**, in which the two methyl groups are in the *meta*,*para* positions, had the best affinity and selectivity for the D_4 DAR, whereas the *o*-methyl substitution, occurring in four other possible regioisomers, caused a decrese in the binding affinity.

The present results for PPEs **2a**−**d** show that the *o*-monomethyl derivative **2a** possesses a significantly lower affinity than its *meta* and *para* analogues **2b**,**c**, thus confirming the detrimental effect of a methyl moiety in this position.

We hypothesized that the aromatic moiety of these derivatives may interact with a lipophilic pocket at the level of the binding site of the D_4 DAR. This is consistent with the decreased affinity for the D_4 DAR of 3-PPP, which has a polar hydroxy moiety on the phenyl ring.

The fact that compound **2d**, which lacks methyl substituents, had a similar affinity profile to that of monomethyl-substituted PPEs **2b**,**c** and dimethyl-substituted PPE **1** indicates that the presence of the methyl substituents in the *meta* and/or *para* positions of the phenyl ring may not play a crucial role for the interaction with the hypothesized lipophilic binding pocket. On the contrary, the methyl substituent in the *ortho* position might hinder the locking of the aromatic moiety of PPEs within the binding pocket.

The interaction of these compounds with the hypothesized lipophilic pocket could have been of $\pi-\pi$ type with an aromatic receptor moiety or of a simple hydrophobic nature. Completely aliphatic CHPEs **3a**−**d** generally showed an affinity for the D_4 DAR even higher than their corresponding aromatic PPE analogues: this result is consistent with a non $\pi-\pi$ type hydrophobic interaction.

In the aliphatic series of CHPEs **3a**−**d** the affinity of the 2′-monomethyl substituted compound (**3a**) was not so different from those of **3b**−**d**.

Among all the new PPEs and CHPEs herein reported, completely aliphatic compounds **3b**−**d** showed the best biological properties in terms of affinity and selectivity for the D_4 DAR. However, it is important to note that compounds **3a**−**c** were tested as mixtures of diastereomers. Therefore, estimates of their binding affinities are useful only for making a crude comparison. Considering the structural simplicity of the nonmethylated compound **3d**, which, unlike **3b** and **3c**, can exist only as a single diastereoisomer, we chose this compound, together with PPE **1**, for further functional evaluation.

The observation of high- and low-affinity components in the displacement curves of PPE and CHPE (see Figure 1), which is typical of the interaction of agonists with receptors that couple to GTP binding proteins, may indicate that PPE and CHPE are agonists. To evaluate this hypothesis we carried out competition binding assays in the presence of the nonhydrolyzable GTP analogue GMP-PNP.

The results illustrated in Figure 2 are consistent with the idea that **1**, **3d**, and the reference compound PD 168 077 are agonists which, upon binding to the D4 DARs, promote GDP/GTP exchange by the G-protein α subunit. The α subunit with bound GMP-PNP has low affinity for the receptor, causing the dissociation of the ternary complex $\alpha\beta\gamma$. The change of receptor conformation following dissociation from the α-subunit is characterized by a state of low affinity for agonists, and the competition curves became monophasic, with a single binding component with a low affinity.

The observation that for PPE and CHPE the high-affinity site was less than 30% of total binding, lower than the 60% estimated for PD 168 077, which is a selective agonist at the D_4 DARs, may indicate that at the functional level PPE and CHPE are agonists at the retinal D_4 DAR, although with a lower efficacy than the agonist PD 168 077. To test this hypothesis, the ability of **1** and **3d** to inhibit the forskolin-induced synthesis of melatonin was compared with that of PD 168 077. Data in Figure 3 indicate that **1** and **3d** have a lower efficacy than PD 168 077 in inhibiting the synthesis of melatonin induced by FRSK. Coapplication of PD 168 077 with either **1** or **3d** brought about a reduction of PD 168,077 efficacy in blocking FRSK-induced melatonin synthesis. These results indicate that compounds **1** and **3d** are partial agonists at the D_4 DAR.

It is important to note that we previously found that the two enantiomers of PPE **1** have a similar binding profile for the D_4-DAR;[27] furthermore, both enantiomers have biphasic competition curves, which are converted to monophasic in the presence of GMP-PNP (unpublished). These observations suggest that the partial agonism found for racemic **1** may not be ascribed to different agonist/antagonist properties of the two enantiomers. Considering the overall similarity between **1** and **3d** in the binding and functional profile, as well as in the molecular structure, the same conclusion may apply also to racemic **3d**.

In conclusion, we have reported here compound **3d** as the first completely aliphatic compound able to selectively bind the D_4 DARs and promote G-protein activation followed by adenylyl cyclase inhibition. An intriguing property of **3d**, as well as of **1**, is their partial agonism, which may provide new pharmacological tools to investigate the role of D_4 DAR activation in the control of cognitive functions and emotional states in health and disease.

Experimental Section

Chemistry. Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. NMR spectra were obtained with a Varian Gemini 200 MHz spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane and referenced from solvent references. Electron impact (EI) mass spectra were obtained on a HP-5988A mass spectrometer at 70 eV. The elemental compositions of the compounds agreed to within ±0.4% of the calculated value. Chromatographic separations were performed on silica gel columns by flash (Kieselgel 40, 0.040−0.063 mm; Merck) or gravity column (Kieselgel 60, 0.063−0.200 mm; Merck) chromatography. Reactions were followed by thin-layer chromatography (TLC) on Merck aluminum silica gel (60 F_{254}) sheets that were visualized under a UV lamp (254 nm). Evaporation was performed in vacuo (rotating evaporator). Sodium sulfate was always used as the drying agent. 3-Arylpyridines **4a**,[28] **4b**,[18] **4c**,[29] **4d**,[30] and *N*-*n*-propyl-3-arylpiperidines **2b**,**d**,[18] were prepared as reported in the literature.

Preparation of 3-Arylpiperidine Hydrochlorides 5a,c. General Procedure. A solution of the appropriate 3-arylpyridine (**4a**[28] or **4c**[18]) (4.9 mmol) in methanol (50 mL) and 37% aqueous HCl (1.5 mL), in the presence of 0.10 g (0.44 mmol) of platinum dioxide, was submitted to hydrogenation under 1 atm of molecular hydrogen for 4 h at room temperature. The mixture was filtered through a Celite pad and the filtrate was concentrated under a vacuum. The residue was dissolved in diethyl ether and treated with gaseous HCl. The precipitate was collected by filtration and recrystallized from EtOH/Et_2O.

3-(2-Methylphenyl)piperidine hydrochloride (5a): 0.64 g (3.0 mmol, 62% yield); mp 155−157 °C; ^{1}H NMR (D_2O) δ 1.55−2.12 (m, 4H), 2.35 (s, 3H), 2.58−3.68 (m, 5H), 7.02−7.55 (m, 4H) MS (EI, 70 eV) *m/e* 175 (M^+ − HCl). Anal. $C_{12}H_{17}N\cdot$HCl (C, H, N).

3-(4-methylphenyl)piperidine hydrochloride (5c): 0.77 g (3.6 mmol, 75% yield); mp 166−168 °C; ^{1}H NMR (D_2O) δ 1.44−2.18 (m, 4H), 2.31 (s, 3H), 2.71−3.69 (m, 5H), 7.20−7.24 (m, 4H); MS (EI, 70 eV) *m/e* 175 (M^+ − HCl). Anal. $C_{12}H_{17}N\cdot$HCl (C, H, N).

Preparation of 3-Cyclohexylpiperidine Hydrochlorides 6a−d. General Procedure. A solution of the appropriate arylpyridine hydrochloride **4a**,[28] **4b**,[18] **4c**,[29] or **4d**,[30] (4.9 mmol) in methanol (50 mL), in the presence of 0.54 g (2.4 mmol) of platinum dioxide, was submitted to hydrogenation under 1 atm of molecular hydrogen for 24 h at room temperature. The mixture was filtered through a Celite pad, and the filtrate was concentrated under a vacuum. The residue was dissolved in diethyl ether and treated with gaseous HCl. The precipitate was collected by filtration and recrystallized from MeOH/Et_2O.

3-(2-Methylcyclohexyl)piperidine hydrochloride (6a): 0.75 g (3.5 mmol, 71% yield); mp 162−164 °C; ^{1}H NMR (D_2O) δ 0.83 (d, 3H, J = 7.2 Hz), 1.08−2.04 (m, 15H), 2.59 (t, 1H, J = 12.3 Hz), 2.84 (td, 1H, J = 12.5, 2.6 Hz), 3.21−3.48 (m, 2H); MS (EI, 70 eV) *m/e* 181 (M^+ − HCl). Anal. $C_{12}H_{23}N\cdot$HCl (C, H, N).

3-(3-Methylcyclohexyl)piperidine hydrochloride (6b): 0.53 g (2.4 mmol, 50% yield); mp 150−152 °C; ^{1}H NMR (D_2O) δ 0.83 (d, 3H, J = 6.8 Hz), 0.92−1.94 (m, 15H), 2.71 (t, 1H, J = 12.3 Hz), 2.83 (td, 1H, J = 12.6, 3.2 Hz), 3.29−3.39 (m, 2H); MS (EI, 70 eV) *m/e* 181 (M^+ − HCl). Anal. $C_{12}H_{23}N\cdot$HCl (C, H, N).

3-(4-Methylcyclohexyl)piperidine hydrochloride (6c): 0.88 g (4.0 mmol, 82% yield); mp 143−145 °C; ^{1}H NMR (D_2O) δ 0.82 (d, 3H, J = 6.4 Hz, minor diastereomer), 0.87 (d, 3H, J = 7.0 Hz, major diastereomer), 0.93−1.96 (m, 15H), 2.66 (t,

1H, J = 12.1 Hz), 2.84 (td, 1H, J = 12.5, 2.7 Hz), 3.20−3.48 (m, 2H); MS (EI, 70 eV) *m/e* 181 (M^+ − HCl). Anal. $C_{12}H_{23}N$·HCl (C, H, N).

3-Cyclohexylpiperidine hydrochloride (6d): 0.65 g (3.2 mmol, 66% yield); mp 160−162 °C; ^{1}H NMR (D_2O) δ 1.06−2.01 (m, 16H), 2.60−2.93 (m, 2H), 3.18−3.62 (m, 2H); MS (EI, 70 eV) *m/e* 167 (M^+ − HCl). Anal. $C_{11}H_{21}N$·HCl (C, H, N).

Preparation of *N*-*n*-Propyl-3-arylpiperidine Hydrochlorides 2a,c and *N*-*n*-Propyl-3-cyclohexylpiperidine Hydrochlorides 3a−d. General Procedure. A solution of the appropriate arylpiperidine hydrochloride **5a**,**c** or cyclohexylpiperidine hydrochloride **6a**−**d** (0.94 mmol) in methanol (10 mL) was treated at room temperature with propionic aldehyde (0.13 g, 2.2 mmol) and $NaBH_3CN$ (0.16 g, 2.3 mmol). The mixture was stirred at room temperature for 24 h, and then the reaction was quenched with water (50 mL). The resulting mixture was partially concentrated under a vacuum (to remove most of the methanol), and the remaining aqueous suspension was extracted with ethyl acetate. The organic phase was dried over sodium sulfate and concentrated under a vacuum. The residue was dissolved in Et_2O and treated with gaseous HCl. The precipitate was collected by suction filtration and recrystallized from MeOH/Et_2O.

***N*-*n*-Propyl-3-(2-methylphenyl)piperidine hydrochloride (2a):** 0.040 g (0.16 mmol, 17% yield); mp 179−181 °C; ^{1}H NMR (D_2O) δ 0.92 (t, 3H, J = 7.2 Hz),1.63−2.18 (m, 6H), 2.35 (s, 3H), 2.90−3.69 (m, 7H), 7.15−7.33 (m, 4H); MS (EI, 70 eV) *m/e* 217 (M^+ − HCl). Anal. $C_{15}H_{23}N$·HCl (C, H, N).

***N*-*n*-Propyl-3-(4-methylphenyl)piperidine hydrochloride (2c):** 0.078 g (0.31 mmol, 33% yield); mp 187−189 °C; ^{1}H NMR (D_2O) δ 1.00 (t, 3H, J = 7.2 Hz), 1.51−2.19 (m, 6H), 2.31 (s, 3H), 2.52−3.68 (m, 7H), 7.05−7.13 (m, 4H); MS (EI, 70 eV) *m/e* 217 (M^+ − HCl). Anal. $C_{15}H_{23}N$·HCl (C, H, N).

***N*-*n*-Propyl-3-(2-methylcyclohexyl)piperidine hydrochloride (3a):** 0.117 g (0.45 mmol, 48% yield); mp 169−171 °C; ^{1}H NMR (D_2O) δ 0.86 (d, 3H, J = 6.8 Hz), 0.90 (t, 3H, J = 7.2 Hz), 1.08−2.46 (m, 19H), 2.92−3.16 (m, 2H), 3.49−3.67 (m, 2H); MS (EI, 70 eV) *m/e* 223 (M^+ − HCl). Anal. $C_{15}H_{29}N$·HCl (C, H, N).

***N*-*n*-Propyl-3-(3-methylcyclohexyl)piperidine hydrochloride (3b):** 0.085 g (0.33 mmol, 35% yield); mp 162−164 °C; ^{1}H NMR (D_2O) δ 0.81 (d, 3H, J = 6.6 Hz), 0.94 (t, 3H, J = 7.3 Hz), 1.12−2.37 (m, 19H), 2.60−3.12 (m, 2H), 3.35−3.68 (m, 2H); MS (EI, 70 eV) *m/e* 223 (M^+ − HCl). Anal. $C_{15}H_{29}N$·HCl (C, H, N).

***N*-*n*-Propyl-3-(4-methylcyclohexyl)piperidine hydrochloride (3c):** 0.109 g (0.42 mmol, 45% yield); mp 174−176 °C; ^{1}H NMR (D_2O) δ 0.80−0.98 (m, 6H), 1.09−2.32 (m, 19H), 2.59−2.97 (m, 2H), 3.24−3.45 (m, 2H); MS (EI, 70 eV) *m/e* 223 (M^+ − HCl). Anal. $C_{15}H_{29}N$·HCl (C, H, N).

***N*-*n*-Propyl-3-cyclohexylpiperidine hydrochloride (3d):** 0.035 g (0.14 mmol, 15% yield); mp 188−190 °C; ^{1}H NMR (D_2O) δ 0.99 (d, 3H, J = 7.3 Hz), 1.05−1.24 (m, 6H), 1.64−2.00 (m, 12H), 2.22−2.57 (m, 2H), 2.83−2.94 (m, 2H), 3.40−3.56 (m, 2H); MS (EI, 70 eV) *m/e* 209 (M^+ − HCl). Anal. $C_{14}H_{27}N$·HCl (C, H, N).

Radioligand Binding. 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 48 000 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.

Retinas 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)

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

or a two-site model, (eq 2)

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

by nonparametric fitting using a modified Lowenberg−Marquardt algorithm implemented in the data analysis program Microcal Origin, version 6.0 (Microcal Software, Inc., Northampton, MA 01060). The acceptance of a two-site model versus a single-site model was performed in accordance with the criteria described by Munson and Rodbard.[31] Briefly, the statistical significance of a two-site versus a one-site model was assessed by the F-test, in accordance with the relation:

$$F = \frac{\dfrac{(SS2 - SS1)}{SS1}}{\dfrac{(df2 - df1)}{df1}} \quad (3)$$

where SS1 and SS2 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 (dfl and df2) 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 (df2 − df1) and df1 degrees of freedom. K_i values were obtained from IC_{50} by the Cheng−Prusoff equation:[32]

$$K_i = \frac{IC_{50}}{\left(1 + \dfrac{[L]}{K_d}\right)} \quad (4)$$

where IC_{50} is the drug concentration inhibiting 50% of specific binding. K_d values were 2 and 0.4 nM for [^{3}H]-SCH23390 and [^{3}H]-YM-09-151-2, respectively.

Data in Figure 2 display the results of experiments carried out in the absence (control) and in the presence of 100−200 μM of guanosine 5′-[β,γ-imido]triphosphate (GMP-PNP), an analogue of GTP that is resistant to the intrinsic GTPase activity of the α-subunit of GTP binding protein. The use of this nonhydrolyzable analogue of GTP aims to stabilize the α-subunit of G-proteins in a form separated from the $\beta\gamma$ subunits and the receptor as well. The receptor dissociated from the α-subunit has a low affinity for agonists; therefore, the conversion of a competition curve from biphasic to monophasic is an indication that the competing compound acts like an agonist, promoting GDP−GMP−PNP exchange. The ensuing

dissociation of the receptor from the α-subunit stabilizes the state of the receptor characterized by a low affinity for agonists.

Melatonin Synthesis. Tests for the agonist activity of compounds were carried out by exploiting the reported inhibition of melatonin synthesis by the mammalian retina in response to D_4 DAR activation.[33] Guinea-pig rather than bovine retinas were used, to improve tissue viability by avoiding the hypoxia associated with the transfer of tissue from the slaughterhouse to the lab (1 h). Tissue viability is critical because the synthesis of melatonin depends on activation by cAMP of transcription of the serotonin *N*-acetyl transferase (NAT) gene (unpublished). Note that retinal DARs have similar characteristics in bovine and guinea-pig tissue.[34]

Adult albino guinea-pigs (250−400 g) obtained from a local supplier (Stefano Morini S.a.S., S. Polo d′Enza, Italy) were kept on a 12:12 light:dark cycle, and reared in accordance with the rules of the local Animal Welfare Committee for the Care and Use of Laboratory Animals. On the day of the experiment, the animal was dark-adapted for 1 h and then anaesthetized by an initial intraperitoneal injection of 35 mg kg^{-1} pentothal sodium (Gellini S.p.A., Aprilia, Italy). After enough anaesthetic had been provided to fully suppress corneal reflexes, the eye was quickly (6 min) enucleated in dim red light and the animal killed by an intraperitoneal lethal dose of anaesthetic (350 mg kg^{-1} pentothal sodium). For each experimental session, from two to three adult male guinea-pigs (200−400 g) were used.

After enucleation, the anterior pole was discarded and the posterior pole (eyecup) was bathed in 250 μL of Locke's solution, the composition of which was (mM) NaCl, 140; KCl, 3.6; $CaCl_2$, 1.2; $MgCl_2$, 2.4; glucose, 10; Hepes, 10; pH 7.6 with NaOH). Each eyecup with 250 μL of Locke's solution was positioned in a custom-made chamber obtained by cutting the bottom of a 12 × 75 mm polystyrene tube; the chambers were positioned in a light-proof container and incubated at 35 °C in a thermostated water-shaking bath for the duration of the experiment. Owing to its lipophilic nature, melatonin diffuses across the plasma membrane of photoreceptor cells; a change in melatonin synthesis will thus translate into a concentration change in the medium where the eyecup is incubated. Starting at 13:00 h, 50 μL of incubating medium was collected every 30 min, followed by the addition of 50 μL of fresh Locke's solution. For each retina, the first five samples were pooled and the melatonin content of this 250 μL (1st interval) was used to obtain a baseline for normalization between retinas and treatment. Ten more 50 μL samples were collected from 15:30 to 20:00 h and pooled in groups of five samples, which are indicated as 2nd and 3rd interval. Compounds were added at 15:30 at 5× concentrations, and care was taken to add subsequent aliquots containing the desired compound(s) at the final concentration. Data in Figure 3 report the ratio of melatonin measured in the 3rd to that in the 1st interval. Melatonin in the samples was measured using a commercial immunoassay kit (ICN, Italy), in accordance with the manufacturer's instruction. Briefly, samples were desalted by three elutions with water through C18 columns, and melatonin was finally eluted from columns by methanol. Samples were then dried under nitrogen and reconstituted in 50 μL of H_2O. Samples were assayed by a competition enzyme-linked immunosorbent assay (ELISA), and the photometric measurement at 405 nm was converted to absolute melatonin concentration by a calibration curve that used authentic melatonin.

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JM021019A