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Original article

Efficient microwave combinatorial synthesis of novel indolic arylpiperazine derivatives as serotoninergic ligands

Francesco Frecentese [a], Ferdinando Fiorino [a], Elisa Perissutti [a], Beatrice Severino [a], Elisa Magli [a], Antonella Esposito [a], Francesca De Angelis [a], Paola Massarelli [b], Cristina Nencini [b], Barbara Viti [b], Vincenzo Santagada [a,*], Giuseppe Caliendo [a]

[a] Dipartimento di Chimica Farmaceutica e Tossicologica, Università di Napoli «Federico II», Via D. Montesano, 49, 80131 Naples, Italy
[b] Dipartimento di Farmacologia "G. Segre" Università di Siena, Via delle Scotte, 6, 53100 Siena, Italy

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ABSTRACT

An easy and convenient microwave-assisted synthesis of a small library of indolic arylpiperazine derivatives is described. Parallel and mixed pool combinatorial methods are reported and compared. The described reactions are nucleophilic substitutions of several aromatic piperazines in presence of K_2CO_3. Good yields and short reaction times are the main aspect of these procedures. Binding assays shed additional light on the influence of the LCAPs on the 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors affinity and allowed to disclose three interesting compounds as 5-HT_{2C}, mixed 5-HT_{2A}/5-HT_{2C} and 5-HT_{1A}/5-HT_{2C} ligands (**4i**, **4l** and **4d**, respectively), with potential antiepileptic, anxiolytic or atypical antipsychotic agent therapeutical profiles.

1. Introduction

Over the last decade, microwave-assisted chemistry has become an important tool that can accelerate drug discovery within industry and academia. Shifting the focus from screening mixtures to a single compound libraries, it proved to be a powerful technique for medicinal chemists for either hit identification or lead refinement [1]. Long-chain arylpiperazines (LCAPs) are a class of molecules of considerable pharmaceutical interest. They bind to many classes of G-protein-coupled receptors (serotoninergic, dopaminergic, adrenergic) and produce a variety of pharmacological responses. In the past years our outmost attention was focused on 5-HT_{1A} receptor [2], since its role in the pathology of such mental disorders as anxiety or depression has been well-established [3]. In particular, 5-HT_{1A} receptor, belonging to the superfamily of G-protein-coupled receptors and negatively coupled to adenylyl cyclase, was found in high concentration in the limbic system, where it is thought to play a role in emotional processes and represents a major target for research and drug development due to its implication in the pathophysiology and treatment of major neuropsychiatric disorders, including depression, schizophrenia and anxiety. Moreover, 5-HT_{2A} receptor is known to play a key role in the action of psychedelics as well as being a therapeutic target for the treatment of schizophrenia [4]; finally 5-HT_{2C} receptor is considered to be an attractive target for the design of novel drugs for treatment of CNS-related diseases such as obesity, obsessive compulsive disorder, and sexual dysfunction, even if few 5-HT_{2C} selective agonists are known so far [5].

Structure-activity relationship (SAR) studies, performed with numerous generations of arylpiperazine derivatives, showed that CNS activity and receptor affinity and selectivity depend on the N-1-aryl substituent, the terminal fragment and the alkyl spacer length. To get direct and easy access to this class of molecules, we developed and optimized a microwave-assisted synthesis of a 12-members arylpiperazine small library characterized by an indolic nucleus. The indole substructure is a basic element for a number of biologically active natural and synthetic products. In fact, until today, there have been more than 400 drugs and 3000 patents in which the indole motif has been present; the range of applications for these therapeutically relevant compounds includes anti-inflammatory drugs, protein kinase C inhibitors, 5-HT agonists and antagonists, melatonin agonists and glucocorticoid receptor modulators [6].

In order to explore its influence on the serotoninergic activity, 5-hydroxy-1H-indole-2-carboxylic acid has been linked to some of

* Corresponding author. Tel.: +39 81 678648; fax: +39 81 678649.
E-mail address: santagad@unina.it (V. Santagada).

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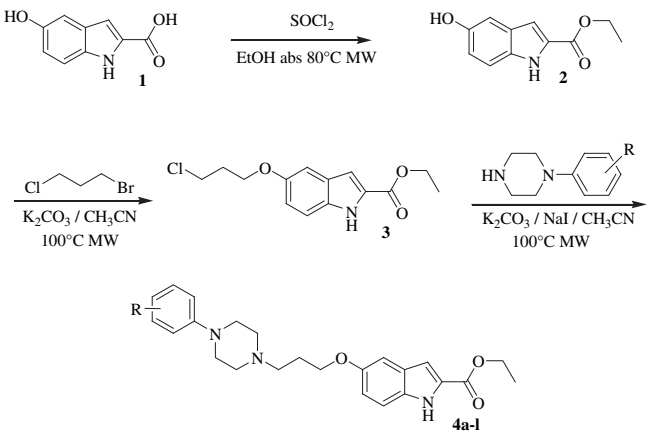
the most thoroughly studied aryl-substituted piperazines via a three methylene spacing unit. In recent years our interest has been devoted to the application of microwave in the syntheses of pharmacologically active peptidomimetic [7] or heterocyclic derivatives [8]. In this paper we describe the application of this technology for the fast generation of a small library of indolic arylpiperazines in parallel and mixed pool combinatorial methods. The application of microwave energy to organic compounds in order to perform synthetic reactions at highly accelerated rates has become a well-known technique [9]. Microwave technology, indeed, has become very popular among synthetic organic chemists both to improve classical organic reactions, shortening reaction times and/or improving yields, as well as to promote novel efficient synthetic routes. The developed procedure, particularly, in the last step, allows the obtaining of the complete arylpiperazines library with a single reaction and purification step and this can facilitate the quick screening of a greater number of synthetic analogues in the identification of a lead structure.

2. Chemistry

The synthesis of compounds **4a–l** is summarized in Scheme 1.

5-Hydroxy-1H-indole-2-carboxylic acid **1** was converted to the corresponding ethyl ester **2** by treatment with thionyl chloride in ethanol under reflux; the reaction with 1-bromo-3-chloropropane, in presence of K_2CO_3 in acetonitrile, gave the corresponding chloro-alkyl indolic derivative **3**. These reactions were carried out using a microwave oven (ETHOS 1600, Milestone®) especially designed for organic synthesis placing reagents and solvents in a sealed reactor specific for high pressure reactions. The subsequent condensation of compound **3** with the opportune substituted arylpiperazine, performed in acetonitrile in presence of K_2CO_3 and NaI, was carried out in parallel using sealed tubes fitted in the 36 positions of a multiPREP rotor (Milestone®); the remaining tubes were filled with the same amount of the reaction solvent (acetonitrile). The synthetic procedure was performed following a microwave program which was composed by appropriate ramping and holding steps. The temperature of the stirred reaction mixture was monitored by an IR probe and rotation of the rotor, irradiation time and power were monitored with the "easyWAVE" software package. The reactions provided the final compounds **4a–l**.

The piperazine coupling reaction was performed at 100 °C with 300 W, in acetonitrile for a total time of 1 h. This condition was found to be the optimized one because higher temperatures, times or power gave no increase in the obtained yields or resulted in decomposition of the reagents. The aryl-substituted piperazines (**a–l**) have been selected in order to consider different steric, hydrophobic and electronic features useful to develop a small library of indolic arylpiperazine derivatives with different physicochemical properties. The structures of the employed arylpiperazines are depicted in Fig. 1.



Scheme 1. Synthetic procedure for the synthesis of **4a–l**.

The main advantage of this synthetic route is that a short irradiation time of the reaction mixtures provided the compounds **4a–l** as the major products.

As starting point for a combinatorial mixed pool procedure, a small amount of pure previously obtained compounds **4a–l** was mixed and several analytical RP-HPLC elution gradients were evaluated to optimize a complete separation. The best condition for analytical determination was found to be the following one, carried out by two solvent systems: A: 0.05% TFA (v/v) in acetonitrile; B: 0.05% TFA in H_2O (linear gradient from 20% to 40% A over 60 min, UV detection at 254 nm, flow rate 1 mL/min). The obtained chromatographic profile showed sufficient resolution between the compounds to undergo preparative purification (Fig. 2).

On the basis of the obtained results, a mixed pool synthetic procedure was developed as a one-pot reaction: the chloro-alkyl ethyl ester **3** was reacted with the twelve arylpiperazines **a–l** in acetonitrile in presence of K_2CO_3 and NaI, heating the mixture by microwave irradiation with 300 W at 100 °C, for a total event time of 90 min. The reaction mixture was filtered to remove the potassium carbonate, subjected to an extraction with brine and was purified by preparative RP-HPLC applying the same gradient used for the analytical determinations. The crude analytical HPLC chromatographic profile was similar to the previously obtained (Fig. 3) with sufficient separations of the final compounds. High-reaction-temperature conditions achieved by microwave irradiation in acetonitrile allowed the synthesis to be carried out in one-pot to obtain the desired compounds **4a–l**.

3. Results and discussion

Results relative to the synthesis of the compounds **4a–l>** are summarized in Table 1 and show that there is not a significant difference between yields obtained in parallel or mixed pool method; a small reduction in yields is evidenced in mixed pool synthesis, but it can be addressed to the more complexity of the crude mixture in which the piperazine reactivity plays a dominant role.

On the basis of these results it's possible to state that the application of microwave irradiation and the simultaneous presence of K_2CO_3 and NaI improve the yields and significantly reduce reaction times in the synthesis of arylpiperazine derivatives, either in parallel or mixed pool combinatorial methods and this procedure could be applied in future for the generation of larger libraries of arilpiperazine derivatives to be evaluated for their biological properties.

The twelve piperazine derivatives (**4a–l**) were evaluated for their activity and selectivity towards 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors and some of the molecules showed affinity in nanomolar range towards these receptors (Table 2). Moreover, the multi-receptor profiles of promising derivatives were also evaluated in terms of binding affinities for dopaminergic (D_1, D_2) and adrenergic (α_1, α_2) receptors (Table 3).

The outstanding 5-HT_{1A} receptor affinity of compound **4g** (K_i = 35.8 nM), that showed a good nanomolar affinity on the 5-HT_{1A} receptor in conjunction with a good selectivity on the 5-HT_{2A} and 5-HT_{2C} receptors, is of particular interest; compound **4i** (K_i = 50.4 nM) showed the most interesting selectivity profile with a good affinity towards 5-HT_{2C} receptor, whereas compound

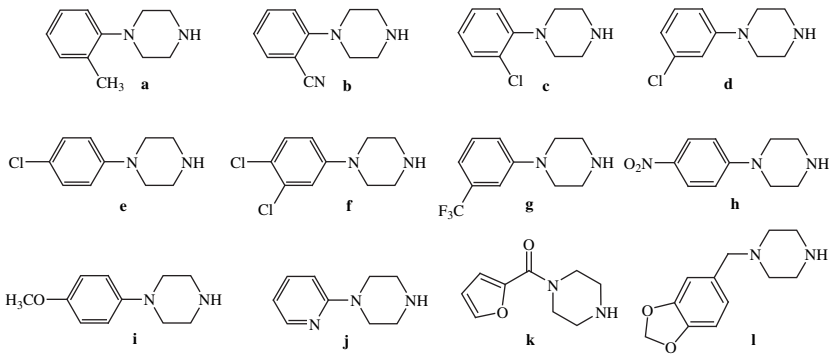


Fig. 1. Arylpiperazines used for the generation of the library.

4l presented a mixed 5-HT_{2A}/5-HT_{2C} affinity (K_i = 26.1 and 43.6 nM, respectively) and **4d** a mixed 5-HT_{1A}/5-HT_{2C} affinity (K_i = 39 and 1.28 nM, respectively). Successively, the affinity of the most active compounds (**4i**, **4d** and **4l**) on several other receptors (α_1 and α_2 adrenergic and D_1 and D_2 dopaminergic receptors) was examined in order to verify their selectivity. Results are summarized in Table 3. All the compounds proved highly selective against dopaminergic receptors, with K_i values of above 10^4 nM or no affinity except for compound **4d**, which exhibited an high K_i value of 1070 nM on the D_1 receptor. Regarding α_1 and α_2 receptors, only compound **4d** showed quite moderate affinity (32 nM) towards α_1 receptors. These results further support the choice of the indolic nucleus not only for the preparation of serotoninergic ligands endowed with a 5-HT_{1A} affinity, but also with a mixed 5-HT_{2A}/5-HT_{2C} and 5-HT_{1A}/5-HT_{2C}/α_1 activity, as well as compounds with an interesting 5-HT_{2C} affinity and selectivity.

The high differences in binding results observed between the derivatives **4a**–**l**, in accordance to the previously described series of LCAPs derivatives [2b–2c], demonstrate that the heterocyclic fragment as the terminal part of the LCAPs and the substituents on the N-4 of the piperazine moiety represent critical structural features in determining 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors affinity and selectivity.

The introduction of substituents characterized by different steric, hydrophobic and electronic features on the N-4 position of the piperazine moiety allowed us to evaluate the influence of the nature and the position of these on the aromatic ring. Analysis of the results suggests, in accordance to data already reported in literature [10], that m-Cl and m-CF_3 are favorable substituents in order to obtain compounds with nanomolar affinity on the 5-HT_{1A} receptor.

In summary, ethyl 5-(3-(4-(4-methoxyphenyl)piperazin-1-yl)propoxy)-1H-indole-2-carboxylate (**4i**, K_i = 50.4 nM) was the most active and selective derivative for the 5-HT_{2C} receptor; this result is particularly interesting even because of the few evidences regarding the interaction of the LCAPs structures on the 5-HT_{2C} receptor. In fact, only some piperazine analogues were disclosed as potent and selective 5-HT_{2C} agonists, but unfortunately no supporting *in vivo* data were reported [11]. Nanomolar interesting affinity on the 5-HT_{2C} receptor was showed by compounds **4c**, **4e** and **4l** too, but with lower affinity/selectivity profile. Additionally, the mixed 5-HT_{2A}/5-HT_{2C} affinity (K_i values of 7.48/5.16 nM respectively) and the mixed 5-HT_{1A}/5-HT_{2C}/α_1 affinity (K_i values of 39/1.28/32.9 nM respectively) showed by compounds **4l** and **4d** are of particular interest and outline a potential atypical antipsychotic profile for these derivatives.

4. Conclusion

Microwave combinatorial chemistry was adopted in the synthesis of a new series of N-4-substituted piperazines linked to an indolic nucleus via three methylene spacing unit and, even in this case, has shown its potential as technological tool able to improve on the reaction condition allowing the synthesis of the final compounds **4a**–**l** with high yields in both the combinatorial approaches.

The binding data presented in this paper shed additional light on the influence of the LCAPs on the 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors affinity and selectivity. In fact, the selected N-4-substituted piperazines are not tolerated, even when indolic nucleus has been used as terminal fragment, in order to obtain derivatives with a good affinity/selectivity profile on 5-HT_{1A} receptor with the only exception represented by compound **4g** (K_i = 35.8 nM), where a moderate nanomolar affinity on the 5-HT_{1A}

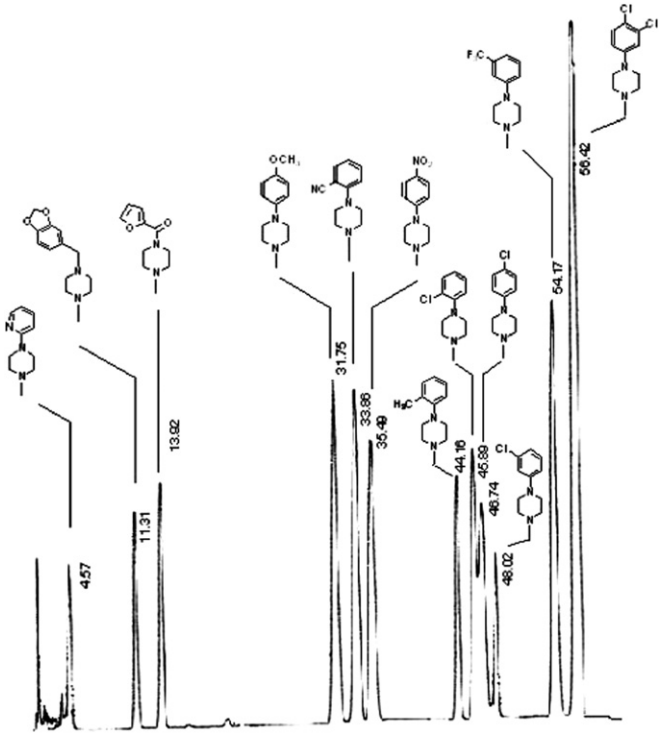


Fig. 2. – HPLC records of separation of compounds obtained by parallel method **4a**–**l**.

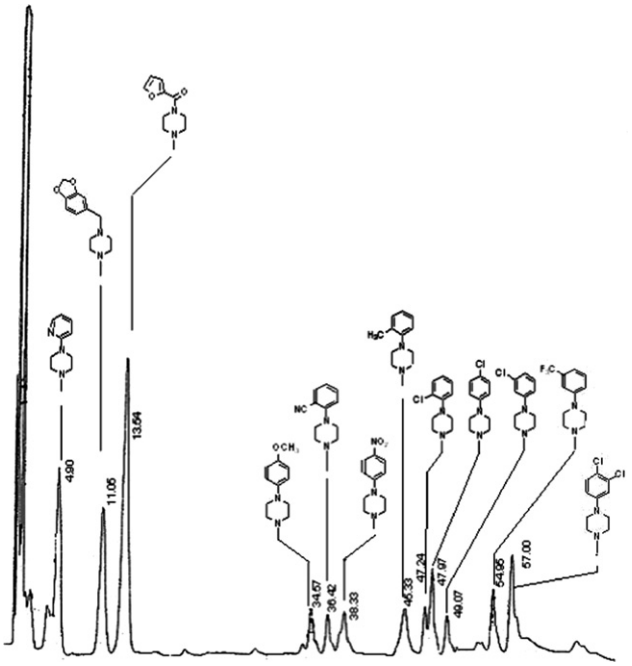


Fig. 3. – HPLC records of the crude mixed pool reaction mixture.

receptor is associated to a concomitant selectivity on the 5-HT_{2A} and 5-HT_{2C} receptors. Simultaneously, we have disclosed three interesting compounds as 5-HT_{2C}, mixed 5-HT_{2A}/5-HT_{2C} and 5-HT_{1A}/5-HT_{2C}/α_1 ligands (**4i**, **4l** and **4d** respectively), with a potential therapeutical profile as antiepileptic, anxiolytic or atypical antipsychotic agents.

5. Experimental

5.1. Synthesis

5.1.1. General procedures

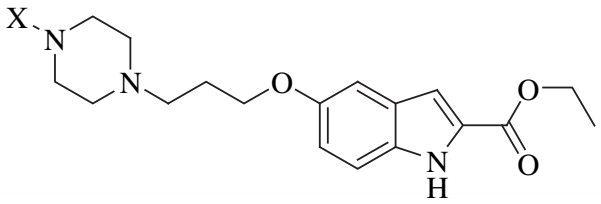
All synthetic reagents were purchased from Aldrich (Milan, Italy) and were used without further purification. Solvents were analytical reagent grade or higher purity and were used as supplied. All reactions were performed, either in parallel or in mixed pool procedure, using a microwave oven (ETHOS 1600, Milestone®) especially designed for organic synthesis. Thin layer chromatography was performed on Macherey–Nagel DURASIL silica gel plates

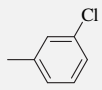
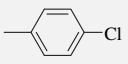
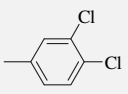
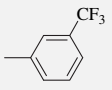
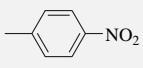
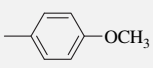
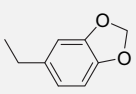
Table 1
Indolic arylpiperazine derivatives by combinatorial microwave irradiation.

Entry	Parallel synthesis	Mixed pool synthesis
	Yield[a](%)	Yield[a](%)
4a	51	48
4b	49	47
4c	59	55
4d	62	58
4e	56	56
4f	48	45
4g	53	49
4h	51	50
4i	51	50
4j	78	73
4k	71	69
4l	83	79

[a] All the reactions were performed 3 times and the reaction yields given are the average values.

Table 2
Affinities of compounds **4a-n** for 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors.



Compd	Substituents	Receptor affinity $K_i \pm$ SD (nM)[a]		
	X	5-HT_{1A} [^{3}H]8OH-DPAT	5-HT_{2A} [^{3}H]Ketanserin	5-HT_{2C} [^{3}H]Mesulergine
4a		$>10^4$	No affinity	No affinity
4b		$>10^4$	No affinity	No affinity
4c		$>10^4$	537 ± 11	39.7 ± 5.2
4d		39.0 ± 0.8	$>10^4$	1.28 ± 0.1
4e		14.0 ± 0.9	$>10^4$	45.1 ± 1.7
4f		$>10^4$	No affinity	$>10^4$
4g		35.8 ± 1.2	3610 ± 192	No affinity
4h		No affinity	$>10^4$	247 ± 47
4i		$>10^4$	$>10^4$	50.4 ± 2.4
4j		No affinity	344 ± 51	104 ± 19
4k		No affinity	452 ± 27	309 ± 16
4l		$>10^4$	26.1 ± 3.3	43.6 ± 55

[a] For purpose of comparison, 8-OH-DPAT, Ketanserine and Mesulergine bind 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors with values of 0.80, 0.85 and 1.90 nM, respectively, under these assay conditions.

Table 3
Affinities of compounds **4i**, **4l** and **4d** for D_1, D_2, α_1 and α_2 receptors.

Compd	Receptor affinity $K_i \pm$ SD (nM)			
	D_1	D_2	α_1	α_2
	[^{3}H]SCH-23390	[^{3}H]spiperone	[^{3}H]prazosin	[^{3}H]yohimbine
4d	1070 ± 122	No affinity	32.9 ± 1.69	1350 ± 169
4i	No affinity	No affinity	514 ± 9.2	6480 ± 533
4l	$>10^4$	No affinity	338 ± 14.6	231 ± 14.8

with fluorescent indicator and the plates were visualized with UV light (254 nm). Kieselgel 60 was used for column chromatography. Melting points, determined using a Buchi Melting Point B-540 instrument, are uncorrected and represent values obtained on recrystallized or chromatographically purified material. Elemental analyses were carried out on a Carlo Erba model 1106; analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values. A Buchi R-114 rotavapor was utilized for the removal of the solvents *in vacuo*. Analytical RP-HPLC determinations were performed with a Phenomenex C18-column (5 μm, 4.6 × 150 mm, spherical). Preparative RP-HPLC purifications were performed on a Waters Delta Prep 4000 system equipped with a Waters 484 multi-wavelength detector on a Vydac C-18 column (15–20 μm, 22 × 5000 mm) adopting the same gradient used for the analytical determinations. The operational flow rate was 30 mL/min. Structures were verified spectroscopically by proton ^{1}H NMR, ^{13}C NMR and ESI-MS. NMR spectra were recorded on Varian Mercury Plus 400 instrument. Chemical shifts are referred to Me_4Si as internal standard. Mass spectra of the final products were performed on Applied Biosystem API 2000 triple-quadrupole mass spectrometer.

*5.1.2. Ethyl 5-hydroxy-1H-indole-2-carboxylate (**2**)*

1.9 mL of $SOCl_2$ were added dropwise to a solution of 5-hydroxy-1H-indole-2-carboxylic acid **1** (16.9 mmol, 3 g) in 20 mL of anhydrous ethanol. A gentle reflux occurred spontaneously and was maintained transferring the reaction in a Milestone microwave oven at 80 °C using 150 W power for 30 min. The mixture was then allowed to cool to room temperature; the solvent was removed and the crude mixture taken up in ethyl acetate was extracted with a 5% $NaHCO_3$ aqueous solution and with brine (twice each). The organic phase was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness to afford 2.3 g of pure compound **2** as a white powder (68% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.86 (bs, NH), 7.31 (d, 1H, J = 8.8 Hz), 7.13 (s, 1H), 7.09 (s, 1H), 6.99 (d, 1H, J = 8.8 Hz), 4.40 (q, 2H, J = 7.2 Hz), 1.41 (t, 3H, J = 7.2 Hz). ^{13}C NMR ($CDCl_3$): 162.1, 154.0, 151.6, 131.2, 128.1, 117.4, 113.0, 108.4, 104.1, 61.2, 14.6. ESI-MS: 206 (MH^+), 228 (M − Na), 244 (M − K). Anal. ($C_{11}H_{11}NO_3$) C, H, N, O. m.p. 146–148 °C.

*5.1.3. Ethyl 5-(3-chloropropoxy)-1H-indole-2-carboxylate (**3**)*

In a two neck flask, K_2CO_3 (1.57 mmol, 216 mg) was suspended in acetonitrile and heated by microwave under stirring at 70 °C. Ethyl 5-hydroxy-1H-indole-2-carboxylate **2** (1.57 mmol, 323 mg) was then added followed, after 10 min, by 1-bromo-3-chloropropane and the reaction mixture was further stirred at 70 °C for 1 h. Afterwards the mixture was cooled to room temperature, concentrated to dryness and the residue diluted in water (40 mL). The solution was extracted several times with CH_2Cl_2. The combined organic layers were dried on anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography (dichloromethane/methyl alcohol 95:5 (v/v)). The combined and evaporated product fractions were crystallized from diethyl ether/hexane, affording 312 mg (71% yield) of the desired compound **3** as a yellow pale solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.86 (bs, NH), 7.31 (d, 1H, J = 8.8 Hz), 7.13 (s, 1H), 7.09 (s, 1H), 6.99 (d, 1H, J = 8.8 Hz), 4.40 (q, 2H, J = 7.2 Hz), 4.15 (t, 2H, J = 6 Hz), 3.78 (t, 2H, J = 6 Hz), 2.26 (quint., 2H, J = 6 Hz), 1.41 (t, 3H, J = 7.2 Hz). ^{13}C NMR ($CDCl_3$): δ 162.1, 154.0, 151.6, 131.2, 128.1, 117.4, 113.0, 108.4, 104.1, 65.1, 61.2, 41.9, 32.6, 14.6. ESI-MS: 282 (MH^+), 304 (M-Na), 320 (M-K). Anal. ($C_{14}H_{16}ClNO_3$) C, H, N, O. m.p. 177–179 °C.

*5.1.4. General parallel synthetic procedure for compounds **4a–l***

Using a multiPREP rotor (Milestone®) with 36 reaction tubes, ethyl 5-(3-chloropropoxy)-1H-indole-2-carboxylate **3** (1.78 mmol, 500 mg) and the appropriate substituted arylpiperazine (1.78 mmol) were added to each reaction tube and were dissolved in acetonitrile. K_2CO_3 (2.67 mmol, 615 mg) and NaI (2.67 mmol, 400 mg) were added and then the tubes were closed. The reactions were heated in a Milestone microwave oven at 100 °C using 300 W power for 1 h. After 3 min of ventilation, the tubes were opened and each reaction was filtered to remove the excess of K_2CO_3. Each reaction mixture was then transferred into a separating funnel and washed with brine. The organic phases were collected, dried over anhydrous Na_2SO_4 and evaporated to dryness. Purification by silica gel column chromatography afforded the pure compounds **4a–l**.

*5.1.5. General mixed pool synthetic procedure for compounds **4a–l***

Ethyl 5-(3-chloropropoxy)-1H-indole-2-carboxylate **3** (21.36 mmol, 6 g) and 12 commercially available arylpiperazines (1.78 mmol each) were dissolved in a two neck flask in acetonitrile. K_2CO_3 (32.04 mmol, 7.38 g) and NaI (32.04 mmol, 4.8 g) were added and then the reaction was transferred in the microwave oven. The reaction was heated and stirred for 90 min at 100 °C using 300 W of power. After 3 min of ventilation, the reaction was filtered to remove the excess of K_2CO_3 and washed with brine. The crude mixture was dried over anhydrous Na_2SO_4 and evaporated to dryness. Purification by preparative RP-HPLC afforded the pure compounds **4a–l**.

*5.1.6. Ethyl 5-(3-(4-o-tolylpiperazin-1-yl)propoxy)-1H-indole-2-carboxylate (**4a**)*

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.77 (bs, NH), 7.30 (d, 1H, J = 8.8 Hz), 7.25 (t, 1H, J = 7.8 Hz), 7.14–7.16 (m, 3H), 7.12 (s, 1H), 7.09 (s, 1H), 7.00 (d, 1H, J = 8.8 Hz), 4.39 (q, 2H, J = 7.2 Hz), 4.07 (t, 2H, J = 6 Hz), 2.95–2.98 (m, 6H), 2.62–2.66 (m, 4H), 2.30 (s, 3H), 2.04 (quint., 2H, J = 6 Hz), 1.39 (t, 3H, J = 7.2 Hz). ^{13}C NMR ($CDCl_3$): δ 160.9, 153.3, 147.3, 132.1, 129.9, 127.2, 126.7, 125.3, 119.2, 114.2, 111.7, 109.7, 108.4, 102.9, 66.8, 61.1, 55.5, 53.3, 48.8, 27.1, 15.8, 14.6. ESI-MS: 422 (MH^+), 444 (M-Na), 460 (M-K). Anal. ($C_{25}H_{31}N_3O_3$) C, H, N, O. m.p. 130–132 °C.

*5.1.7. Ethyl 5-(3-(4-(2-cyanophenyl)piperazin-1-yl)propoxy)-1H-indole-2-carboxylate (**4b**)*

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.76 (bs, NH), 7.55 (t, 1H, J = 7.8 Hz), 7.47 (d, 1H, J = 7.8 Hz), 7.30 (d, 1H, J = 8.8 Hz), 7.27 (d, 1H, J = 7.8 Hz), 7.23 (s, 1H), 7.12 (s, 1H), 7.08 (s, 1H), 7.00 (d, 1H, J = 8.8 Hz), 4.39 (q, 2H, J = 7.2 Hz), 4.07 (t, 2H, J = 6 Hz), 3.23–3.25 (m, 6H), 2.63–2.71 (m, 4H), 2.03 (quint., 2H, J = 6 Hz), 1.40 (t, 3H, J = 7.2 Hz). ^{13}C NMR ($CDCl_3$): δ 160.8, 153.1, 150.2, 134.0, 133.1, 132.3, 129.7, 125.4, 119.2, 116.0, 115.3, 111.7, 109.7, 108.4, 102.9, 96.5, 66.7, 61.0, 55.5, 53.3, 48.8, 27.0, 14.5. ESI-MS: 433 (MH^+), 455 (M-Na), 471 (M-K). Anal. ($C_{25}H_{28}N_4O_3$) C, H, N, O. m.p. 172–173 °C.

*5.1.8. Ethyl 5-(3-(4-(2-chlorophenyl)piperazin-1-yl)propoxy)-1H-indole-2-carboxylate (**4c**)*

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.82 (bs, NH), 7.35 (d, 1H, J = 7.6 Hz), 7.30 (d, 1H, J = 8.8 Hz), 7.26 (d, 1H, J = 7.6 Hz), 7.20 (t, 1H, J = 7.6 Hz), 7.13 (s, 1H), 7.09 (s, 1H), 7.00 (d, 1H, J = 8.8 Hz), 6.95 (t, 1H, J = 7.6 Hz), 4.39 (q, 2H, J = 7.2 Hz), 4.07 (t, 2H, J = 6 Hz), 3.20–3.23 (m, 6H), 2.60–2.64 (m, 4H), 2.04 (quint., 2H, J = 6 Hz), 1.41

(t, 3H, $J = 7.2$ Hz). ^{13}C NMR ($CDCl_3$): δ 162.1, 154.2, 151.4, 132.5, 129.8, 128.3, 127.8, 123.4, 119.7, 115.7, 112.9, 111.7, 109.7, 108.4, 103.9, 66.9, 61.2, 55.5, 53.3, 48.8, 27.1, 14.6. ESI-MS: 442 (MH^+), 464 (M-Na), 480 (M-K). Anal. ($C_{24}H_{28}ClN_3O_3$) C, H, N, O. m.p. 133–134 °C.

*5.1.9. Ethyl 5-(3-(4-(3-chlorophenyl)piperazin-1-yl)propoxy)-1H-indole-2-carboxylate (**4d**)*

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.81 (bs, NH), 7.31 (d, 1H, $J = 8.8$ Hz), 7.16 (t, 1H, $J = 8.0$ Hz), 7.12 (s, 1H), 7.08 (s, 1H), 7.05 (s, 1H), 7.00 (d, 1H, $J = 8.8$ Hz), 6.79 (m, 2H), 4.39 (q, 2H, $J = 7.2$ Hz), 4.07 (t, 2H, $J = 6$ Hz), 3.20–3.23 (m, 6H), 2.60–2.64 (m, 4H), 2.04 (quint., 2H, $J = 6$ Hz), 1.41 (t, 3H, $J = 7.2$ Hz). ^{13}C NMR ($CDCl_3$): δ 162.1, 154.2, 151.4, 132.5, 129.9, 128.3, 128.1, 119.4, 117.5, 115.9, 112.9, 111.7, 109.7, 108.4, 103.9, 66.9, 61.2, 55.5, 53.3, 48.8, 27.1, 14.6. ESI-MS: 442 (MH^+), 464 (M-Na), 480 (M-K). Anal. ($C_{24}H_{28}ClN_3O_3$) C, H, N, O. m.p. 140–142 °C.

*5.1.10. Ethyl 5-(3-(4-(4-chlorophenyl)piperazin-1-yl)propoxy)-1H-indole-2-carboxylate (**4e**)*

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.80 (bs, NH), 7.30 (d, 1H, $J = 8.8$ Hz), 7.20 (d, 2H, $J = 8.0$ Hz), 7.13 (s, 1H), 7.09 (s, 1H), 7.00 (d, 1H, $J = 8.8$ Hz), 6.84 (d, 2H, $J = 8.0$ Hz), 4.39 (q, 2H, $J = 7.2$ Hz), 4.07 (t, 2H, $J = 6$ Hz), 3.20–3.23 (m, 6H), 2.60–2.64 (m, 4H), 2.04 (quint., 2H, $J = 6$ Hz), 1.41 (t, 3H, $J = 7.2$ Hz). ^{13}C NMR ($CDCl_3$): δ 162.3, 154.1, 151.6, 132.1, 128.5, 123.2, 119.5, 115.5, 112.7, 111.5, 110.0, 108.5, 103.9, 66.9, 61.2, 55.4, 53.3, 48.8, 27.2, 14.7. ESI-MS: 442 (MH^+), 464 (M-Na), 480 (M-K). Anal. ($C_{24}H_{28}ClN_3O_3$) C, H, N, O. m.p. 128–130 °C.

*5.1.11. Ethy-5-(3-(4-(3-(trifluoromethyl)phenyl)piperazin-1-yl) propoxy)-1H-indole-2-carboxylate (**4f**)*

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.95 (bs, NH), 7.36 (t, 1H, $J = 8.0$ Hz), 7.31 (d, 1H, $J = 8.8$ Hz), 7.12 (m, 2H), 7.07 (m, 2H), 7.05 (s, 1H), 6.97 (d, 1H, $J = 8.8$ Hz), 4.39 (q, 2H, $J = 7.2$ Hz), 4.08 (t, 2H, $J = 6$ Hz), 3.36–3.39 (m, 6H), 2.89–2.93 (m, 4H), 2.15 (quint., 2H, $J = 6$ Hz), 1.41 (t, 3H, $J = 7.2$ Hz). ^{13}C NMR ($CDCl_3$): 162.1, 153.9, 151.5, 132.5, 131.9, 129.9, 128.3, 128.1, 124.2, 119.3, 117.3, 116.9, 113.0, 110.1, 108.4, 104.0, 66.4, 61.2, 55.4, 52.8, 48.1, 26.0, 14.6. ESI-MS: 476 (MH^+), 498 (M-Na), 514 (M-K). Anal. ($C_{25}H_{28}F_3N_3O_3$) C, H, N, O. m.p. 131–132 °C.

*5.1.12. Ethyl 5-(3-(4-(3,4-dichlorophenyl)piperazin-1-yl)propoxy)-1H-indole-2-carboxylate (**4g**)*

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.77 (bs, NH), 7.30 (d, 1H, $J = 8.8$ Hz), 7.12 (s, 1H), 7.09 (s, 1H), 7.00 (d, 1H, $J = 8.8$ Hz), 6.95 (s, 1H), 6.75 (d, 1H, $J = 8.0$ Hz), 6.73 (d, 1H, $J = 8.0$ Hz), 4.38 (q, 2H, $J = 7.2$ Hz), 4.07 (t, 2H, $J = 6$ Hz), 3.18–3.20 (m, 6H), 2.62–2.66 (m, 4H), 2.04 (quint., 2H, $J = 6$ Hz), 1.41 (t, 3H, $J = 7.2$ Hz). ^{13}C NMR ($CDCl_3$): δ 161.2, 152.1, 151.6, 134.3, 132.7, 129.7, 128.2, 125.3, 122.9, 116.1, 115.3, 112.7, 109.7, 108.4, 103.9, 66.7, 61.1, 55.5, 53.3, 48.8, 27.1, 14.6. ESI-MS: 477 (MH^+), 499 (M-Na), 515 (M-K). Anal. ($C_{24}H_{27}Cl_2N_3O_3$) C, H, N, O. m.p. 154–156 °C.

*5.1.13. Ethyl 5-(3-(4-(4-nitrophenyl)piperazin-1-yl)propoxy)-1H-indole-2-carboxylate (**4h**)*

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.79 (bs, NH), 8.12 (d, 2H, $J = 7.6$ Hz), 7.31 (d, 1H, $J = 8.8$ Hz), 7.12 (s, 1H), 7.09 (s, 1H), 6.99 (d, 1H, $J = 8.8$ Hz), 6.83 (d, 2H, $J = 7.6$ Hz), 4.39 (q, 2H, $J = 7.2$ Hz), 4.08 (t, 2H, $J = 6$ Hz), 3.42–3.45 (m, 6H), 2.62–2.66 (m, 4H), 2.04 (quint., 2H, $J = 6$ Hz), 1.41 (t, 3H, J = 7.2 Hz). ^{13}C NMR ($CDCl_3$): δ 159.9, 155.7, 152.4, 137.9, 132.0, 130.8, 125.2, 122.0, 115.2, 111.7, 109.7, 108.5, 102.2, 66.6, 60.9, 52.6, 50.4, 48.8, 27.8, 14.1. MS: 453 (MH^+), 475 (M-Na), 491 (M-K). Anal. ($C_{24}H_{28}N_4O_5$) C, H, N, O. m.p. 183–185 °C.

*5.1.14. Ethyl 5-(3-(4-(4-methoxyphenyl)piperazin-1-yl)propoxy)-1H-indole-2-carboxylate (**4i**)*

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.81 (bs, NH), 7.30 (d, 1H, $J = 8.8$ Hz), 7.12 (s, 1H), 7.09 (s, 1H), 7.00 (d, 1H, $J = 8.8$ Hz), 6.91 (d, 2H, $J = 8.0$ Hz), 6.84 (d, 2H, $J = 8.0$ Hz), 4.39 (q, 2H, $J = 7.2$ Hz), 4.07 (t, 2H, $J = 6$ Hz), 3.77 (s, 3H, $-OCH_3$), 3.10–3.12 (m, 6H), 2.62–2.66 (m, 4H), 2.04 (quint., 2H, $J = 6$ Hz), 1.41 (t, 3H, $J = 7.2$ Hz). ^{13}C NMR ($CDCl_3$): δ 162.2, 154.1, 151.6, 132.7, 129.7, 128.2, 119.2, 117.3, 116.0, 115.3, 112.7, 108.4, 103.9, 66.9, 61.2, 57.6, 55.5, 53.3, 48.8, 27.1, 14.6. ESI-MS: 438 (MH^+), 460 (M-Na), 476 (M-K). Anal. ($C_{25}H_{31}N_3O_4$) C, H, N, O. m.p. 165–167 °C.

*5.1.15. Ethyl 5-(3-(4-(pyridin-2-yl)piperazin-1-yl)propoxy)-1H-indole-2-carboxylate (**4j**)*

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.77 (bs, NH), 8.19 (d, 1H, $J = 8.00$), 7.48 (t, 1H, $J = 8.00$), 7.30 (d, 1H, $J = 8.8$ Hz), 7.12 (s, 1H), 7.09 (s, 1H), 6.99 (d, 1H, $J = 8.8$ Hz), 6.55 (d, 1H, $J = 8.0$ Hz), 6.18 (d, 1H, $J = 8.0$ Hz), 4.39 (q, 2H, $J = 7.2$ Hz), 4.07 (t, 2H, $J = 6$ Hz), 3.53–3.57 (m, 6H), 2.62–2.66 (m, 4H), 2.06 (quint., 2H, $J = 6$ Hz), 1.41 (t, 3H, $J = 7.2$ Hz). ^{13}C NMR ($CDCl_3$): δ 159.9, 154.2, 152.5, 148.2, 138.3, 132.0, 130.8, 125.2, 113.3, 111.7, 109.9, 109.7, 108.4, 102.1, 66.8, 60.9, 52.5, 50.5, 48.7, 27.9, 14.2. ESI-MS: 409 (MH^+), 431 (M-Na), 447 (M-K). Anal. ($C_{23}H_{28}N_4O_3$) C, H, N, O. m.p. 155–157 °C.

*5.1.16. Ethyl 5-(3-(4-(2-furoyl)piperazin-1-yl)propoxy)-1H-indole-2-carboxylate (**4k**)*

^{1}H NMR (400 MHz, $CDCl_3$): δ 9.00 (bs, NH), 7.41 (s, 1H), 7.30 (d, 1H, $J = 8.8$ Hz), 7.12 (s, 1H), 7.07 (s, 1H), 7.00 (d, 1H, $J = 8.8$ Hz), 6.98 (t, 1H, $J = 8.0$ Hz), 6.46 (s, 1H), 4.39 (q, 2H, $J = 7.2$ Hz), 4.05 (t, 2H, $J = 6$ Hz), 3.82 (t, 2H, $J = 7.2$ Hz), 2.53–2.58 (m, 8H), 2.00 (quint., 2H, $J = 6$ Hz), 1.40 (t, 3H, $J = 7.2$ Hz). ^{13}C NMR ($CDCl_3$): δ 159.3, 154.1, 152.4, 147.1, 146.0, 132.4, 128.0, 117.4, 116.6, 113.0, 111.5, 108.4, 103.8, 66.7, 61.2, 55.4, 53.6, 50.4, 47.9, 27.0, 14.7. ESI-MS: 426 (MH^+), 448 (M-Na), 464 (M-K). Anal. ($C_{23}H_{27}N_3O_5$) C, H, N, O. m.p. 129–131 °C.

*5.1.17. Ethyl 5-(3-(4-((benzo[d][1,3]dioxol-5-yl)methyl)piperazin-1-yl)propoxy)-1H-indole-2-carboxylate (**4l**)*

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.76 (bs, NH), 7.30 (d, 1H, $J = 8.8$ Hz), 7.12 (s, 1H), 7.06 (s, 1H), 6.97 (d, 1H, $J = 8.8$ Hz), 6.85 (s, 1H), 6.84 (d, 2H, $J = 8.0$ Hz), 6.74 (s, 1H), 5.93 (s, 2H), 4.39 (q, 2H, $J = 7.2$ Hz), 4.04 (t, 2H, $J = 6$ Hz), 3.45 (s, 2H), 2.57–2.63 (m, 6H), 2.05 (quint., 2H, $J = 6$ Hz), 1.40 (t, 3H, $J = 7.2$ Hz). ^{13}C NMR ($CDCl_3$): δ 159.9, 154.1, 148.5, 147.3, 138.3, 132.4, 131.6, 128.1, 117.5, 112.9, 109.8, 108.4, 108.1, 103.8, 101.2, 66.8, 62.8, 61.2, 55.4, 53.2, 52.6, 26.7, 14.7. ESI-MS: 466 (MH^+), 488 (M-Na), 504 (M-K). Anal. ($C_{25}H_{29}N_3O_5$) C, H, N, O. m.p. 106–108 °C.

5.2. Pharmacology

5.2.1. General procedures

The newly synthesized compounds were tested for *in vitro* affinity for serotonin 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors by radioligand binding assays. All the compounds were dissolved in ethanol or in 5% DMSO. The following specific radioligands and tissue sources were used: (a) serotonin 5-HT_{1A} receptor, [^{3}H]-8-OH-DPAT, rat brain cortex; (b) serotonin 5-HT_{2A} receptor, [^{3}H]ketanserin, rat brain cortex; (c) serotonin 5-HT_{2C} receptor, [^{3}H]mesulergine, rat brain cortex; (d) dopamine D_1 receptor [^{3}H]SCH-23390, rat striatum; (e) dopamine D_2 receptor [^{3}H]spiperone, rat striatum; (f) $\alpha_1$adrenergic receptor [^{3}H]prazosin, rat brain cortex; (g) α_2 adrenergic receptor [^{3}H]yohimbine, rat brain cortex.

Non-specific binding was determined as described in the experimental section, and specific binding as the difference between total and non-specific binding. Blank experiments were carried out to determine the effect of 5% DMSO on the binding and no effects were observed. Competition experiments were analyzed by the "Easy Fit" program [12] to obtain the concentration of unlabeled drug that caused 50% inhibition of ligand binding (IC_{50}), with six concentrations of test compounds, each performed in

triplicate. The IC_{50} values obtained were used to calculate apparent inhibition constants (K_i) by the method of Cheng and Prussoff [13], from the following equation: $K_i = IC_{50}/(1 + S/K_D)$ where S represents the concentration of the hot ligand used and K_D its receptor dissociation constant (K_D values, obtained by Scatchard analysis [14], were calculated for each labeled ligand).

5.2.2. 5-HT_{1A} binding assay

Radioligand binding assays were performed following a published procedure [15]. Cerebral cortex from male Sprague–Dawley rats (180–220 g) was homogenized in 20 volumes of ice-cold Tris–HCl buffer (50 mM, pH 7.7 at 22 °C) with a Polytron PT10, Brinkmann Instruments (setting 5 for 15 s), and the homogenate was centrifuged at 50,000 *g* for 10 min at 0 °C. The resulting pellet was then resuspended in the same buffer, incubated for 10 min at 37 °C, and centrifuged at 50,000 *g* for 10 min. The final pellet was resuspended in 80 volumes of the Tris–HCl buffer containing 10 μM pargyline, 4 mM $CaCl_2$, and 0.1% ascorbate. To each assay tube was added the following: 0.1 mL of the drug dilution (0.1 mL of distilled water if no competing drug was added), 0.1 mL of [^{3}H]-8-hydroxy-2-(di-n-propylamino)tetralin ([^{3}H]-8-OH-DPAT) (170.0 Ci/mmol, Perkin Elmer Life Sciences, Boston, MA, USA) in the same buffer as above to achieve a final assay concentration of 0.1 nM, and 0.8 mL of resuspended membranes. The tubes were incubated for 30 min at 37 °C, and the incubations were terminated by *vacuum* filtration through Whatman GF/B filters (Brandel Biomedical Research and Laboratories Inc., Gaithersburg, MD, USA). The filters were washed twice with 5 mL of ice-cold Tris–HCl buffer, and the radioactivity bound to the filters was measured by liquid scintillation spectrometer (Packard TRI-CARB® 2000CA - Packard BioScience s.r.l., Pero, Milan, Italy). Specific [^{3}H]-8-OH-DPAT binding was defined as the difference between binding in the absence and presence of 5-HT (10 μM).

5.2.3. 5-HT_{2A} and 5-HT_{2C} binding assays

Radioligand binding assays were performed as previously reported by Herndon et al. [16]. Briefly, frontal cortical regions of male Sprague–Dawley rats (180–220 g) were dissected on ice and homogenized (1:10 w/v) in ice-cold buffer solution (50 mM Tris HCl, 0.5 mM EDTA, and 10 mM $MgCl_2$ at pH 7.4) with a Polytron PT10 (setting 5 for 15 s) and centrifuged at 3000 *g* for 15 min. The pellet was resuspended in buffer (1:30 w/v), incubated at 37 °C for 15 min and then centrifuged twice more at 3000 *g* for 10 min (with resuspension between centrifugations). The final pellet was resuspended in buffer that also contained 0.1% ascorbate and 10^{-5} M pargyline.

Assays were performed in triplicate in a 2.0 mL volume containing 5 mg wet weight of tissue and 0.4 nM [^{3}H]ketanserin hydrochloride (88.0 Ci/mmol; Perkin Elmer Life Sciences, Boston, MA, USA) for 5-HT_{2A} receptor assays, and 10 mg wet weight of tissue and 1 nM [^{3}H]mesulergine (87.0 Ci/mmol; Amersham Biosciences Europe GmbH) for 5-HT_{2C} receptor assays. Cinanserin (1.0 μM) was used to define non-specific binding in the 5-HT_{2A} assay. In the 5-HT_{2C} assays, mianserin (1.0 μM) was used to define non-specific binding, and 100 nM spiperone was added to all tubes to block binding to 5-HT_{2A} receptors. Tubes were incubated for 15 min at 37 °C, filtered on Schliecher and Schuell (Keene, NH, USA) glass fibre filters presoaked in polyethylene imine, and washed with 10 mL of ice-cold buffer. Filters were counted at an efficiency of 50%.

5.2.4. D_1 dopaminergic binding assay

The binding assay for D_1 dopaminergic receptors was that described by Billard et al. (Billard et al., 1985) [17] Corpora striata were homogenized in 30 vol. (w/v) ice-cold 50 mM Tris–HCl buffer (pH 7.7 at 25 °C) using a Polytron PT10 (setting 5 for 20 s). Homogenates were centrifuged twice for 10 min at 50,000 *g* with resuspension of the pellet in fresh buffer. The final pellet was resuspended in 50 mM ice-cold Tris–HCl containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% ascorbic acid and 10 μM pargyline (pH 7.1 at 37 °C). Each assay tube contained 50 μL [^{3}H]SCH-23390 (85.0 Ci/mmol, Perkin Elmer Life Sciences, Boston, MA, USA) to achieve a final concentration of 0.4 nM, and 900 μL resuspended membranes (3 mg fresh tissue). The tubes were incubated for 15 min at 37 °C and the incubation was terminated by rapid filtration under vacuum through Whatman GF/B glass fibre filters. The filters were washed three times with 5 mL ice-cold 50 mM Tris–HCl buffer (pH 7.7 at 25 °C). The radioactivity bound to the filters was measured by a liquid scintillation counter. Specific [^{3}H]SCH-23390 binding was defined as the difference between binding in the absence or in the presence of 0.1 μM piflutixol.

5.2.5. D_2 dopaminergic binding assay

The procedure used in the radioligand binding assay was reported in detail by Creese et al. (Creese et al., 1977) [18] Corpora striata were homogenized in 30 vol. (w/v) ice-cold 50 mM Tris–HCl buffer (pH 7.7 at 25 °C) using a Polytron PT10 (setting 5 for 20 s). Homogenates were centrifuged twice for 10 min at 50,000 *g* with resuspension of the pellet in fresh buffer. The final pellet was resuspended in 50 mM ice-cold Tris–HCl containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% ascorbic acid and 10 μM pargyline (pH 7.1 at 37 °C). Each assay tube contained 50 μL [^{3}H]spiperone (15.7 Ci/mmol, Perkin Elmer Life Sciences, Boston, MA, USA) to achieve a final concentration of 0.4 nM, and 900 μL resuspended membranes (3 mg fresh tissue). The tubes were incubated for 15 min at 37 °C and the incubation was terminated by rapid filtration under *vacuum* through Whatman GF/B glass fibre filters. The filters were washed three times with 5 mL ice-cold 50 mM Tris–HCl buffer (pH 7.7 at 25 °C). The radioactivity bound to the filters was measured by a liquid scintillation counter. Specific [^{3}H]spiperone binding was defined as the difference between binding in the absence or in the presence of 1 μM (+)-butaclamol.

5.2.6. α_1 Adrenergic binding assay

The procedure used in the radioligand binding assay has been reported in detail by Greengrass and Bremner [19]. Brain cortex was homogenized in 30 vol. (w/v) ice-cold 50 mM Tris–HCl buffer, (pH 7.2 at 25 °C) using a Polytron PT10 (setting 5 for 20 s). Homogenates were centrifuged twice for 10 min at 50,000 *g* with resuspension of the pellet in fresh buffer. The final pellet was resuspended in 50 mM ice-cold Tris–HCl, (pH 7.4 at 25 °C). Each assay tube contained 50 μL drug solution, 50 μL [^{3}H]prazosin (80.5 Ci/mmol, Perkin Elmer Life Sciences, Boston, MA, USA) to achieve a final concentration of 0.4 nM, and 900 μL resuspended membranes (10 mg fresh tissue). The tubes were incubated for 30 min at 25 °C and the incubation was terminated by rapid filtration under *vacuum* through Whatman GF/B glass fibre filters. The filters were washed three times with 5 mL ice-cold 50 mM Tris–HCl, buffer (pH 7.2 at 25 °C). The radioactivity bound to the filters was measured by a liquid scintillation counter. Specific [^{3}H]prazosin binding was defined as the difference between binding in the absence or in the presence of 10 μM phentolamine.

5.2.7. α_2 Adrenergic binding assay

The procedure used in the radioligand binding assay was reported in detail by Perry and U'Prichard [20]. Brain cortex was homogenized in 30 vol. (w/v) ice-cold 5 mM Tris–HCl, 5 mM EDTA buffer (pH 7.3 at 25 °C) using a Polytron PT10 (setting 5 for 20 s). Homogenates were centrifuged three times for 10 min at 50,000 *g* with resuspension of the pellet in fresh buffer. The final pellet was

resuspended in 50 mM ice-cold Tris–HCl, 0.5 mM EDTA (pH 7.5 at 25 °C). Each assay tube contained 50 μL drug solution, 50 μL [^{3}H]yohimbine (80.5 Ci/mmol, Perkin Elmer Life Sciences, Boston, MA, USA) to achieve a final concentration of 1 nM, and 900 μL resuspended membranes (10 mg fresh tissue). The tubes were incubated for 30 min at 25 °C and the incubation was terminated by rapid filtration under *vacuum* through Whatman GF/B glass fibre filters. The filters were washed three times with 5 mL ice-cold 50 mM Tris–HCl, 0.5 mM EDTA buffer (pH 7.5 at 25 °C). The radioactivity bound to the filters was measured by a liquid scintillation counter. Specific [^{3}H]yohimbine binding was defined as the difference between binding in the absence or in the presence of 10 μM phentolamine.

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