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Design, Synthesis and Pharmacological Profile of Novel Dopamine D2 Receptor Ligands

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Abstract—The present study describes the synthesis and pharmacological profile of three novel heterocyclic compounds originally designed, on the basis of bioisosterism, as dopamine D2 receptor ligands: 1-[1-(4-chlorophenyl)-1H-pyrazol-4-ylmethyl]-4-phenyl-piperazine (LASSBio-579), 1-phenyl-4-(1-phenyl-1H-[1,2,3]triazol-4-ylmethyl)-piperazine (LASSBio-580) and 1-[1-(4-chloro-phenyl)-1H-[1,2,3]triazol-4-ylmethyl]-4-phenyl-piperazine (LASSBio-581). Binding studies performed on brain homogenate indicated that all three compounds bind selectively to D2 receptors. In addition, electrophysiological studies carried out in cultured hippocampal neurons suggested that LASSBio-579 and 581 act as D2 agonists, whereas LASSBio-580 acts as a D2 antagonist.

Introduction

Schizophrenia is a devastating mental disorder that affects 1–2% of the population worldwide.[1] The first signs of schizophrenia typically emerge in adolescence or young adulthood,[2] and include difficulties in organizing thoughts, which lead to hallucinations, delusions, disordered thinking, and unusual speech or behavior. Schizophrenia is the single most destructive disease to young people.

It has been hypothesized that decreased dopaminergic tonus in prefrontal cortical areas accounts for the negative symptoms of schizophrenia, including apathy and social withdraw, and that increased dopaminergic activity in the striatum is associated with the positive symptoms of the disease, including delusions and hallucinations.[3–5] The fact that all antipsychotic drugs at clinically relevant concentrations block dopaminergic receptors[6] lends support to the concept that the dopaminergic system is involved in schizophrenia and in the mechanism of action of neuroleptics. To date, six types of dopaminergic receptors have been cloned from brain tissue.[7] These receptors are sub-divided into two families, referred to as D1 and D2. The family of D1 receptors includes the D1A and D1B subtypes (D1B is also known as D5 in humans). The family of D2 receptors includes the D2S, D2L, D3 and D4 subtypes.

Positive symptoms of schizophrenia respond well to treatment with classical antipsychotic drugs such as haloperidol, which act primarily as D2 receptor antagonists.[3] In contrast, the negative symptoms of schizophrenia tend to worsen upon treatment with classical antipsychotics.[8] Clozapine (**1**), an atypical antipsychotic agent that effectively controls the positive and

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some of the negative symptoms of schizophrenia, in addition to binding to D2 receptors, also binds to D4 receptors with an apparent affinity that is approximately 5-fold higher than that for D2 receptors (see Fig. 1).[9,10] It was originally suggested that the therapeutic efficacy of clozapine was related to its ability to block D4 receptors.[11] However, continued studies came to indicate that the therapeutic efficacy of this atypical neuroleptic is likely due to its ability to induce high levels of D2-receptor occupancy in the cerebral cortex.[12] By and large, atypical neuroleptics cause much less extrapyramidal side effects than classical neuroleptics. Nevertheless, pharmacological intervention with currently available (classical and atypical) antipsychotics is not effective against all symptoms of the disease.[6] There is still need for development of more effective antipsychotic drugs.

In search for drugs that have better therapeutic efficacy and less side effects than those that are currently being used for treatment of schizophrenic patients, new molecular templates that present functional selectivity have been described. These include L-741 (**2**),[13] a selective ligand for D2 receptors ($K_i = 2.4$ nM) (see Fig. 1).

In the scope of a research program aimed at drug development for treatment of neurological disorders, we describe in the present study the synthesis and pharmacological evaluation of new *N*-phenylpiperazine derivatives **3**, **4** and **5**, which were originally designed to be selective D2 or D4 receptor ligands. These substances resulted from hybridization of the lead compounds clozapine (**1**) and L-741 (**2**). In designing compounds **3**, **4** and **5**, the central b ring of clozapine was contracted raising a pyrazole or 1,2,3-triazole ring, which received an aryl group on the position 1 to prevent the typical prototropy of these unsubstituted azaheterocyclic system.[14] A *para*-chloro substituent was introduced in the *N*-phenyl rings of **3** and **5** in order to increase the structural similarity with **1**. Finally, the d ring of **1** was transposed to the distal nitrogen atom of the piperazine c ring mimicking the structural subunit A present in **2** (see Fig. 1).

Chemistry

The synthetic route planned to achieve the new *N*-phenylpiperazine derivatives **3**–**5** (Scheme 1) explored, in the key step, the reductive amination of the 1-(4-chlorophenyl)pyrazole-4-carbaldehyde (**8**), 1-phenyl-1,2,3-triazole-4-carbaldehyde (**9**) and 1-phenyl-1,2,3-triazole-4-carbaldehyde (**10**).

Substituted 1,2,3-triazole-4-carbaldehyde derivatives **9** and **10** were prepared in 80 and 76% yield, respectively, employing the reaction of diazomalonaldehydes with appropriate aromatic amines in acetic acid, according to the procedure described by Arnold and coworkers.[15] The physical and spectroscopic properties of heteroaromatic aldehydes **9** and **10** are in full agreement with previously described by L'abbé and coworkers.[16] On the other hand, condensation of 4-chlorophenylhydrazine (**6**) with 1,1,3,3-tetramethoxipropane in the presence of hydrochloric acid furnished 1-(4-chlorophenyl)-pyrazole (**7**)[17] which after regioselective formylation[18] with phosphorus oxychloride and DMF yielded 1-(4-chlorophenyl)-pyrazole-4-carbaldehyde (**8**).

Finally, sodium cyanoborohydride reduction[19] of the imine aducts formed from treatment of the heterocyclic

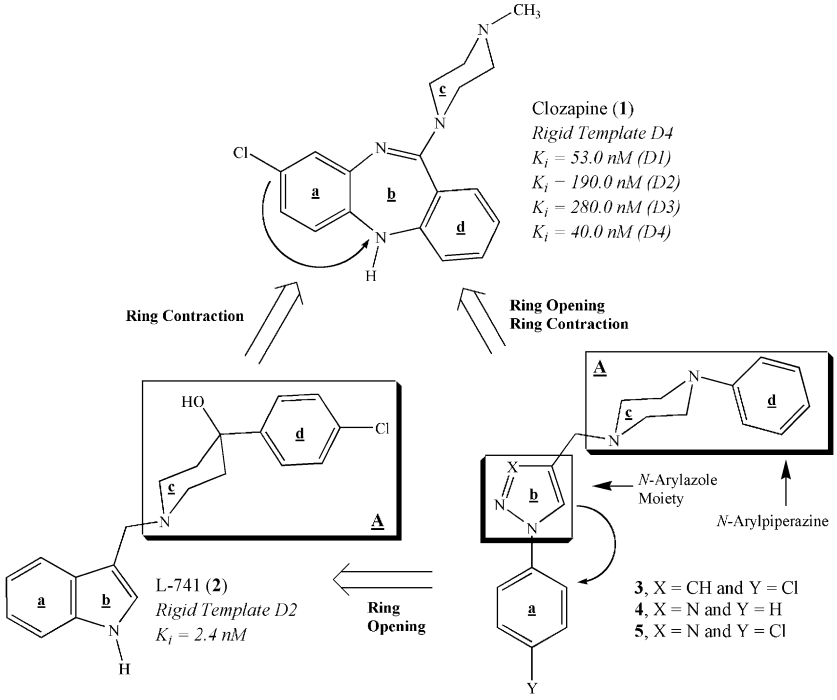
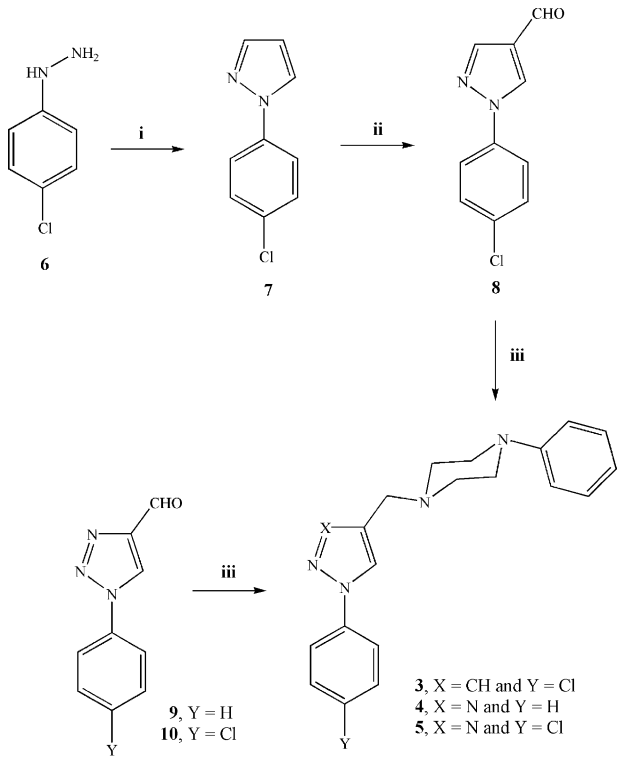


Figure 1. Structural design concept of new *N*-phenylpiperazine derivatives **3**–**5**.



Scheme 1. Reagents and conditions: (i) 1,1,3,3-tetramethoxipropane (1 equiv), concd HCl, reflux, 1 h, 86%; (ii) phosphorus oxychloride (4 equiv), DMF (4 equiv), 70 °C, 12 h, 78%; (iii) *N*-phenylpiperazine (1 equiv), acetic acid, methanol, sodium cyanoborohydride (5.7 equiv), 4 h, **3** (79%), **4** (72%) and **5** (78%).

aldehydes **8**–**10** with *N*-phenylpiperazine in dry methanol containing catalytic amount of acetic acid give target-compounds **3**, **4**, and **5**, herein referred to as LASSBio-579, -580 and -581, respectively.

Pharmacology

Receptor binding selectivity of LASSBio-579, -580 and -581 was assayed according to a protocol recently developed to allow for rapid screening of binding of different compounds to various neurotransmitter receptors in a single membrane preparation from rat brain. The following radioactive ligands were used: [^{3}H]quinuclidinyl benzilate (0.4 nM, selective for muscarinic receptors[20]) [^{3}H]epibatidine (10 nM, selective for α4β2 nicotinic receptors[21]); [^{3}H]SCH-23390 (3 nM, selective for D1 receptors[22]); and [^{3}H]YM-09151-2 (2.5 nM, selective for D2 receptors[23]). The concentrations of the radioactive ligands were selected such that their specific binding corresponded to 82–96% of the total binding.[24] At 10 μM, LASSBio-579, -580 and -581 were unable to displace binding of quinuclidinyl benzilate to muscarinic receptors, epibatidine to nicotinic receptors and SCH-233390 to D1 receptors (Fig. 2). However, at the same concentration, all three compounds effectively displaced the binding of YM-0915-2 to D2 receptors (Fig. 2).

Analysis of the concentration-response relationship for displacement of [^{3}H]YM-09151-2 binding to the rat brain membranes revealed that the D2 receptor binding

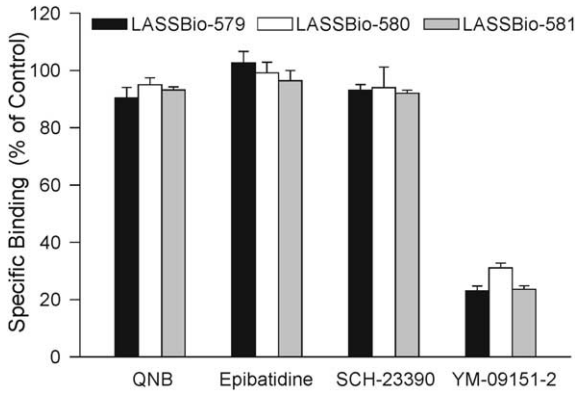


Figure 2. Displacement by LASSBio-579, -580 and -581 of binding of quinuclidinyl benzilate, epibatidine, SCH-23390 and YM-09151-2 to muscarinic, nicotinic, D1 and D2 receptors, respectively, in rat brain membranes.

affinities of LASSBio-579 and -581 were the same (Fig. 3). In contrast, the D2 receptor binding affinity of LASSBio-580 was approximately 5-fold lower than that of LASSBio-579 and -581 (Fig. 3).

An electrophysiological assay was used to determine whether the novel compounds act as D2 receptor agonists or antagonists. Numerous studies have demonstrated that D2 receptor agonists interacting with presynaptically located D2 receptors can reduce glutamate release in the ventral tegmental area, the striatum and the hippocampus.[25,26] Thus, to determine whether LASSBio-579, -580 and -581 can alter glutamate release, spontaneous postsynaptic currents (PSCs) were recorded by means of the patch-clamp technique from cultured hippocampal neurons before, during and after their exposure to physiological solution containing one of the test compounds. All PSCs were recorded from neurons continuously perfused with external solution containing the $GABA_A$ receptor antagonist picrotoxin (100 μM). Under this experimental condition, as shown in previous studies from our laboratory, the PSCs are

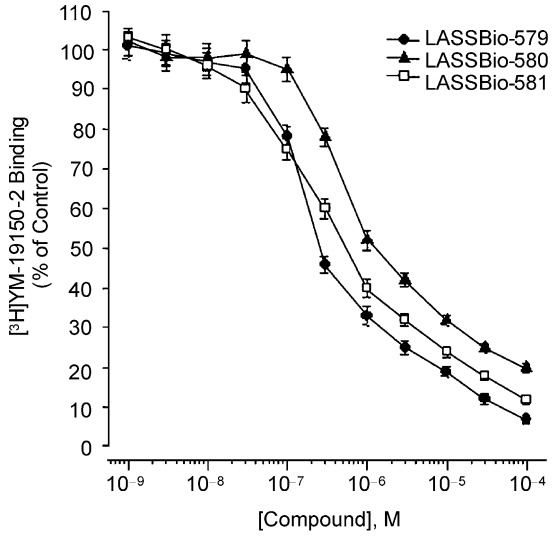


Figure 3. Concentration–response relationship for binding of LASSBio-579, 580- and -581 to D2 receptors in rat brain membranes.

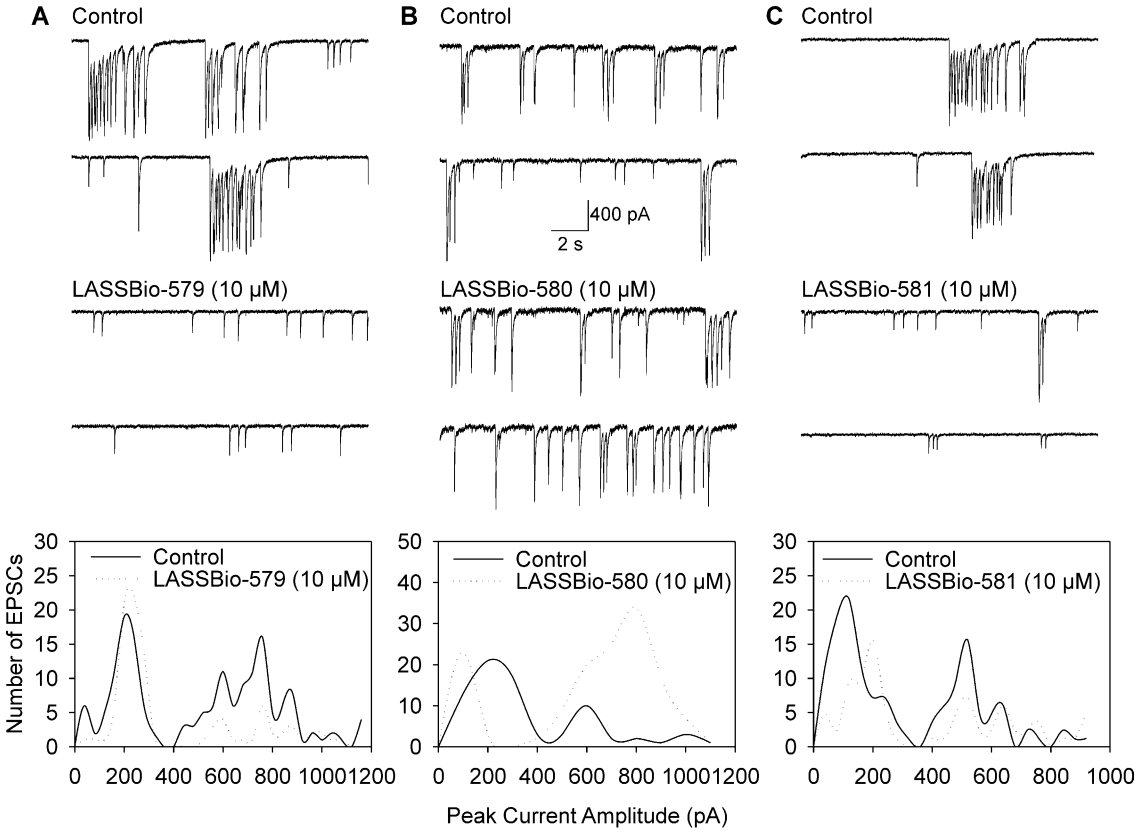


Figure 4. Effects of LASSBio-579, -580 and -581 on frequency and amplitude of spontaneous EPSCs recorded from cultured hippocampal neurons.

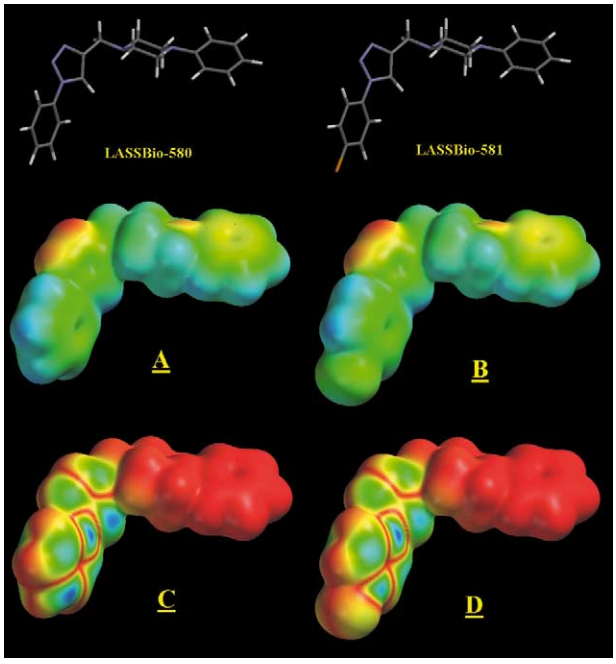


Figure 5. Minimum energy conformers of the derivatives LASSBio-580 and LASSBio-581. Molecular electrostatic maps of LASSBio-580 (A) and LASSBio-581 (B). LUMO distribution maps of LASSBio-580 (C) and LASSBio-581 (D-).

the result of activat ion of postsynaptic AMPA receptors by glutamate released from glutamatergic neurons synapsing onto the neurons under study. The frequency and the amplitude of these excitatory PSCs (herein referred to as EPSCs) were reduced during exposure of the neurons to LASSBio-579 and -581 (Fig. 4). At 10 μM, each compound reduced by 65 and 68%, respectively, the area-under-the-curve of histograms of number of EPSCs versus amplitude (Fig. 4). In contrast, EPSC frequency and amplitudes were increased by LASSBio-580. At 10 mM, LASSBio-580 increased by 54% the area-under-the-curve of histograms of number of EPSCs versus amplitude (Fig. 4). On average, it took 40 s for the onset of the effects of LASSBio-579, -580 and -581 on glutamatergic transmission. In addition, the effects were fully reversible after 1 min of perfusion of the neurons with drug-free physiological solution. In agreement with the concept that the effects were mediated by the interaction of the test compounds with presynaptically located dopaminergic receptors, were the findings that LASSBio-579, -580 and -581 were unable to affect the frequency or amplitude of spontaneous EPSCs recorded from neurons continuously perfused with physiological solution containing the dopamine receptor antagonist spiperone (10 μM, data not shown). These results taken together indicate that LASSBio-579 and 581- act as D2 receptor agonists, whereas LASSBio-580 acts as a D2 receptor antagonist.

Molecular Modeling

To elucidate the possible structural reasons of the dopamine receptor ligand profile of these new azaheterocyclic derivatives, we submitted representative compounds

LASSBio-580 and LASSBio-581 to theoretical study using semiempirical AM1 method[27] at the SCF-MO level, with full geometry optimization implemented on a Pentium III 900 MHz computer.

From this studies we were able to observe that the minimal energy conformer of LASSBio-580 and LASSBio-581 presented a very closed extended shape, with piperazine group adopting a chair orientation (Fig. 5). Additionally, the measurement of the torsional angles (θ_1) between 1,2,3-triazole ring and its phenyl attached group of LASSBio-580 and LASSBio-581, that is −25.4 and −24.1° respectively, indicated that no significative effect of the chloro substituent on the conformation was evidenced. On the other hand, chloro substituent of LASSBio-581 contributes to the increase of its molar volume in ca. 23 Å^3 and to the significant decrease of the overall dipole moment.

The comparative analysis of the electrostatic potential surface maps of the minimal energy conformers of LASSBio-580 (Fig. 5, A) and LASSBio-581 (Fig. 5, B), obtained after single-point ab initio calculation using 3-21G* basis set[28] with SPARTAN 1.0.5 program,[29] did not allow to evidence significative differences in the overall charge distribution. Despite this, plot of the LUMO delineate areas of LASSBio-580 (Fig. 5, C) and LASSBio-581 (Fig. 5, D), indicated that chloro atom increase significantly the charge density on its phenyl-attached ring and additionally in the C-5 of 1,2,3-triazole ring, which could be evidenced by the attenuation of 'blue' color in the assigned regions.

This distinct electronic distribution profile associated with steric effect promoted by introduction of *para*-chloro substituent in the compound LASSBio-581 suggested to us that intrinsic activity could be modulated by its different molecular recognition by dopamine D2 receptors, probably due to modifications in charge-transfer type and/or hydrophobic interations with complementary aminoacid residues.

As concluding remarks, our results suggest that this new series of heterocyclic *N*-phenylpiperazine derivatives **3**–**5** designed by applying molecular hybridization and simplification approach on clozapine (**1**) are selective dopamine D2 receptor ligands, which presents its intrinsic agonist/antagonist activity modulated by introduction of a chloro atom on the heterocyclic-attached phenyl ring. Additionally, the dopamine D2 receptor agonist LASSBio-581 was selected for further in vivo pharmacological investigations[30] to well characterize its potential use for treatment of schizophrenia.

Experimental

Chemistry

All melting points were determined with a Thomas-Hoover apparatus and are uncorrected. Proton magnetic resonance (^{1}H NMR), unless otherwise stated, was determined in deuterated chloroform containing ca. 1% tetramthylsilane as an internal standard with Bruker AC 200, Bruker DRX 300 spectrometers at 200 MHz, respectively. Splitting patterns are as follows: s, singlet; d, doublet; dd, double doublet; m, multiplet. Carbon magnetic resonance (^{13}C NMR) was determined with the same spectrometer described above at 50 MHz, using deuterated chloroform containing ca. 1% tetramethylsilane. Infrared (IR) spectra were obtained with a Nicolet-55a Magna spectrophotometer by using potassium bromide plates. The ultraviolet spectra were obtained with a Hitachi U-2000 Spectrophotometer by using methanol with solvent and an internal standard. Microanalysis data was obtained with Perkin–Elmer 240 analyzer, using Perkin-Elmer AD-4 balance.

The progress of all reactions was monitored by TLC performed on 2.0 cm × 6.0 cm aluminum sheets pre-coated with silica gel 60 (HF-254, Merck) to a thickness of 0.25 mm. The developed chromatograms were viewed under ultraviolet light at 254 nm. For column chromatography Merck alumine was used. The usual workup means that the organic extracts prior to concentration, under reduced pressure, were treated with a saturated aqueous sodium chloride solution, referred to as brine, dried over anhydrous sodium sulfate and filtered.

1-(4-Chlorophenyl)-*1H*-pyrazole (7). A solution of 1,1,3,3-tetramethoxypropane (2.54 g, 15.5 mmol) and 4-chlorophenylhydrazine (**6**) (2.21 g, 15.5 mmol) in 95% ethanol (15.5 mL) containing hydrochloric acid (0.8 mL) was refluxed until TLC analysis indicated the total consumption of the starting materials (ca. 1 h). Then, after cooling at room temperature the reaction mixture was neutralized with 10% aq $NaHCO_3$ and extracted with CH_2Cl_2 (3 × 20 mL). After usual workup of the organic extracts, the precipitate was crystallized from hexanes to give 2.37 g (86%) of the compound **7**, as yellow crystals, mp 57–58 °C (lit.,[16] 54 °C), R_f = 0.62 (CH_2Cl_2/MeOH 95:5): IR (KBr) cm^{-1}: 3101–3069 (ν C–H), 1523–1498 (ν C=C and C=N), 1095 (ν C–Cl); ^{1}H NMR (200 MHz, $CDCl_3$): δ 6.49 (1H, s, H-4), 7.43 (2H, d, J = 8.9 Hz, H-3′ and H-5′), 7.66 (2H, d, J = 8.9 Hz, H-2′ and H-6′), 7.74 (1H, s, H-3), 7.90–7.91 (1H, m, H-5); ^{13}C NMR (50 MHz, $CDCl_3$): δ 108.1 (C-4), 120.5 (C-2′ and C-6′), 126.8 (C-5), 129.7 (C-3′ and 5′), 132.0 (C-4′), 138.9 (C-1′), 141.5 (C-3).

1-(4-Chlorophenyl)-*1H*-pyrazole-4-carbaldehyde (8). Phosphorus oxychloride (1.04 mL, 1.72 g, 11.22 mmol) was added dropwise to dry *N*,*N*-dimethylformamide (0.86 mL, 0.82 g, 11.22 mmol) at −10 °C. Then, 1-(4-chlorophenyl)-1*H*-pyrazole (**7**) (0.5 g, 2.8 mmol) was added to the Vilsmeier–Haack complex and the reaction mixture heated at 70 °C for 12 h. After neutralization with 10% aq $NaHCO_3$, the precipitate was filtered out and recrystallised from hexanes furnishing derivative **8** (0.42 g, 78%) as a white crystals, mp 113–114 °C, R_f = 0.38 (CH_2Cl_2/MeOH 95:5): IR (KBr) cm^{-1}: 3123–3067 (ν C–H), 1681 (ν C=O), 1630–1501 (ν C=C and C=N), 1099 (ν C–Cl); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.47–7.62 (2H, m, H-3′ and 5′), 7.51–7.79 (3H, m, H-3, H-2′ and H-6′), 8.56 (1H, s, H-5), 10.22 (1H, s, CHO); ^{13}C NMR (50 MHz, $CDCl_3$): δ 120.8. (C-4′), 121.0 (C-2′

and C-6′), 123.3 (C-4), 130.0 (C-5), 130.1 (C-3′ and C-5′), 136.4 (C-1′), 148.3 (C-3), 185.2 (CHO).

General procedure for the preparation of compounds 3–5

A solution of the corresponding heterocyclic aldehyde derivative **8–10** (0.7 mmol) and *N*-phenylpiperazine (0.11 g, 0.7 mmol) in dry methanol (2.5 mL) was adjusted to pH 6.0 by dropwise addition of concentrated acetic acid. Then, sodium cyanoborohydride (0.25 g, 4 mmol) was added and the resultant mixture stirred at 60 °C for 2–4 h. After removal of the solvent under reduced pressure, the residue was partitioned between dichloromethane and 10% aqueous potassium phosphate. The organic layer was separated and submitted to usual workup to furnish a crude precipitate, which was purified by recrystallization in ethanol/water.

1-[1-(4-Chlorophenyl)-1H-pyrazol-4-ylmethyl]-4-phenylpiperazine (3). Derivative **3** was obtained in 77% yield, as a white solid, mp 122 °C, R_f = 0.38 (CH_2Cl_2/MeOH 95:5): IR (KBr) cm^{-1}: 3106–3022 (ν C–H), 1598–1497 (ν C=C and C=N), 1093 (ν C–Cl); ^1H NMR (200 MHz, $CDCl_3$) δ 2.67–2.71 (4H, m, Ar-$CH_2N(C\underline{H}_2-CH_2)_2NPh$), 3.21–3.26 (4H, m, Ar-$CH_2N(CH_2-C\underline{H}_2)_2NPh$), 3.59 (2H, s, Ar-$C\underline{H}_2N(CH_2-CH_2)_2NPh$), 6.83–6.95 (3H, m, H-2″, H-4″ and H-6″), 7.23–7.30 (2H, m, H-3″ and H-5″), 7.42 (2H, d, J = 8.9 Hz, H-3′ and H-5′), 7.64 (2H, d, J = 8.9 Hz, H-2′ and H-6′), 7.68 (1H, s, H-3), 7.90 (1H, s, H-5); ^{13}C NMR (50 MHz, $CDCl_3$): δ 49.2 (Ar-$CH_2N(CH)_2-\underline{C}H_2)_2NPh$), 52.7 (Ar)-$\underline{C}H_2N(CH)_2-CH_2)_2NPh$), 53.1 (Ar-$CH_2N(\underline{C}H_2-CH_2)_2NPh$), 116.3 (C-2″ and C-6″), 119.9 (C-4), 120.2 (C-2′ and C-6′), 126.6 (C-5), 129.3 (C-3′ and C-5′), 129.7 (C-3″ and C-5″), 132.0 (C-4′), 138.8 (C-1′), 142.3 (C-3), 151.4 (C-1″); UV (MeOH) λ_{max}: 255 (log ε = 4.47). Anal. calcd for $C_{20}H_{21}ClN_4$: C, 68.08%; H, 6.00%; N, 15.88%. Found: C, 66.99%; H, 6.15%; N, 15.80%.

1-Phenyl-4-(1-phenyl-1H-[1,2,3]triazol-4-ylmethyl)-piperazine (4). Derivative **4** was obtained in 72% yield, as a white solid, mp 150 °C, R_f = 0.48 (CH_2Cl_2/MeOH 95:5): IR (KBr) cm^{-1}: 3083–3035 (ν C–H), 1601–1501 (ν C=C and C=N); ^1H NMR (200 MHz, $CDCl_3$): δ 2.73–2.78 (4H, m, Ar-$CH_2N(C\underline{H}_2-CH_2)_2NPh$), 3.21–3.26 (4H, m, Ar-$CH_2N(CH_2-C\underline{H}_2)_2NPh$), 3.84 (2H, s, Ar-$C\underline{H}_2N(CH_2-CH_2)_2NPh$), 6.82–6.95 (3H, m, H-2″, H-4″ and H-6″), 7.22–7.30 (2H, m, H-3″ and H-5″), 7.44–7.54 (3H, m, H-3′, H-4′ and H-5′), 7.73–7.77 (2H, m, H-2′ and H-6′), 7.98 (1H, s, H-5); ^{13}C NMR (50 MHz, $CDCl_3$): δ 49.2 (Ar)-$\underline{C}H_2N(CH)_2-CH_2)_2NPh$), 53.2 (Ar)-$\underline{C}H_2N(CH)_2-CH_2)_2NPh$), 53.4 (Ar)-$\underline{C}H_2N(CH)_2-CH_2)_2NPh$), 116.3 (C-2″ and C-6″), 120.0 (C-4″), 120.6 (C-2′ and C-6′), 121.1 (C-5), 128.9 (C-4′), 129.3 (C-3′ and C-5′), 130.0 (C-3″ and C-5″), 137.2 (C-1′), 145.1 (C-4), 151.4 (C-1″); UV (MeOH) λ_{max}: 246 (log ε = 4.29). Anal. calcd for $C_{19}H_{21}N_5$: C, 71.45%; H, 6.63%; N, 21.93%. Found: C, 71.56%; H, 6.54%; N, 21.77%.

1-[1-(4-Chlorophenyl)-1H-[1,2,3]triazol-4-ylmethyl]-4-phenyl-piperazine (5). Derivative **5** was obtained in 77% yield, as a white solid, mp 151 °C, R_f = 0.48 (CH_2Cl_2/MeOH 95:5): IR (KBr) cm^{-1}: 3094–3067 (ν C–H), 1599–1501 (ν C=C and C=N), 1097 (ν C–Cl); ^1H NMR (200 MHz, $CDCl_3$): δ 2.72–2.77 (4H, m, Ar-$\underline{C}H_2N(CH)_2-CH_2)_2NPh$), 3.20–3.25 (4H, m, Ar-$\underline{C}H_2N(CH)_2-CH_2)_2NPh$), 3.83 (2H, s, Ar-$\underline{C}H_2N(CH)_2-CH_2)_2NPh$), 6.82–6.95 (3H, m, H-2″, H-4″ and H-6″), 7.22–7.30 (2H, m, H-3″ and H-5″), 7.50 (2H, d, J = 8.7 Hz, H-3′ and H-5′), 7.71 (2H, d, J = 8.7 Hz, H-2′ and H-6′), 7.95 (1H, s, H-5); ^{13}C NMR (50 MHz, $CDCl_3$): δ 49.2 (Ar-$CH_2N(CH_2-\underline{C}H_2)_2NPh$), 53.2 (Ar-$CH_2N(\underline{C}H_2-CH_2)_2NPh$), 53.4 Ar-$\underline{C}H_2N(CH)_2-CH_2)_2NPh$, 116.3 (C-2″ and C-6″), 120.0 (C-4″), 121.0 (C-5), 121.8 (C-2′ and C-6′), 129.3 (C-3′ and C-5′), 130.1 (C-3″ and C-5″), 134.6 (C-4′), 135.7 (C-1′), 145.4 (C-4), 151.3 (C-1″); UV (MeOH) λ_{max}: 252 (log ε = 4.34). Anal. calcd for $C_{19}H_{20}ClN_5$: C, 64.49%; H, 5.70%; N, 19.79%. Found: C, 64.61%; H, 5.59%; N, 19.75%.

Pharmacology

Rat brain membrane preparation. For each experiment, one male Sprague–Dawley rat (200–300 g) was anesthetized with CO_2 and decapitated by guillotine. The whole brain was rapidly removed onto ice. Then, using a glass Teflon Potter-Elvehjem homogenizer (10 strokes at 600 rpm), the brain was homogenized in 20 volumes of ice-cold Tris–Krebs Ringer containing 118 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM EDTA (pH, 7.4). The homogenate was centrifuged at 40,000 *g* for 20 min at 4 °C. The resultant pellet (P1) was resuspended in ice-cold 20 mM Tris–HCl pH 7.4 by homogenization with a Brinkmann PT-10 Polytron at a setting of 5 s, and subsequently centrifuged at 40,000*g* for 20 min at 4 °C. This washing step was repeated four times with ice-cold 20 mM Tris–HCl buffer pH 7.5. The final pellet was resuspended in the homogenization buffer and kept on ice until assayed. Protein content was determined using BCA reagents (Pierce Chemical, Rockford, IL, USA) with bovine serum albumin as a standard.

Binding of radioactive ligands to neurotransmitter receptors. All radioactive ligands were purchased from New England Nuclear. Nonspecific binding was determined as the amount of radioactive ligand bound in the presence of a high concentration of a displacer that would inhibit all nonspecific binding. Binding of selective radiolabeled ligands to their receptors was measured by a filtration assay. Rat brain membranes were incubated for 60 min with the radiolabeled ligand in a total volume of 250 mL buffer in the absence or presence of the specific displacer (i.e., binds to the same receptor and saturates all sites). The most selective radioactive ligand and displacer were used under optimum incubation conditions.[24] All incubations were terminated by vacuum filtration over Whatman GF/B glass-fiber filters (presoaked in 0.05% polyethylenimine for at least 30 min to eliminate nonspecific filter binding), followed by three 4-mL washes with ice-cold 0.9% NaCl solution. Radioactivity was counted by liquid scintillation spectroscopy.

Cultured hippocampal neurons. Primary cultures of neurons dissociated from the hippocampi of 16–19-day-old

rat fetuses (Sprague–Dawley) were prepared according to the procedure described in Pereira et al.[31] Experiments were performed on hippocampal neurons cultured for 15–20 days.

Electrophysiological recordings. Postsynaptic currents were recorded according to the whole-cell mode of the patch-clamp technique[32] using an LM-EPC-7 amplifier (List Electronics, Darmstadt, FRG). Signals were filtered at 1–2 kHz, and either stored in a VCR for later analysis or directly sampled by a Pentium-III computer using the PCLAMP6 program (Axon Instruments, Foster City, CA, USA). The composition of the external solution used to bathe the neurons was (in mM): NaCl 165, KCl 5, $CaCl_2{\cdot}2H_2O$ 1, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 5, dextrose 10 (pH was adjusted to 7.3 with NaOH; 340 mOsM). The internal solution used to fill the patch pipettes had the following composition (in mM): CsCl 80, CsF 80, ethyleneglycoltetraacetic acid (EGTA) 10, CsOH 22.5 and HEPES 10 (pH was adjusted to 7.3 with CsOH; 340 mOsM). Results were not used when the access resistance changed significantly during the experiment.

Data analysis. Peak amplitude and frequency of PSCs were analyzed using the Continuous Data Recording program.[33]

Computational chemistry

The geometry optimization of LASSBio-580 and LASSBio-581 was performed using the AM1Hamiltonian[27] within SPARTAN 1.0.5 program[29] on a Pentium III 900 MHz. In order to better evaluate the electronic properties of the AM1 minimal energy conformations of LASSBio-580 and LASSBio-581, they were submitted to a single point ab-initio calculation with a 3-21G* basis set[28] with the same program described above. Molecular electrostatic potential maps (MEPs) and LUMO density isosurfaces were generated and plotted employing default parameters in SPARTAN.

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