0026-895X/96/040752-11$3.00/0

MOLECULAR PHARMACOLOGY, 50:752–762 (1996).

5-Hydroxytryptamine-moduline, a New Endogenous Cerebral Peptide, Controls the Serotonergic Activity via Its Specific Interaction with 5-Hydroxytryptamine$_{1B/1D}$ Receptors

O. MASSOT, J.-C. ROUSSELLE, M.-P. FILLION, B. GRIMALDI, I. CLOËZ-TAYARANI, A. FUGELLI, N. PRUDHOMME, L. SEGUIN, B. ROUSSEAU, M. PLANTEFOL, R. HEN, and G. FILLION

Unité de Pharmacologie Neuro-Immuno-Endocrinienne, Institut Pasteur, F75724 Paris, Cedex 15, France (O.M., J.-C.R., M.-P.F., B.G., I.C.-T., A.F., N.P., L.S., M.P., G.F.), Service des Molécules Marquées, Biologie Cellulaire et Moléculaire, Commisariat à l'Energie Atomique/Centre d'Etudes Nucléaires de Saclay, F91191 Gif-sur-Yvette, France (B.R.), and Columbia University, College of Physicians and Surgeons, Center for Neurobiology and Behavior, New York, New York 10032 (R.H.)

Received January 16, 1996; Accepted June 21, 1996

SUMMARY

The serotonergic system controls the activity of neurotransmissions involved in numerous physiological functions. It is also thought to be crucially implicated in various pathologies, including psychiatric disorders such as depression, anxiety, and aggressiveness. The properties of 5-hydroxytryptamine (5-HT)-moduline, a novel endogenous peptide, have been tested *in vitro* and *in vivo*. Binding studies have shown that the peptide specifically interacts with 5-HT$_{1B/1D}$ receptors via a noncompetitive mechanism corresponding to a high apparent affinity ($EC_{50} = 10^{-10}$ M). The interaction was shown in rat and guinea pig brain tissues and in cells transfected with either 5-HT$_{1B}$ or 5-HT$_{1D\beta}$ receptor gene. [^{3}H]5-HT-moduline binds to a single population of sites in mammalian brain (K_d = 0.4 nM in rat, K_d = 0.8 nM in guinea pig) as well as in transfected cells expressing the 5-HT$_{1B}$ or the 5-HT$_{1D\beta}$ receptors (K_d = 0.2 and 0.6 nM, respectively). Furthermore, the binding is clearly specific of the LSAL sequence. Autoradiographic studies showed an heterogeneous brain distribution of this site. The interaction of 5-HT-moduline with the 5-HT$_{1B/1D}$ receptor corresponds to a decrease in the functional activity of the receptor (i.e., a decrease in the inhibitory effect of a 5-HT$_{1B}$ agonist on the evoked release of [^{3}H]5-HT from synaptosomal preparation). It was also shown that 5-HT-moduline possess an *in vivo* effect in the social interaction test in mouse. Finally, it was demonstrated that 5-HT-moduline was released from brain synaptosomal preparation by a K^+/Ca^{2+}-dependent mechanism. In conclusion, 5-HT-moduline is a novel endogenous peptide regulating the serotonergic activity via a direct action at presynaptic 5-HT receptor. It may play an important role in the physiological mechanisms involving the serotonergic system, particularly in mechanisms corresponding to the elaboration of an appropriate response of the central nervous system to a given stimulus.

5-HT is a neurotransmitter that is involved in numerous physiological (e.g., sleep, thermoregulation, behavior, pain, locomotion, appetite, sexual activity) and pathological (e.g., stress, anxiety, depression, aggressiveness, bulimia, anorexia, schizophrenia, migraine, digestive disorders) events (1). The serotonergic neurotransmission essentially regulates the activity of other neuronal circuitries without being constitutively part of them (2); its regulatory role is exerted via numerous serotonergic terminals along the axons, increasing its ability to interact with other neurotransmissions. The regulatory effects of the 5-HT system are mediated by a large number of receptors: four families, including 10 subtypes that were characterized as functional receptors (5-HT$_1$ through 5-HT$_4$), and three additional families that are potential receptors (5-ht$_5$ through 5-ht$_7$). The genes coding for each of them have been cloned (3).

Interestingly, two receptors (5-HT$_{1A}$ and 5-HT$_{1B/1D}$) are either postsynaptic receptors (heteroreceptors) located on nonserotonergic neurons or presynaptic receptors (autoreceptors) located on dendrites and soma (5-HT$_{1A}$) or terminals (5-HT$_{1B/1D}$) of serotonergic neurons. 5-HT$_{1A}$ receptors regulate the frequency of discharge of the serotonergic neurons, whereas 5-HT$_{1B/1D}$ receptors regulate the release of 5-HT from the terminals. Thus, these receptors are thought to play a crucial role in the control of the 5-HT neuronal activity.

5-HT$_{1B/1D}$ receptors correspond to the 5-HT$_{1B}$ subtype in rodents and the 5-HT$_{1D\beta}$ subtype in other species; the genes coding for these two receptors have 95% identity. Essentially, the mutation of a single amino acid (human Thr355/rat Asn351) confers the major pharmacological difference be-

This work was supported by Pasteur Institute Comité Consultatif d'Aide à la Recherche and by Direction des Recherches et Techniques (93/062).

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; 5-CT, 5-carboxytryptamine; CHO, Chinese hamster ovary; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography.

tween these receptors because 5-HT_{1B} receptors recognize β-adrenergic antagonists with a high affinity, whereas 5-$HT_{1Dβ}$ receptors do not (4). Otherwise, transduction systems, regional distributions in brain, and cellular functions of the two receptors are similar; i.e., they inhibit the release of neurotransmitter from terminals and, in particular, mediate the negative feedback mechanism that controls the release of 5-HT. Accordingly, these receptors are thought to play an important role in all physiopathological events involving a serotonergic control (5).

On the basis of experimental evidences, we previously proposed the hypothesis suggesting that an endogenous ligand may be present in cerebral tissue and interacts with the 5-HT_1 receptors function (6). Accordingly, we recently purified and isolated an endogenous peptide characterized as a tetrapeptide Leu-Ser-Ala-Leu (LSAL) from rat and bovine brains (7). The purification of the peptide from rat brain extract was carried out because of its ability to inhibit the binding of [^{3}H]5-HT to its 5-HT_1 receptors. It was shown that the synthetic peptide and the purified active fraction had the same pharmacological specificity; i.e., they inhibited the binding of [^{3}H]5-HT to 5-HT_{1B} receptors and did not interact with the binding of specific radioligands to other serotonergic (5-HT_{1A}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_2, 5-HT_3) or nonserotonergic (α- and β-adrenergic, dopaminergic, histaminergic, muscarinic, benzodiazepine, and opiate) receptors. They did not affect the uptake of 5-HT in synaptosomal preparations. This peptide was called 5-HT-moduline because it potentially modulated the serotonergic activity. We present the results of biochemical and pharmacological studies that were performed to further characterize this peptide.

Experimental Procedures

Materials

[^{3}H]5-HT (3.66 TBq/mmol) and ^{125}I-cyanopindolol (74 TBq/mmol) were from Amersham International (Buckinghamshire, UK). [^{3}H]5-HT-moduline (4.14 TBq/mmol) was synthesized by the Service des Molécules Marquées, CEA/Saclay (Gif-sur-Yvette, France). Mice (male Swiss NMRI, 3–4 weeks old) and rats (adult male Wistar) were obtained from Iffa Credo (L'Arbresele, France). Adult guinea pigs were purchased from Elevage Lebeaux (Gambais, France). Synthetic peptides were synthesized by Neosystem (Strasbourg, France). The C_{18} Ultrabase reverse-phase column was from SFCC-Shandon (Life Science International, Manchester, UK).

Membrane Preparations

Brain membranes. Rat brain or guinea pig cortices were dissected on ice and homogenized for 30 sec with an Ultra-Turrax apparatus in 5 volumes (v/w) of a 50 mM Tris-acetate buffer, pH 7.4, containing 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 5 IU/liter aprotinin. The homogenate was then diluted in 30 volumes (v/w) of the same medium and centrifuged (17,500 × *g* at 4° for 5 min). The resulting pellet was resuspended in 30 volumes of the same buffer completed with 0.3 mg/ml bacitracin and 0.1% bovine serum albumin, incubated for 10 min at 37° to remove endogenous ligands, and centrifuged as described above. The homogenate was washed two additional times, and the pellet was resuspended in the appropriate buffer. Protein equivalent was determined according to the method of Lowry (8).

Cell membranes. NIH 3T3 or CHO cells (10^6/dish) were cultured for 48 hr in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 0.3 mg/ml geneticin, 10 IU/liter penicillin, and 10 μg/ml streptomycin. Cells were collected and extensively washed in 50 mM Tris-acetate buffer, pH 7.4, before homogenization. Membranes were then prepared as described above.

Synaptosomes. Rat brain synaptosomes (hippocampus or total brain) were prepared according to the method of Cotman and Matthews (9).

[^{3}H]5-HT Binding Experiments

Saturation experiments. Rat brain membranes (500 μg of protein in a final volume of 500 μl) were incubated for 30 min at 25° in 50 mM Tris-acetate buffer, pH 7.4, containing 4 mM $CaCl_2$, 0.1% ascorbic acid, 10 μM pargyline, 0.1 μM 8-hydroxy-2[di-*n*-propylamino]tetralin, and 0.1 μM mesulergine, with increasing concentrations (1–30 nM) of [^{3}H]5-HT in the absence or presence of 5-HT-moduline (1 nM). Nonspecific and 5-$HT_{1E/1F}$ bindings were determined in the presence of 20 nM 5-CT (10). Specific 5-$HT_{1B/1D}$ binding was calculated as the difference between total binding and residual binding measured in the presence of 5-CT. Because 5-CT has a high affinity for 5-ht_7 receptors, it cannot be excluded that a minor part of the calculated binding involves these receptors (11). At the end of the incubation period, the tubes were cooled on ice for 10 min and filtered with Whatmann GF/B filters using a cell harvester.

Dose-response curves. Rat or guinea pig cortical membranes or NIH 3T3 cell membranes were incubated (250 μg of protein/tube) for 30 min at 25° with [^{3}H]5-HT (20 nM) and increasing concentrations of 5-HT-moduline (0.1 pM to 1 μM). Nonspecific binding was determined in the presence of 20 nM 5-CT.

^{125}I-Cyanopindolol Binding

Rat brain membranes (25 μg of protein in 200 μl of final volume) were incubated in 10 mM Tris-acetate buffer, pH 7.7, containing 157 mM NaCl, 20 μM pargyline, 0.1 μM 8-hydroxy-2[di-*n*-propylamino]-tetralin, and 30 μM isoproterenol for 60 min at 37° with increasing concentrations (20–350 pM) of ^{125}I-cyanopindolol in the absence or presence of 5-HT-moduline (1 nM). Nonspecific binding was determined in the presence of 10 μM 5-HT. At the end of the incubation period, the tubes were cooled and filtered with Whatmann GF/B filters.

[^{3}H]5-HT-moduline Stability

Next, 1 nM [^{3}H]5-HT-moduline was incubated overnight at 4° in 50 mM Tris-acetate buffer, pH 7.4, in the presence of brain membranes as described above. The incubate was then centrifuged (12,500 × *g* for 10 min at 4°), and the supernatant was analyzed by HPLC on a C_{18} reverse-phase column (Ultrabase; 150 × 4 mm) equilibrated in 50 mM ammonium acetate buffer, pH 5. Elution was run at 1 ml/min by a 3-min isocratic step followed by a 30-min linear gradient of acetonitrile (0–30%). Forty fractions of 1 min were collected, and radioactivity content was measured by liquid scintillation counting. The elution pattern of native [^{3}H]5-HT-moduline was also determined under the same chromatographic conditions.

[^{3}H]5-HT-moduline Binding

Saturation experiments. Rat or guinea pig cortical membranes or NIH 3T3 wild-type or transfected and CHO-transfected cell membranes (250 μg of protein in a total volume of 1 ml) were incubated overnight at 4° in 50 mM Tris-acetate buffer, pH 7.4, in the presence of increasing concentrations (0.1–4 nM) of [^{3}H]5-HT-moduline. Nonspecific binding was determined in the presence of 1 μM nonlabeled 5-HT-moduline. At the end of the incubation period, the tubes were filtered as described.

Structural specificity of [^{3}H]5-HT-moduline binding. Membranes were incubated overnight at 4° with [^{3}H]5-HT-moduline (1.5 nM) in the presence of increasing concentrations (0.1 pM to 10 μM) of nonlabeled 5-HT-moduline or other compounds (i.e., modified 5-HT-moduline, scrambled peptides, dipeptide and tripeptide derivatives, constitutive amino acids, nonrelated peptides, and 5-HT).

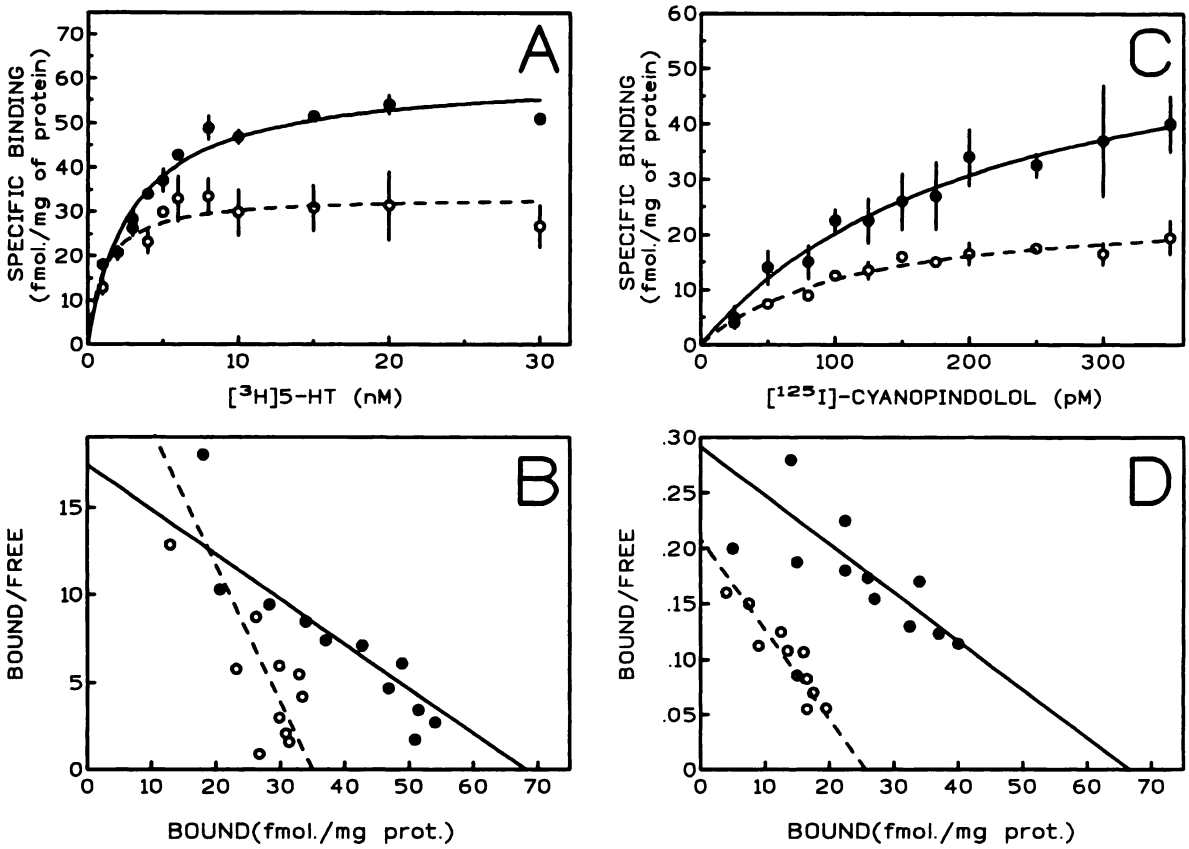

Fig. 1. Binding interactions of 5-HT-moduline on 5-HT_{1B} receptors. A, [^{3}H]5-HT binding. Rat brain membranes were incubated for 30 min at 25° with increasing concentrations (1–30 nM) of [^{3}H]5-HT in the absence (●) or presence (○) of 5-HT-moduline (1 nM). Nonspecific and 5-$HT_{1E/1F}$ bindings were determined in the presence of 20 nM 5-CT. Each value represents the mean ± standard error of triplicate determinations. Data represent a typical experiment with the corresponding Scatchard plot (B). C, ^{125}I-Cyanopindolol binding. Rat brain membranes were incubated for 60 min at 37° with increasing concentrations (20–350 pM) of ^{125}I-cyanopindolol in the absence (●) or presence (○) of 5-HT-moduline (1 nM). Nonspecific binding was determined in the presence of 10 μM 5-HT. *Points*, mean ± standard error of triplicate determinations. Data represent a typical experiment with the corresponding Scatchard plot (D).

Comparison of [^{3}H]5-HT and [^{3}H]5-HT-moduline Bindings in CHO Cells Transfected with the 5-$HT_{1D\beta}$ Receptor Gene

[^{3}H]5-HT binding. Cell membranes (200 μg of protein in a total volume of 2 ml) were incubated for 60 min at 25° with increasing concentrations of [^{3}H]5-HT (1–40 nM) in 50 mM Tris-acetate buffer, pH 7.4, containing 4 mM $CaCl_2$, 0.1% ascorbic acid, and 10 μM pargyline. Nonspecific binding was determined in the presence of 10 μM nonlabeled 5-HT. At the end of the incubation period, the tubes were filtered as described.

[^{3}H]5-HT-moduline binding. Cell membranes (200 μg of protein in a total volume of 10 ml) were incubated for 60 min at 25° with increasing concentrations of [^{3}H]5-HT-moduline (0.1–4 nM) in 80 mM HEPES buffer, pH 7.4. Nonspecific binding was determined in the presence of 1 μM nonlabeled [^{3}H]5-HT-moduline. At the end of the incubation period, the tubes were filtered as described.

Autoradiographic Studies

Sections (20-μm) of frozen rat brain were cut using a Reichert-Jung cryostat at −20°, thaw-mounted onto warm gelatin-coated glass slides, and immediately freeze-dried at −20°. After drying under a cold air stream, the sections were preincubated in 50 mM Tris-acetate buffer, pH 7.4, containing 0.3 mg/ml bacitracin, 0.1% bovine serum albumin, 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 5 IU/liter aprotinin for 10 min and rinsed in the same buffer for 5 min. Sections were incubated for 15 hr at 4° in the presence of 1 nM [^{3}H]5-HT-moduline in 50 mM Tris-acetate buffer, pH 7.4, rinsed twice in the incubation buffer at 4° for 30 sec, and dried in a cold air stream. Nonspecific binding was determined in the presence of 1 μM nonlabeled 5-HT-moduline. Tritium-sensitive films were exposed to slide-mounted sections in X-ray cassettes for 3 months.

Synaptosomal Release of 5-HT-moduline

Synaptosomes from six rat brains were diluted in 30 ml of a 37° oxygenated Krebs-Ringer buffer, pH 7.4, without $CaCl_2$. A 4-min K^+ stimulation (30 mM) was then applied to the synaptosomes in the presence or absence of $CaCl_2$ (1.22 mM). A control stimulation (without K^+ but with $CaCl_2$) was also carried out. The tubes were then immediately centrifuged (17,500 × *g* for 25 min at 4°), and the supernatants were lyophilized. The dried extracts were then resuspended in 1 ml of 50 mM ammonium acetate buffer, pH 5, and injected into a C_{18} Ultrabase reverse-phase column (250 × 10 mm). Elution was run at 4 ml/min under the chromatographic conditions described previously. For each extract, fractions 18–22 were lyophilized, resuspended in 400 μl of incubation buffer, and then tested for their abilities to displace the [^{3}H]5-HT-moduline binding (1.5 nM).

Synaptosomal Release of [^{3}H]5-HT

Hippocampal synaptosomes were loaded with 30 nM [^{3}H]5-HT, resuspended in a 37° oxygenated Krebs-Ringer buffer, pH 7.4, and dispatched in 200-μl aliquots in a 96-well filtration plate (MAFB

NOB glass-fiber filter type B, Millipore, Bedford, MA). 5-HT-moduline, CGS 12066B, or both were then added and incubated for 3 min with the loaded synaptosomes. At the end of the incubation period, a 5-min K^+ stimulation (15 mM) was applied. The 96-well filtration plate was filtered under vacuum, and the 96 filtrates were recovered. The radioactivity contained in each filtrate was then measured by liquid scintillation counting.

Behavioral Effect of 5-HT-moduline

The social interaction test was used (12). Mice were either housed in groups of five animals or isolated for 1 week. They were tested in pairs (one grouped and one isolated) under a transparent beaker inverted onto a rough-surface glass plate. The number of escape attempts was counted for 2 min and defined as one of the following: 1) the forepaws were placed against the beaker wall, 2) the mouse sniffed at the rim of the beaker, or 3) the mouse scratched the glass floor. Drugs (or sodium chloride for controls) were administered intracerebroventricularly at 45 min before the test for 5-HT-moduline (LSAL) or ALLS and intraperitoneally at 30 min before the test for RU 24969. Five mice were tested in each group, and the experiment was repeated four times. All mice were tested only once.

Mathematical Analysis

Binding experiments were analyzed with Inplot 2.04 (GraphPAD Software, San Diego, CA). For statistical analysis, Student's t test was performed using Instat 2.04.

Results

Binding interaction with 5-$HT_{1B/1D}$ receptors. The interaction of 5-HT-moduline with 5-HT_{1B} receptor binding was studied using agonist ([^{3}H]5-HT) and antagonist (^{125}I-cyanopindolol) radioligands. Analysis of the saturation curves and their Scatchard plots showed that the B_{max} value for [^{3}H]5-HT binding and that for ^{125}I-cyanopindolol were markedly reduced in the presence of 5-HT-moduline (1 nM) (57 ± 3% and 60 ± 5%, respectively; eight measurements), whereas the K_d values were not decreased (the value was slightly increased for [^{3}H]5-HT binding) (Fig. 1). The corresponding IC_{50} value in rat brain membranes was 0.32 ± 0.08 nM (six measurements), indicating a rather high apparent affinity (Fig. 2A). Similar results were observed with 5-HT_{1D} receptors in guinea pig brain membranes (IC_{50} = 0.65 ± 0.23 nM; four measurements) (Fig. 2B). Furthermore, the specific binding to 5-HT_{1B} receptors in NIH 3T3-transfected cells was similarly inhibited by 5-HT-moduline (IC_{50} = 0.43 ± 0.2 nM; five measurements) (Fig. 2C).

Scatchard plots of the saturation curves in the presence of 5-HT-moduline clearly indicated that the interaction did not correspond to a competition but rather suggested that it likely corresponded to a noncompetitive phenomenon.

Stability of 5-HT-moduline. A radiolabeled 5-HT-moduline was prepared by tritiation of the leucine located at the amino terminal using a dehydro-Leu-Ser-Ala-Leu precursor. To test the stability of [^{3}H]5-HT-moduline under the experimental conditions used in the binding studies, chromatographic analysis of the radioactivity content of the incubate was performed at the end of the incubation period. The retention time of [^{3}H]5-HT-moduline determined with the control injection of the peptide on the HPLC system (C_{18} Ultrabase reverse-phase column) was 20.5 min. When the peptide was incubated at 25° in the presence of rat or guinea pig brain membranes, the labeled material found in the supernatant was degraded to a variable degree. The same preparation incubated at 4° overnight was only slightly degraded (19%). The metabolite obtained was identified as the dipeptide LS (Fig. 3). However, using membranes prepared from 5-$HT_{1D\beta}$ CHO-transfected cells, the incubation of [^{3}H]5-HT-moduline at 25° reached equilibrium after 30 min without a significant alteration in the molecule (Fig. 4B, *inset*).

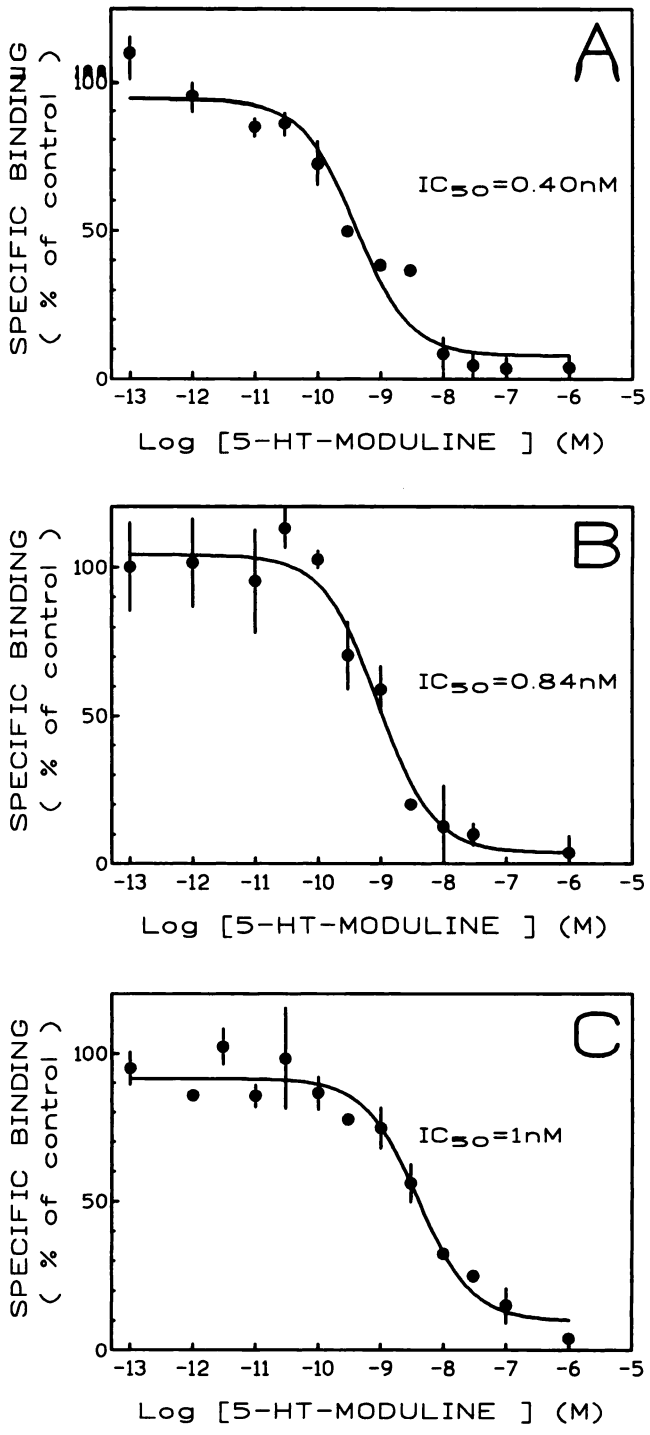

Fig. 2. Dose-response curves of 5-HT-moduline on [^{3}H]5-HT binding. Rat (A) or guinea pig (B) cortical membranes or NIH 3T3-transfected cell membranes (C) were incubated for 30 min at 25° with [^{3}H]5-HT (20 nM) and increasing concentrations of 5-HT-moduline (0.1 pM to 1 μM). Nonspecific and 5-$HT_{1E/1F}$ bindings were determined in the presence of 20 nM 5-CT and represented ~50% of the total binding. *Points*, mean ± standard error of triplicate determinations. Data represent typical experiments.

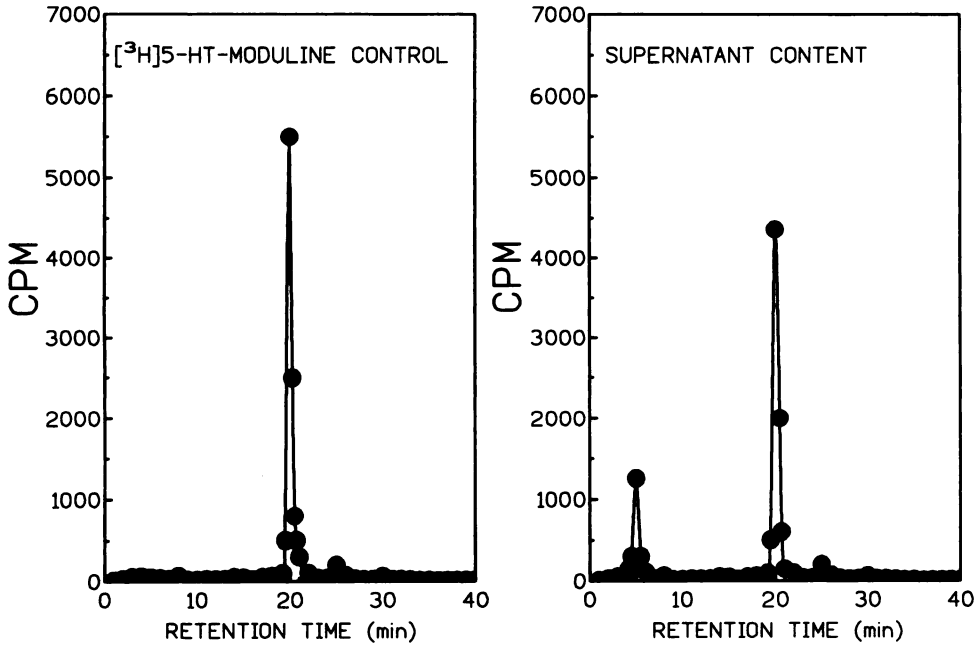

Fig. 3. Stability of [^{3}H]5-HT-moduline; 1 nM [^{3}H]5-HT-moduline was incubated overnight at 4° with rat brain membranes. The incubate was then centrifuged, and the radioactivity content of the supernatant was analyzed by HPLC on a C_{18} reverse-phase column as described in Experimental Procedures. A control injection of [^{3}H]5-HT-moduline was also carried out under the same chromatographic conditions.

5-HT-moduline-specific binding. To ensure that the binding equilibrium was reached at 4° under the our experimental conditions (i.e., 50 mM Tris-acetate, pH 7.4, incubation medium), an association kinetic of [^{3}H]5-HT-moduline (1 nM) was performed. It was then shown that the equilibrium was reached after 10 hr (Fig. 5A, *inset*). Thus, an overnight incubation period was subsequently applied for binding experiments carried out at 4°. The binding of the radioligand was studied on rat and guinea pig brain membrane preparations (Fig. 5) and on cells transfected with either 5-HT_{1B} or 5-$HT_{1D\beta}$ receptor genes (Fig. 6, A and C). [^{3}H]5-HT-moduline bound in a michaelian manner to a single population of sites. Saturation curves indicated that the binding corresponded to a high affinity: K_d = 0.40 ± 0.11 nM in rat (10 measurements) and 0.8 ± 0.12 nM in guinea pig (four measurements), with B_{max} values of 10 ± 2.5 and 22.2 ± 2.3 fmol/mg protein, respectively. The affinity constants of the binding were very similar in NIH 3T3-transfected cells expressing 5-HT_{1B} receptors (K_d = 0.2 ± 0.1 nM; five measurements) and in CHO-transfected cells expressing 5-$HT_{1D\beta}$ receptors (K_d = 0.61 ± 0.1 nM; five measurements); the corresponding B_{max} values were 11 ± 2.9 and 18 ± 4 fmol/mg protein, respectively. NIH 3T3-nontransfected cells as well as CHO cells expressing the 5-ht_6 receptor did not exhibit any reversible binding of [^{3}H]5-HT-moduline (Fig. 6, B and D).

Various derivatives of 5-HT-moduline were tested for their ability to displace the binding of the radiolabeled peptide in guinea pig brain membranes. LSAL was the most efficient chemical structure displacing [^{3}H]5-HT-moduline, with an IC_{50} of 3 nM (K_i = 1 nM), which is in agreement with the value of the affinity constant determined by direct binding (K_d = 0.8 nM). Modification of the carboxyl and amino terminals of the peptide (i.e., amidation or acetylation, respectively) led to a decrease in the binding affinity (IC_{50} = 0.3 and 1 μM, respectively). Scrambled sequences of the tetrapeptide (ALLS or LASL) as well as derived tripeptides (LSA, SAL, or ALS) or dipeptides (LS or AL) exhibited a marked reduction of efficacy (IC_{50} value close to 1 μM). Other unrelated peptides (Leu-enkephalin, Met-enkephalin, Substance P, or thyrotropin-releasing hormone) were also inactive (IC_{50} = >1 μM). Interestingly, 5-HT did not inhibit the binding of the radiolabeled peptide (IC_{50} = >100 μM) under the experimental conditions used in this study (Table 1).

Comparison of [^{3}H]5-HT and [^{3}H]5-HT-moduline bindings in CHO cells transfected with the 5-$HT_{1D\beta}$ receptor gene. With the use of an incubation procedure at 25° in 80 mM HEPES buffer, pH 7.5, the binding of [^{3}H]5-HT-moduline on CHO-transfected cells corresponded to a similar affinity constant (1.11 ± 0.16 nM) as that observed under the previous experimental conditions. However, the B_{max} value increased from 18 ± 4 to 1400 ± 30 fmol/mg protein (three measurements). Comparison of the saturation curves of [^{3}H]5-HT and [^{3}H]5-HT-moduline indicated that the B_{max} values for the two ligands were similar: the B_{max} value for 5-$H_{1D\beta}$ was 1140 ± 30 fmol/mg protein (three measurements) (Fig. 4).

Autoradiographic localization of 5-HT-moduline binding sites. Autoradiographic study revealed that [^{3}H]5-HT-moduline binding sites were heterogeneously distributed in rat brain; several areas exhibited a high density of sites (subiculum, superior colliculus, substantia nigra), whereas other regions showed low density of binding sites. The addition of 1 μM nonlabeled 5-HT-moduline totally suppressed the labeling of these brain areas (Fig. 7).

Brain release of 5-HT-moduline. The potential release of 5-HT-moduline was studied in crude synaptosomal preparations from rat brain. The preparation was underwent or did not undergo a 4-min K^+ stimulation in the presence or absence of Ca^{2+}. After HPLC separation, fractions 18–22, including fraction 20 corresponding to 5-HT-moduline (retention time of 5-HT-moduline = 20.5 min), were tested by radioreceptor assay. It was shown that inhibitions of 60 ± 8%, 20 ± 5%, and 15 ± 2.5% were obtained with fractions 20 corresponding to extracts submitted to K^+/Ca^{2+}, K^+, or Ca^{2+} stimulations, respectively (Fig. 8).

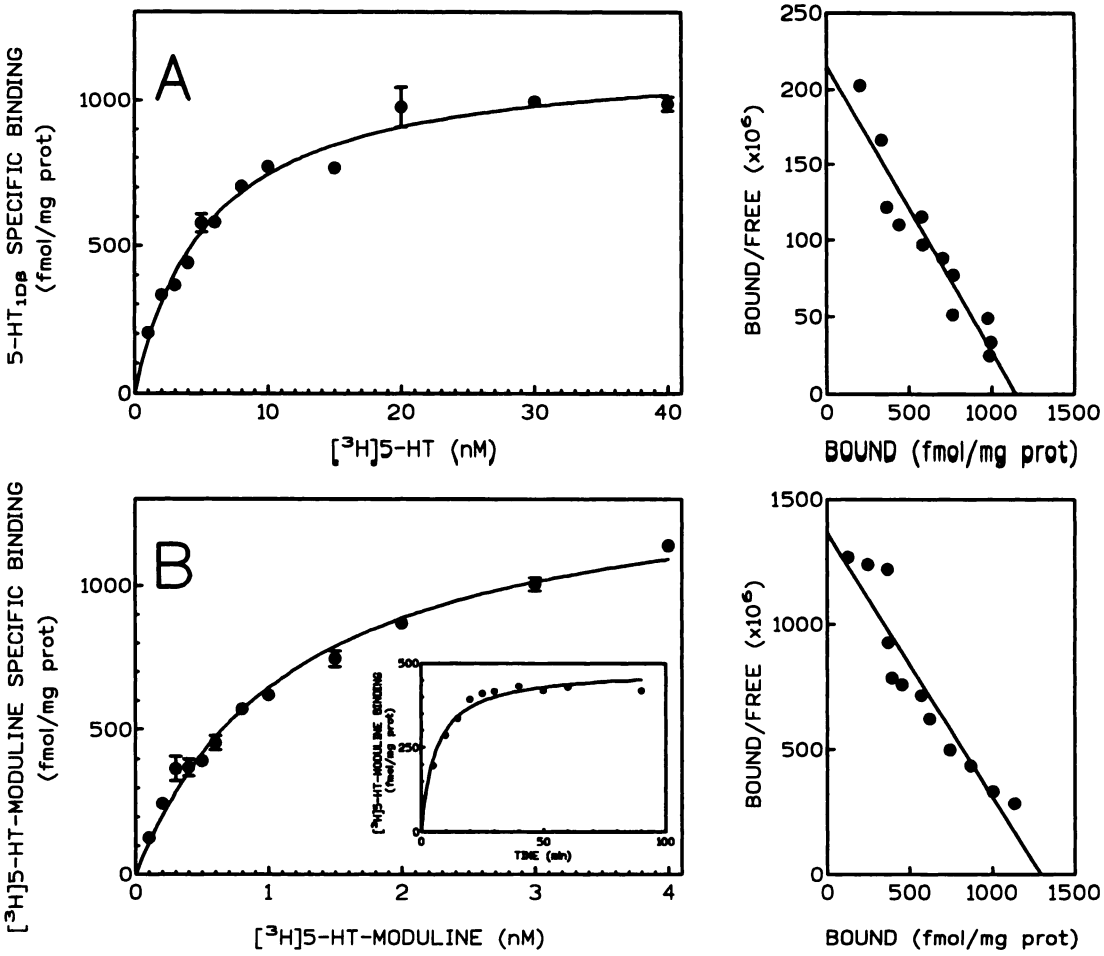

Fig. 4. Comparison of [^{3}H]5-HT and [^{3}H]5-HT-moduline bindings in CHO cells transfected with the gene coding for the 5-HT$_{1D\beta}$ receptor. A, [^{3}H]5-HT binding. Cell membranes (200 μg of protein in a total volume of 2 ml) were incubated for 60 min at 25° in 50 mM Tris-acetate buffer, pH 7.4, containing 4 mM $CaCl_2$, 0.1% ascorbic acid, and 10 μM pargyline. Nonspecific binding was determined in the presence of 10 μM nonlabeled 5-HT and represented ~20% of the total binding at 40 nM [^{3}H]5-HT. B, [^{3}H]5-HT-moduline binding. Cell membranes (200 μg of protein in a total volume of 10 ml) were incubated for 60 min at 25° in a 80 mM HEPES buffer, pH 7.5. Nonspecific binding was determined in the presence of 1 μM nonlabeled 5-HT-moduline and represented ~10% of the total binding at 4 nM [^{3}H]5-HT-moduline. *Inset*, association kinetic of [^{3}H]5-HT-moduline (0.5 nM) at 25° on CHO-transfected cells in the binding conditions described above. *Points*, mean ± standard error of triplicate determinations. Data represent typical experiments with corresponding Scatchard plot.

Functional interaction with 5-HT$_{1B}$ receptors. The cellular effect of 5-HT-moduline was tested *in vitro* by measuring its ability to interact with the effect of a 5-HT$_{1B}$ agonist (CGS 12066B) on the K^+-evoked release of [^{3}H]5-HT from rat brain synaptosomal preparation. CGS 12066B, at a concentration of 50 μM, promoted a 60 ± 8% inhibition of the K^+-evoked release of [^{3}H]5-HT. 5-HT-moduline totally reversed this effect, with an IC_{50} value of 10 nM, in a dose-dependent manner (Fig. 9).

Behavioral studies. The effect of the peptide was measured in *in vivo* experiments using the social interaction test. Isolated mice presented a behavioral deficit because the number of escape attempts was reduced by 55% (27 ± 2 escape attempts for grouped mice versus 15 ± 1.8 for isolated mice). This effect was totally reversed by the administration of a 5-HT$_{1B}$ agonist (RU 24969) (26.5 ± 2.8 escape attempts). 5-HT-moduline totally suppressed the effect of RU 24969, whereas it had no significant effect of its own (14 ± 1 escape attempts). Furthermore, a scrambled peptide (ALLS) was tested under the same experimental conditions to assess the specificity of the LSAL interaction. ALLS did not show any significant effect on either isolated mice or isolated mice treated with RU 24969 (Fig. 10).

Discussion

In the current study, the interaction of 5-HT-moduline with 5-HT$_{1B}$ receptor binding was studied using agonist ([^{3}H]5-HT) and antagonist (^{125}I-cyanopindolol) radioligands. Analysis of the saturation curves and their Scatchard plots suggested that the interaction did not correspond to a competitive but more likely to a complex and noncompetitive mechanism. These results strongly suggest that the interaction of 5-HT-moduline on 5-HT$_{1B/1D}$ receptor likely involves a binding site distinct from that binding 5-HT.

To characterize the potential site able to bind the peptide, a radiolabeled 5-HT-moduline was prepared. The binding of the radioligand was studied on rat and guinea pig brain membrane preparations and on cells transfected with either 5-HT$_{1B}$ or 5-HT$_{1D\beta}$ receptor genes. The results showed that [^{3}H]5-HT-moduline bound to a single population of sites with a high affinity constant (K_d = ~10^{-10} M for the different

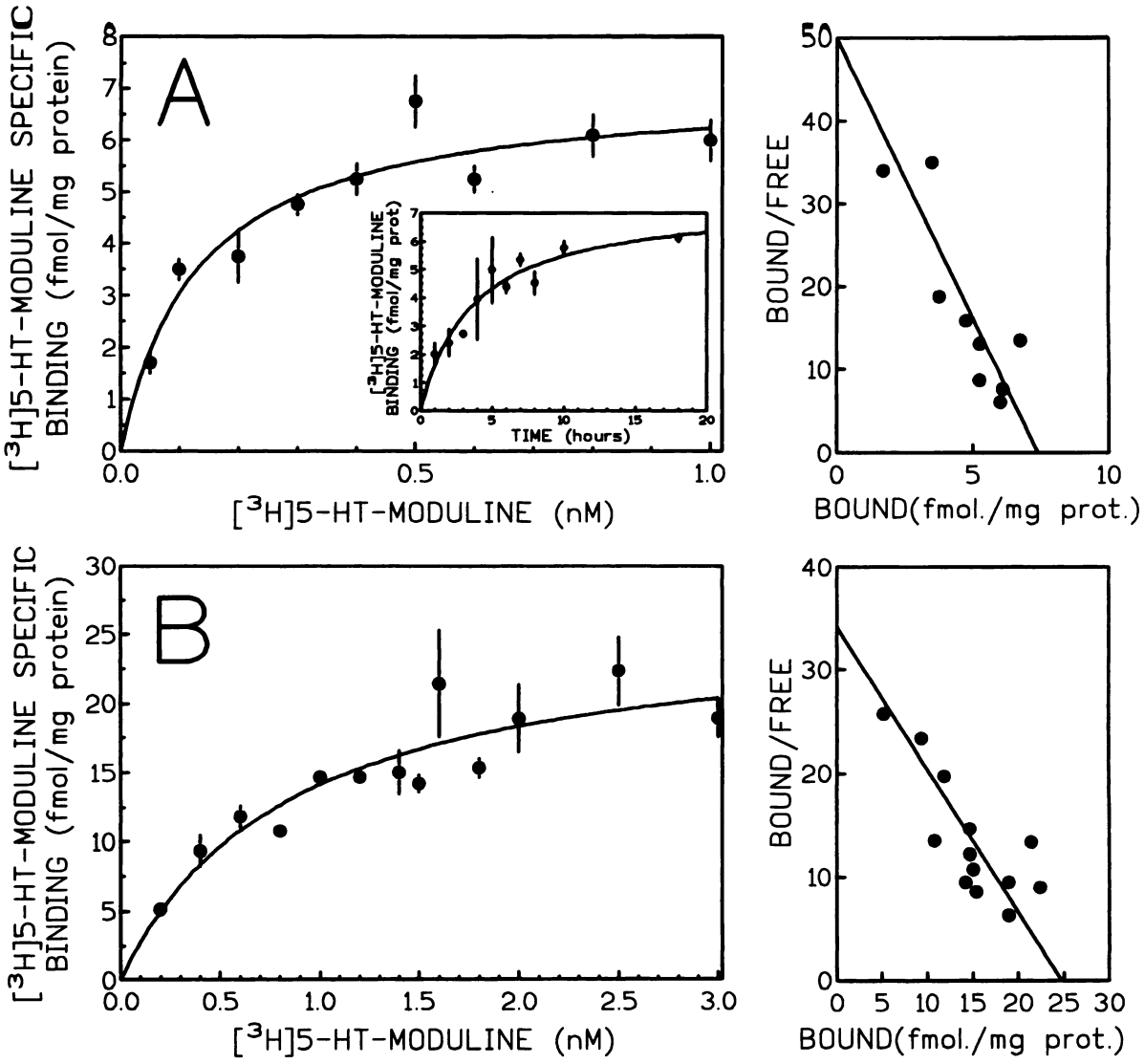

Fig. 5. [^{3}H]5-HT-moduline binding in brain membranes. Rat (A) or guinea pig (B) cortical membranes (250 μg of protein in a total volume of 1 ml) were incubated overnight at 4° in the presence of increasing concentrations (0.1–3 nM) of [^{3}H]5-HT-moduline. Nonspecific binding was determined in the presence of 1 μM nonlabeled 5-HT-moduline and represented ~40%. *Points*, mean ± standard error of triplicate determinations. Data represent typical experiments with corresponding Scatchard plot. *Inset*, association kinetic of [^{3}H]5-HT-moduline (1 nM) on rat brain membranes at 4°.

tissues). The similarity of the affinity constants observed on the various preparations suggests that the site binding 5-HT-moduline is likely the same in these tissues. Furthermore, the fact that it is also found in transfected cells expressing 5-HT$_{1B}$ or 5-HT$_{1D\beta}$ receptors, whereas the binding is absent in the wild-type cells, strongly suggests that the site binding [^{3}H]5-HT-moduline is actually located on the 5-HT$_{1B}$ and 5-HT$_{1D\beta}$ receptor proteins and is distinct but closely related to the site binding [^{3}H]5-HT. However, the number of 5-HT-moduline binding sites determined in a first series of experiments was lower than that of 5-HT$_{1B/1D}$ receptors. This difference was not observed using other incubation procedures involving a different incubation medium (HEPES buffer), duration, and temperature. Under these conditions, CHO-transfected cells (Fig. 4), NIH 3T3-transfected cells, and rat or guinea pig brain membranes (curves not shown) bound the 5-HT-moduline with B_{max} values of 1400 ± 30, 80 ± 10, 65 ± 8, and 168 ± 32 fmol/mg protein, respectively (three measurements), which is similar to values observed for [^{3}H]5-HT (1140 ± 30, 70 ± 8, 91 ± 10, and 131 ± 15, respectively; four measurements). The similarity of the B_{max} values for [^{3}H]5-HT and [^{3}H]5-HT-moduline in these different preparations are in agreement with the hypothesis that 5-HT$_{1B/D}$ receptors actually contain the site binding the peptide. The fact that another incubation procedure led to a lower B_{max} value for 5-HT-moduline indicates that the recognition site for the peptide is highly sensitive to the physicochemical environment. This observation suggests that the binding of 5-HT-moduline to the 5-HT$_{1B/1D}$ receptor, known as an allosteric protein able to exist under various conformational states like other G protein-related receptors (14), depends on the capacity of the receptor to be present in the incubation medium under a state favoring its recognition. In other words, 5-HT-moduline seems to select a conformational state of the receptor that is favored under particular *in vitro* experimental conditions. Thus, these results are in agree-

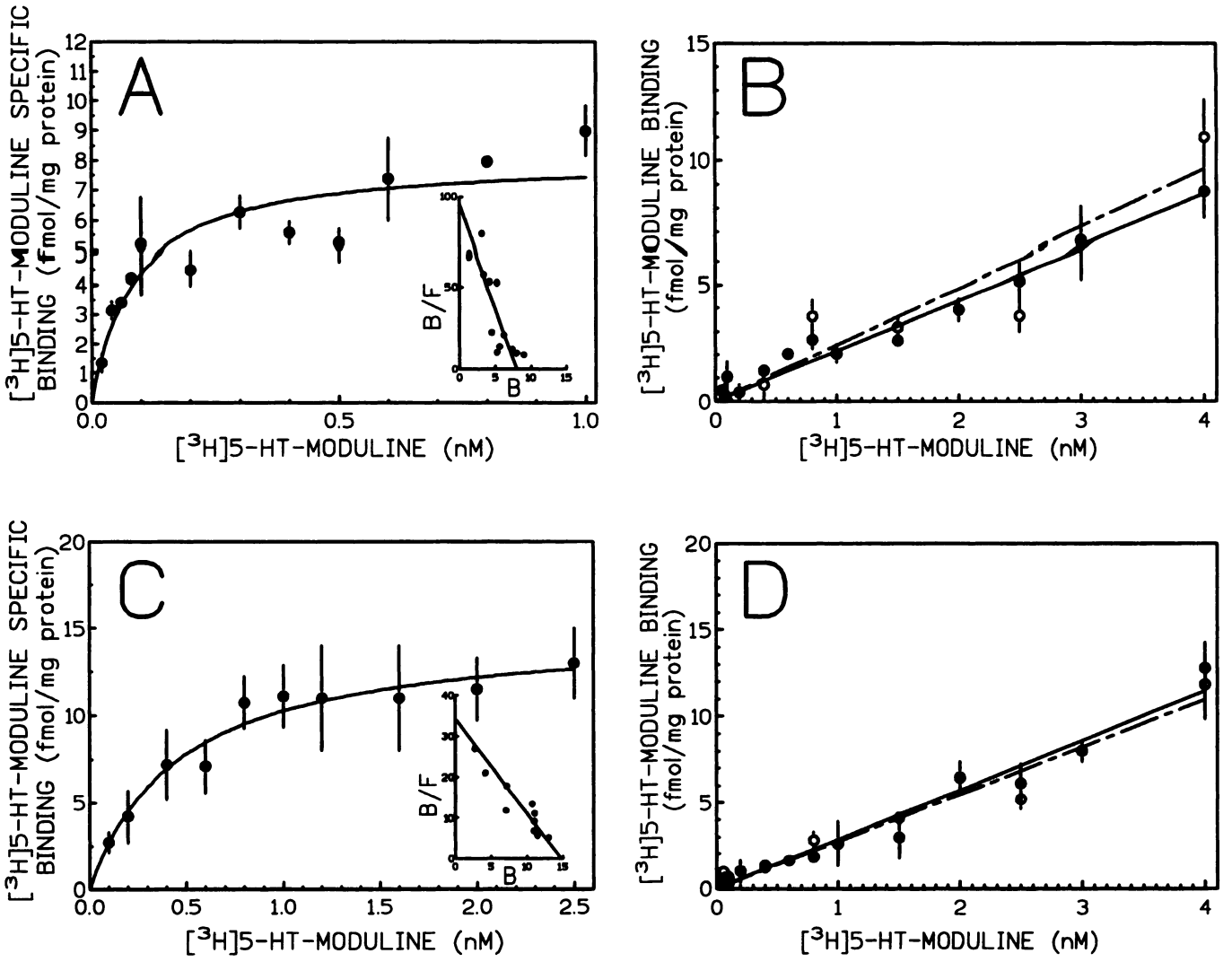

Fig. 6. [^{3}H]5-HT-moduline binding in NIH 3T3 or CHO cell membranes. NIH 3T3- (A) or CHO- (C) transfected cell (expressing 5-HT_{1B} or 5-$HT_{1D\beta}$ receptors, respectively) membranes (250 μg of protein in a total volume of 1 ml) were incubated overnight at 4° in the presence of increasing concentrations (0.1–2.5 nM) of [^{3}H]5-HT-moduline. Nonspecific binding was determined in the presence of 1 μM nonlabeled 5-HT-moduline and represented ~30% of the total binding. Data represent typical experiments. *Insets*, corresponding Scatchard plot. *Points*, mean ± standard error of triplicate determinations. Control experiments with wild-type NIH 3T3- (B) or CHO-transfected cells expressing the 5-ht_6 receptor (D) were carried out. Membranes (250 μg of protein in a total volume of 1 ml) were incubated overnight at 4° in the presence of increasing concentrations (0.1–4 nM) of [^{3}H]5-HT-moduline in the absence (●) or presence (○) of 1 μM nonlabeled 5-HT-moduline. *Points*, mean ± standard error of triplicate determinations.

ment with the hypothesis that 5-HT-moduline acts as an allosteric modulator of the 5-$HT_{1B/1D}$ receptors.

The fact that antibodies raised against a peptide sequence of the third intracellular loop of the 5-HT_{1B} receptor (13) did not block the binding of [^{3}H]5-HT-moduline (data not shown) suggested that the site that recognizes the peptide is not located on this part of the receptor. This is in agreement with the hypothesis presented by Tucek and Proska (14) suggesting that in muscarinic receptor, also a G protein-related receptor, an allosteric site is located in the extracellular part of the receptor.

Various derivatives of 5-HT-moduline were tested for their ability to displace the radiolabeled peptide in guinea pig brain membranes. LSAL was the most efficient chemical structure. Indeed, all chemical modifications in the structure of the peptide (i.e., amidation or acetylation of the extremities, scrambled or partial sequences) led to a decrease in affinity. These results demonstrate that the entire sequence of LSAL is crucial for recognition of this molecule by the receptor protein because a single change in the structure of the molecule considerably reduces its ability to interact with its specific binding site. The fact that 5-HT itself or other peptides, even at a high concentration, did not affect the binding of 5-HT-moduline confirms the strong specificity of this interaction.

A preliminary autoradiographic study revealed that [^{3}H]5-HT-moduline binding sites were heterogeneously distributed in rat brain. Additional studies should be carried out to precisely determine the distribution of 5-HT-moduline binding sites in comparison with that of 5-$HT_{1B/1D}$ (15).

The release of 5-HT-moduline was studied in crude synaptosomal preparations from rat brain. It was shown that 5-HT-moduline was released by a Ca^{2+}-dependent K^+ stimulation, suggesting that 5-HT-moduline is stored in an excit-

TABLE 1

Pharmacological characteristics of [^{3}H]5-HT-moduline binding

[^{3}H]5-HT-moduline (1.5 nM) was incubated overnight at 4° in the presence of increasing concentrations (0.1 pM to 10 μM) of nonlabeled 5-HT-moduline or other compounds (i.e., modified 5-HT-moduline, scrambled peptides, dipeptide and tripeptide derivatives, constitutive amino acids, nonrelated peptides, and 5-HT). Nonspecific binding was determined in the presence of 10 μM 5-HT-moduline and represented ~40% of the total binding. Specific binding was typically 1100 ± 50 dpm at 1.5 nM [^{3}H]5-HT-moduline. Each point of the curves was determined in triplicate, and the presented IC_{50} were the mean ± standard error of three independent experiments.

Compound	IC_{50}
	nM
LSAL	3 ± 1
LSAL-NH_2	300 ± 10
Ac-LSAL	1,000 ± 20
LASL	1,000 ± 18
ALLS	1,000 ± 30
LSA	100 ± 20
ALS	1,000 ± 50
SAL	>1,000
LS	300 ± 10
AL	1,000 ± 50
A	>100,000
L	>100,000
S	>100,000
Leu-enkephalin	>1,000
Met-enkephalin	>1,000
Substance P	>1,000
Thyrotropin-releasing hormone	>1,000
5-HT	>100,000

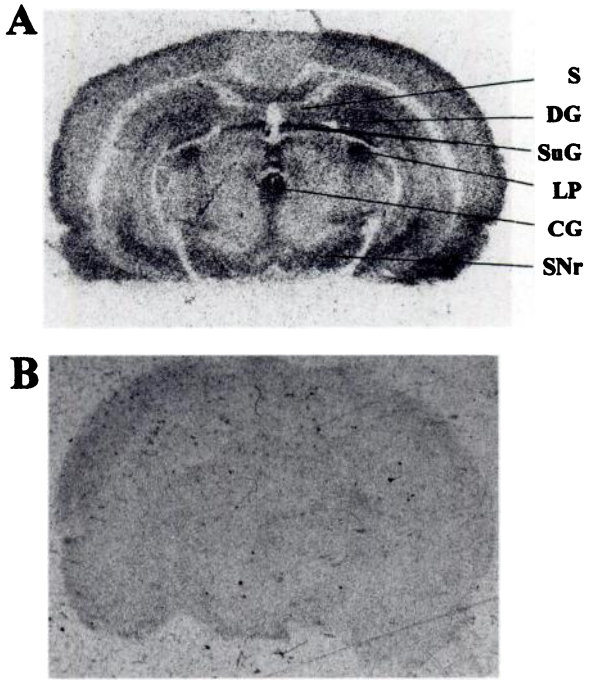

Fig. 7. Autoradiographic localization of [^{3}H]5-HT-moduline binding sites. Autoradiogram of coronal sections (20 μg) labeled with [^{3}H]5-HT-moduline (15 hr at 4°) in the absence (A) or presence (B) of 1 μM nonlabeled 5-HT-moduline. *S*, dorsal subiculum; *DG*, dentate gyrus; *SuG*, superficial gray layer of the superior colliculus; *LP*, lateral posterior nucleus thalamus; *CG*, central gray; *SNr*, substantia nigra, reticular part.

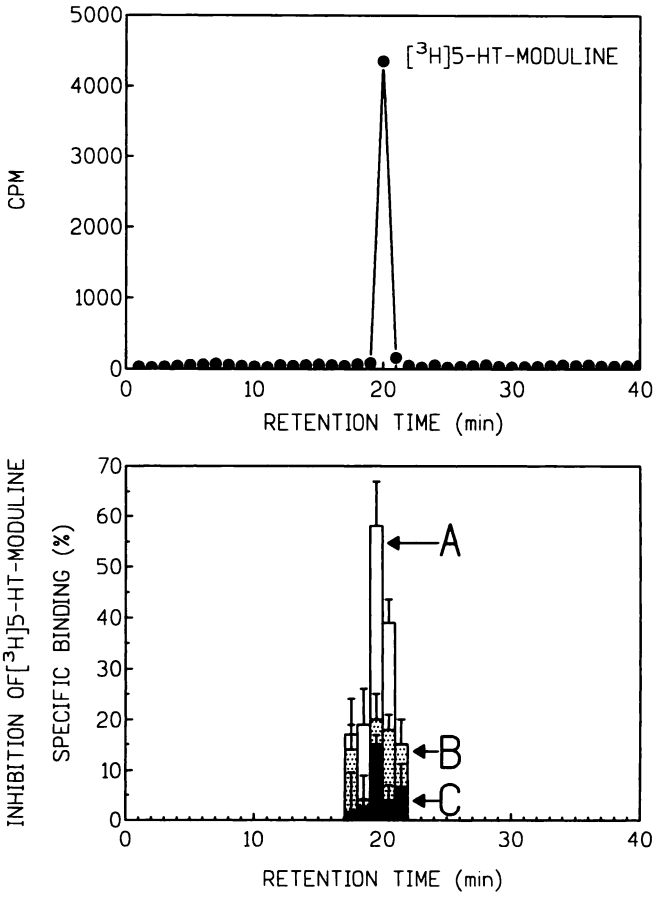

Fig. 8. Brain release of 5-HT-moduline. Synaptosomes were submitted to a 4-min K^+ stimulation (30 mM) in the presence (A) or absence (B) of $CaCl_2$ (1.22 mM). A control stimulation (without K^+ but with $CaCl_2$) was also carried out (C). The tubes were centrifuged, and the supernatants were lyophilized. Each extract was separated by reverse-phase chromatography (C_{18} Ultrabase column) as described in Experimental Procedures, and peptide contents were measured by radioreceptor assay. *Top*, elution pattern of [^{3}H]5-HT-moduline under the same chromatographic conditions. *Bottom*, inhibitory activities of chromatographic fractions (18 to 22) for A, B, and C extracts. *Bars*, mean ± standard error of three independent experiments conducted in quadruplicate.

able tissue. Furthermore, it supports the hypothesis of the endogenous origin of the peptide.

Additional studies were carried out to examine whether the binding of 5-HT-moduline to 5-$HT_{1B/1D}$ receptors had any functional consequences. 5-HT-moduline exerted an antagonistic effect on the 5-HT_{1B} receptor function, decreasing the maximal inhibitory response of the 5-HT_{1B} agonist (CGS 12066B). The shift of the apparent affinity between the IC_{50} value of 5-HT-moduline to inhibit the binding of [^{3}H]5-HT (IC_{50} = 0.1 nM) and that observed on the cellular effect (IC_{50} = 10 nM) may be due to partial degradation of 5-HT-moduline under the experimental conditions used for the release experiments (37°, Krebs-Ringer buffer, absence of antiprotease treatment).

Finally, the effect of the peptide was measured in *in vivo* experiments using the social interaction test (12). Mice were isolated for 8 days; then, when placed in the presence of a normal animal (mouse kept in a group), they showed a behavioral deficit that was totally reversed by the administration of a 5-HT_{1B} agonist (RU 24969). 5-HT-moduline totally suppressed the effect of the 5-HT_{1B} agonist, whereas it had no significant effect of its own. The specificity of this effect was demonstrated by the fact that a scrambled peptide (ALLS) had no effect. This result further demonstrates that

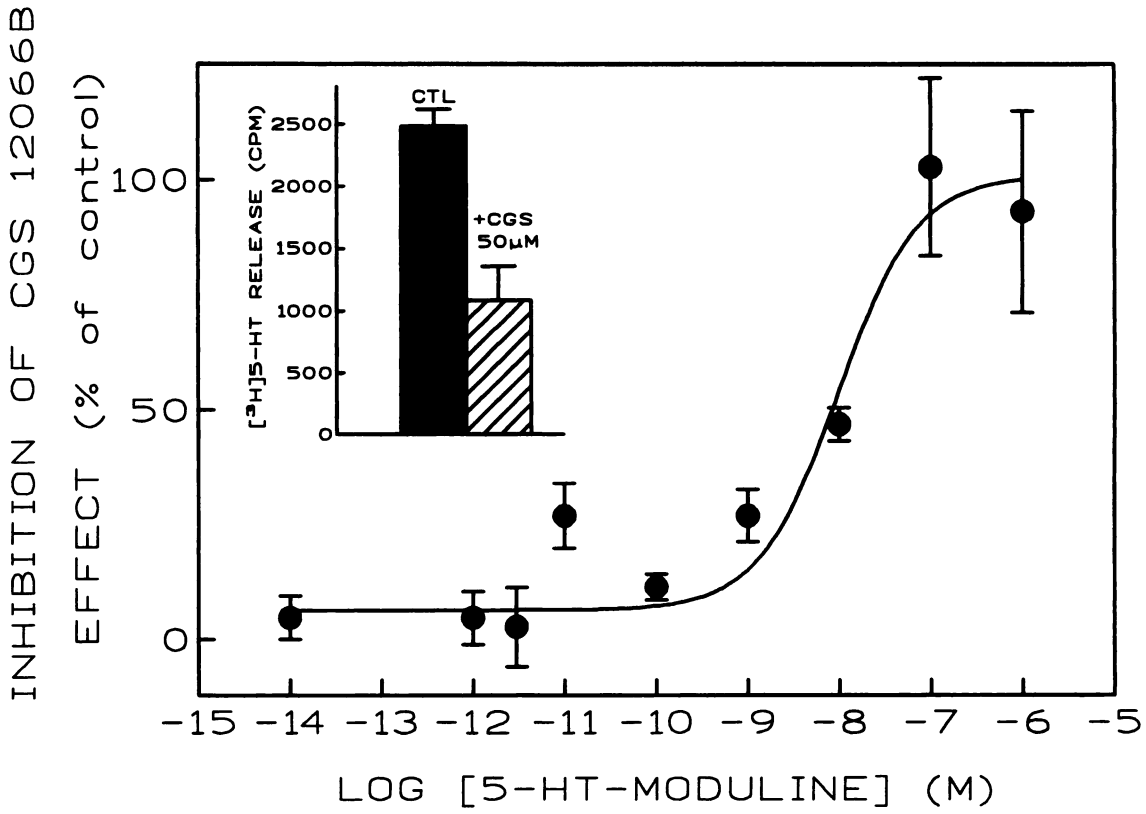

Fig. 9. Effect of 5-HT-moduline on the synaptosomal release of 5-HT. Hippocampal synaptosomes, loaded with 30 nM [^{3}H]5-HT, were incubated for 3 min in the presence of increasing concentrations of 5-HT-moduline (10 fM to 1 μM), CGS 12066B (50 μM), or both. At the end of the incubation period, a 5-min K^+ stimulation (15 mM) was applied, and the released radioactivity was measured by liquid scintillation counting. *Inset*, inhibitory effect of CGS 12066B (*CGS*) (50 μM) on the [^{3}H]5-HT release. *CTL*, control.

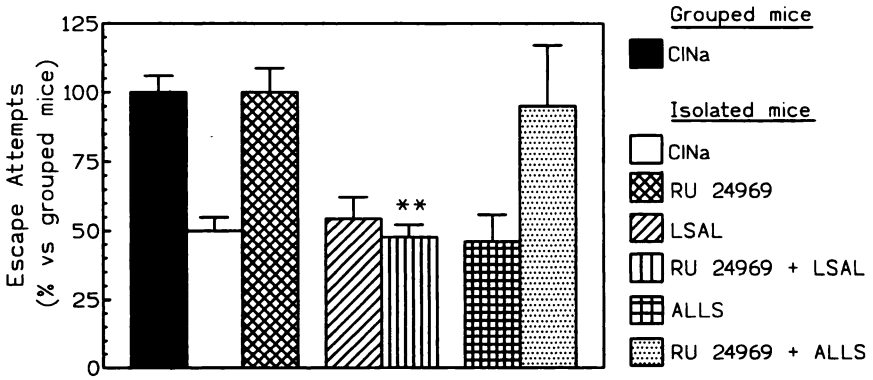

Fig. 10. Behavioral effect of 5-HT-moduline. Mice were tested in the social interaction test as described in Experimental Procedures. RU 24969 (4 mg/kg) was injected intraperitoneally at 30 min before the test, whereas 5-HT-moduline (LSAL) and ALLS were administered intracerebroventricularly at a dose of 50 μg at 45 min before the test. ClNa was injected for controls. Results are expressed as mean ± standard error of escape attempts per mouse. This experiment was repeated three times. Five mice were tested in each group. **, $p < 0.01$.

5-HT-moduline can exert an antagonistic effect on 5-HT_{1B} receptor activity.

Thus, the facts that 5-HT-moduline seems to bind to 5-$HT_{1B/1D}$ receptors in a noncompetitive manner and in parallel reduces the functional activity of the receptor when stimulated by a specific agonist strongly suggest that the interaction of 5-HT-moduline on the receptor corresponds to an allosteric mechanism inducing conformational changes of the protein. The allosteric nature of the G protein-related receptors has been proposed for several of the receptors on the basis of experimental results (14, 16–19). 5-$HT_{1B/1D}$ receptors presumably also belong to that large multigenic family of allosteric proteins. Therefore, it is proposed that peptides, analogous to 5-HT-moduline, may exist and play the role of specific allosteric modulators for particular members of this receptor family. This hypothesis may provide new directions in the field of fundamental neurobiology.

In conclusion, our results demonstrate the existence of an endogenous tetrapeptide, LSAL, that is releasable and able to specifically interact with 5-$HT_{1B/1D}$ receptors. The mechanism involved in the interaction presumably corresponds to an allosteric modulation ultimately leading to the desensitization of the receptor. The detailed mechanisms of the binding of the peptide and its action still need clarification.

Although several endogenous substances have been proposed to interact with the 5-HT system (20), none of them had the characteristics of 5-HT-moduline. Our results may facilitate understanding of the mechanisms involved in the regulation of the serotonergic activity. Because the response of the central nervous system to various stimuli is thought to

be controlled, at least in part, by the serotonergic system, 5-HT-moduline, which regulates the serotonergic activity, may play a crucial role in the adaptive mechanisms of that control. Therefore, 5-HT-moduline binding site may also constitute a new target for drugs potentially involved in all physiopathological events depending on the 5-HT activity, particularly in the domain of psychiatric diseases.

Acknowledgments

We thank Jean-Charles Schwartz for the generous gift of 5-ht_6 CHO-transfected cells.

References

1. Siever, L. J., R. S. Kahn, B. A. Lawlor, T. L. Lawrence, and E. F. Coccaro II. Critical issues in defining Trestman: the role of serotonin in psychiatric disorders. *Pharmacol. Rev.* **43:**509–525 (1991).
2. Baumgarten,. H. G., and Z. Grozdanovic. Neuroanatomy and neurophysiology of central serotonergic systems. *J. Serotonin Res.* **3:**171–179 (1994).
3. Hoyer, D., D. E. Clarke, J. R. Fozard, P. Hartig, G. R. Martin, E. J. Mylecharane, P. R. Saxena, and P. P. A. Humphrey. VII International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (serotonin). *Pharmacol. Rev.* **46:**157–203 (1994).
4. Oksenberg, D., S. A. Marsters, B. F. O'Dowd, H. Jin, S. Havlik, S. J. Peroutka, and A. Ashkenazi. A single amino-acid difference confers a major pharmacological variation between human and rodent 5-HT_{1B} receptors. *Nature (Lond.)* **360:**161–163 (1992).
5. Chopin, P., C. Moret, and M. Briley. Neuropharmacology of 5-hydroxytryptamine$_{1B/D}$ receptor ligands. *Pharmacol. Ther.* **62:**385–405 (1994).
6. Fillion, G., and M.-P. Fillion. Modulation of affinity of postsynaptic serotonin receptors by antidepressant drugs. *Nature (Lond.)* **292:**349–351 (1981).
7. Rousselle, J. C., O. Massot, M. P. Fillion, M. Delepierre, E. Zifa, B. Rousseau, and G. Fillion. Isolation and characterization of an endogenous peptide from rat brain interacting specifically with the 5-HT1B receptors. *J. Biol. Chem.* **271:**726–735 (1996).
8. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275 (1951).
9. Cotman, C. W., and D. A. Matthews. Synaptic plasma membranes from rat brain synaptosomes: isolation and partial characterization. *Biochim. Biophys. Acta* **249:**380–394 (1971).
10. Palacios, J. M., G. Mengod, and D. Hoyer. Brain serotonin receptor subtypes: radioligand binding assays, second messengers, ligand autoradiography and in situ hybridization histochemistry. *Methods Neurosci.* **12:** 238–262 (1993).
11. Roth, B. L., S. C. Craigo, M. S. Choudhary, A. Uluer, F. J. Monsma Jr., Y. Shen, Y. Meltzer, and D. R. Sibley. Binding of typical and atypical antipsychotic agents to 5-hydroxytryptamine-6 and 5-hydroxytryptamine-7 receptors. *J. Pharmacol. Exp. Ther.* **268:**1403–1410 (1993).
12. Francès, H. New animal model of social behavioural deficit: reversal by drugs. *Pharmacol. Biochem. Behav.* **31:**467–470 (1988).
13. Grimaldi, B., I. Cloëz-Tayarani, M.-P. Fillion, J.-C. Mazié, R. Hen, and G. Fillion. Production and characterization of an antibody directed against the mouse 5-HT1B receptor. *Neurosci. Res.* **24:**97–101 (1995).
14. Tucek, S., and J. Proska. Allosteric modulation of muscarinic acetylcholine receptors. *Trends Pharmacol. Sci.* **16:**205–212 (1995).
15. Pazos, A., and J. M. Palacios. Quantitative autoradiographic mapping of serotonin receptors in the rat brain. I. Serotonin-1 receptors. *Brain Res.* **346:**205–230 (1985).
16. Kent, R. S., A. De Lean, and R. J. Lefkowitz. A quantitative analysis of β-adrenergic receptor interactions: resolution of high and low affinity states of the receptor by computer modeling of ligand binding data. *Mol. Pharmacol.* **17:**14–23 (1980).
17. Stiles, G. L., M. G. Caron, and R. J. Lefkowitz. *beta*-Adrenergic receptors: biochemical mechanisms of physiological regulation. *Physiol. Rev.* **64:**661–743 (1984).
18. Stockton, J. M., N. J. M. Birsdall, A. S. V. Burgen, and E. C. Hulme. Modification of the binding properties of muscarinic receptors by gallamine. *Mol. Pharmacol.* **23:**551–557 (1983).
19. Ehlert, F. J. Estimation of the affinities of allosteric ligands using radiolabeled binding and pharmacological null methods. *Mol. Pharmacol.* **33:** 187–194 (1988).
20. Barbaccia, M. L., E. Costa, and A. Guidotti. Endogenous ligands for high-affinity recognition sites of psychotropic drugs. *Annu. Rev. Pharmacol. Toxicol.* **28:**451–476 (1988).

Send reprint requests to: Dr. Gilles Fillion, Unité de Pharmacologie Neuro-Immuno-Endocrinienne, Institut Pasteur, 25–28, rue du Dr. Roux, 75724 Paris Cedex 15, France.