Isolation and Characterization of an Endogenous Peptide from Rat Brain Interacting Specifically with the Serotonergic 1B Receptor Subtypes*

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The existence of endogenous compounds interacting with the serotonergic system was previously postulated. In the present work, rat brain tissues were extracted by acidic and organic procedures. The resulting extract was tested for its capacity to interact with the binding of [3H]5-HT, serotonin (5-HT), to 5-HT1 receptors. Compounds responsible for the observed inhibitory activities were isolated and purified by high pressure liquid chromatography.

A tetrapeptide corresponding to a novel amino acid sequence Leu-Ser-Ala-Leu (LSAL) was identified. It reduces the binding of [3H]5-HT to 5-HT1 receptors at low concentration (IC50 = 10−10 M). This effect corresponds to a specific interaction at 5-HT1B receptors since LSAL does not significantly affect other neurotransmitter bindings. LSAL appears heterogeneously distributed throughout the brain (hippocampus > cerebellum > striatum > brain stem) and in peripheral tissues (kidney > lung > stomach > blood > liver > spleen).

Two other peptides, Leu-Ser (LS) and Ala-Leu (AL), were also purified. They hardly affected [3H]5-HT binding compared with LSAL. They presumably represent degradation products of the functional peptide LSAL. The fact that LSAL interacts specifically with 5-HT1B receptors that inhibit the release of neurotransmitters and particularly that of 5-HT itself suggests that this peptide may be involved in mechanisms controlling 5-HT neurotransmission and, accordingly, may play an important role in pathophysiological functions related to 5-HT activity.

The serotonergic system is thought to play an important role in mental disorders and particularly in depression (1). For a long time, it had been proposed that this pathology was related to a deficit in the serotonergic transmission (2, 3). Accordingly, antidepressant drugs essentially restore a normal level of 5-HT activity. Antidepressant drugs can be classified into groups according to their primary mode of action, i.e. monoamine oxidase inhibitors, tricyclic antidepressants, and selective serotonin reuptake inhibitors (4).

Furthermore, it was also shown that antidepressant drugs could act on 5-HT1 receptors (5–7). The corresponding mechanism of interaction was shown to be noncompetitive suggesting that a site, distinct from that actually binding the amine, existed on these receptors and specifically recognized these drugs and possibly endogenous ligands (5).

Among 5-HT1 receptors, 5-HT1B receptors, located on rat serotoninergic neurons terminals, play a crucial role in regulating the release of the amine (8). In non-rodents, 5-HT1D receptors, which are the species homolog of rodent 5-HT1B, play the same functional role (9, 10). Experiments carried out in rat in vitro assays showed that several antidepressants specifically interacted with 5-HT1B receptor subtypes (11–13) modifying their sensitivity after long term treatment (14–18).

According to these results, the hypothesis of the existence of an endogenous factor acting at 5-HT1 receptors was postulated. Thus, we explored this hypothesis in examining the capacity of various fractions, isolated from brain extracts, to interact with 5-HT1 binding sites.

Herein, we report the isolation and characterization of a cerebral compound, which specifically interacts with 5-HT1B binding sites.

EXPERIMENTAL PROCEDURES

Materials—Male Wistar rats (150–200 g) were obtained from IFFA CREDO (France). Bovine brains were collected at the slaughterhouse. [3H]Bz-OD-PAT (3.7 TBq/mmol), [3H]ketanserin (2.22 TBq/mmold), [3H]BRL 43694 (1.85 TBq/mmold), [3H]DOB (0.37 TBq/mmold), and [3H]chloride (3 TBq/mmold) were purchased from DuPont NEN. [3H]5-HT (3.26 TBq/mmold), 125-I-γ-aminoglutidol (8.4 TBq/mmold), [3H]lspironoridol (0.55 TBq/mmold), [3H]quinidindinyl benzylate (3.33 TBq/mmold), [3H]haloxone (1.48 TBq/mmold), [3H]leprepine (0.74 TBq/mmold), [3H]proazin (2.59 TBq/mmold), [3H]hydroalprenolol (3.33 TBq/mmold), [3H]lunotrazepam (2.22 TBq/mmold), [3H]aminobutyric acid (3.33 TBq/mmold), [3H]dopamine (185 GBq/mmold), and [3H]noradrenaline (444 GBq/mmold) came from Amersham Corp. [3H]LSAL (4.14 TBq/mmold) was synthesized by the Service des Molécules Marquées, CEA-CEN.

The TSK HW 40S column was obtained from Merck, and the Sephadex G25 resin was from Pharmacia Biotech Inc. The C8 ultrabase and Hypercarb column were purchased from SFCC-Shandon. Synthetic peptides came from Bachem for AL and LS and Neosystem for LSAL.

Rat Brain Extracts—Assays were carried out using brain tissue usually prepared from 60 rats (about 90 g). The tissues were lyophilized and homogenized with an Ultraturrax apparatus (Ika Werk) in 10 volumes (v/w) of H2O containing 2 mM EDTA, 5 μl/liter aprotinin, and 0.1 μM phenylmethylsulfonyl fluoride. The homogenate was centrifuged; NR, not retained.

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1 The abbreviations used are: 5-HT, 5-hydroxytryptamine or serotonin; 5-CT, 5-carboxytryptamine; Bz-OD-PAT, 8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino)tetralin; DOB, (±)-1-(4-bromo-2,5-dimethoxyphenyl)-2-aminopropane; BRL 43694, endo-N-(9-methyl-9-azatricyclo[3.3.1]non-3-yl)-1-methyl-1H-indazole-3-carboxyamide; IC50, inhibition time; LSAL, Leu-Ser-Ala-Leu; LS, Leu-Ser; AL, Ala-Leu; HPLC, high pressure liquid chromatography; NR, not retained.
### Table 1: Binding experimental procedures

Table 1 summarizes the different binding conditions used in the pharmacological studies. Binding experiments were carried out in quadruplicate for 30 min at 25°C, except for the [125I]iodocyanopindolol binding, which was performed for 60 min at 25°C (20–32). Positive controls for each receptor are expressed in specific counts per minute. For all tested receptors, specific binding was never less than 40% of the total binding. 

Preparation of rat brain cortical membranes is described under “Experimental Procedures.”

| Receptors | Specific radioligands | Nonspecific binding | Incubation buffers | Specific binding |
|-----------|----------------------|---------------------|--------------------|-----------------|
| S-HT<sub>1A</sub> | [3H]8-OH-DPAT (1 nM) | S-HT | (10 μM) Tris-HCl, pH 7.4, 4 mM CaCl<sub>2</sub>, 0.1% ascorbic acid, 10 μM pargyline | 1300 ± 85 |
| S-HT<sub>1B</sub> | [3H]5-HT | 5-HT | (10 μM) Tris-HCl, pH 7.7, 157 mM NaCl, 20 μM pargyline | 1463 ± 81 |
| 5-HT<sub>1B/D</sub> | [3H]5-HT | 5-HT | (20 μM) Tris-HCl, pH 7.4, 4 mM CaCl<sub>2</sub>, 0.1% ascorbic acid, 10 μM pargyline | 1372 ± 61 |
| 5-HT<sub>1E/F</sub> | [3H]5-HT | 5-HT | (10 μM) Tris-HCl, pH 7.4, 4 mM CaCl<sub>2</sub>, 0.1% ascorbic acid, 10 μM pargyline | 659 ± 29 |
| 5-HT<sub>2A</sub> | [3H]Ketanserin (1 nM) | Methysergide | (10 μM) Tris-HCl, pH 7.4, 120 mM NaCl | 3683 ± 89 |
| 5-HT<sub>3</sub> | [3H]BRL 43694 (1 μM) | S-HT | (10 μM) Tris-HCl, pH 7.4, 5 mM CaCl<sub>2</sub>, 4 μM pargyline | 773 ± 20 |
| Muscarinic cholinergic benzylate | [3H]Acetylcholine (3 nM) | Tripropidide | (10 μM) Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM MgCl<sub>2</sub> | 15631 ± 134 |
| Histaminergic H<sub>1</sub> | [3H]Histamine | Naloxone | (10 μM) Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM MgCl<sub>2</sub> | 519 ± 36 |
| Opiate | [3H]Naloxone | Opiate | (10 μM) Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM MgCl<sub>2</sub> | 1613 ± 81 |
| Dopaminergic D<sub>2</sub> | [3H]Dopamine | (+)-Butaclamol | (10 μM) Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM MgCl<sub>2</sub> | 1363 ± 120 |
| α<sub>1</sub>-Adrenergic | [3H]Phentolamine (1 μM) | Propranolol | (10 μM) Tris-HCl, pH 7.4, 2.5 mM CaCl<sub>2</sub>, 120 mM NaCl, 5 mM KCl, 1 mM MgSO<sub>4</sub>, 50 mM KCl | 1403 ± 71 |
| β<sub>2</sub>-Adrenergic | [3H]Methylphenidate (3 nM) | Diazepam | (10 μM) Tris-HCl, pH 7.4, 4 mM CaCl<sub>2</sub>, 10 μM pargyline | 1090 ± 85 |
| Benzodiazeppine | [3H]Flunitrazepam (3 nM) | Diazepam | (10 μM) Tris-HCl, pH 7.4, 4 mM CaCl<sub>2</sub>, 10 μM pargyline | 10293 ± 121 |

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fuged at 17,500 × g for 40 min at 4°C. The resulting supernatant was kept at 4°C, and the pellet was washed two additional times. The three supernatants were then pooled, lyophilized, and resuspended in 6 volumes (v/weight) of 1 M acetic acid. After a stirring period of 40 min at 4°C, the mixture was centrifuged (17,500 × g for 40 min at 4°C), and the supernatant was lyophilized. An additional extraction was performed in 2 volumes (v/weight) of 75% acetone. After centrifugation (17,500 × g for 20 min at 4°C), the resulting supernatant was evaporated under vacuum at 40°C in a rotovap apparatus (R110, Büchi). The dried extract was then resuspended in 50 ml of H<sub>2</sub>O and ultracentrifuged (120,000 g for 60 min at 25°C). The upper lipid phase was discarded, and the supernatant was lyophilized and stored at −70°C until use.

Localization of Biological Activity—At the completion of each following chromatographic step, an aliquot of each collected fraction (1%) was tested for its ability to displace the binding of [3H]5-HT to the S-HT<sub>1</sub> and S-HT<sub>1E/F</sub> binding sites (20). Rat brain cortical membranes were incubated in a 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% ascorbic acid, 0.1% bovine serum albumin, 4 mM CaCl<sub>2</sub>, 1 μM pargyline, 5 μM 8-OH-DPAT (with 5-HT<sub>1B/D</sub>) or without (5-HT<sub>1E/F</sub>) 0.1% 8-OH-DPAT, in the presence/absence of the various fractions separated by the chromatographic procedure. Incubation was carried out for 30 min at 25°C in a total volume of 1 ml. Nonspecific binding was determined in the presence of 10 μM of 5-HT. At the end of the incubation period, free and bound radioactivities were separated by filtration under vacuum on Whatman GF/B glass fiber filters. Each tested tube was then washed twice with 5 ml of ice-cold incubation buffer. The bound radioactivity retained on the filter was measured by liquid scintillation counting (spectrometer Beta IV, Kontron). Active fractions were then pooled and lyophilized before injection in the next chromatographic column.

Isolation of the Rat F<sub>1</sub> Fraction—The crude extract of rat brains was dissolved in 5 ml of 50 mM ammonium acetate buffer, pH 5. Aliquots (1 ml) were injected into a C<sub>18</sub> Ultrabase reverse phase column (250 × 10 mm), equilibrated in the same buffer. The elution was performed at a flow rate of 4 ml/min with a linear gradient of acetonitrile (0–12%) followed by a 5-min step gradient (50% acetonitrile). The detection wavelength was 240 nm. Thirty fractions of 1 ml were collected. For all the following chromatographic steps, absorbance was measured at 230 nm (except when indicated).

Purification of Rat P Fraction—P fraction obtained from the C<sub>18</sub> Ultrabase chromatographic step was dissolved in 2 ml of a 10 mM ammonium acetate buffer, pH 5, and loaded onto a Sephadex G-25 column (450 × 16 mm; M<sub>r</sub> separation range: 5000–5000). Elution was performed at a flow rate of 0.3 ml/min with the same buffer. The chromatogram was monitored at 280 nm, and 50 fractions of 20 ml were collected. A reverse phase HPLC column (C<sub>18</sub> Ultrabase, 250 × 10 mm) was then used. The equilibrating buffer consisted of a mixture of ammonium acetate (50 mM, pH 5) and acetonitrile (85:15). The elution was performed with a 15-min linear gradient of acetonitrile (15–25%) followed by a 5-min step gradient at 50% acetonitrile at a flow rate of 4 ml/min. 20 fractions of 1 ml were then collected. After lyophilization, the active fraction was injected into a carbon column (Hypercarb, 100 × 3 mm) and eluted with a 50 mM ammonium acetate buffer, pH 5. A 30-min linear gradient of acetonitrile (0–30%) was used. The flow rate was 1 ml/min, and 40 fractions of 1 ml were collected. Final purification step consisted of a reverse phase chromatography using a C<sub>18</sub> Ultrabase column (150 × 4 mm) under isocratic elution conditions (0.5% trifluoroacetic acid, pH 2.5, and acetonitrile (83:17)) at a flow rate of 1 ml/min. The active fraction was collected manually.

Bovine Brain Extract—The same purification procedure was carried out, i.e., acidic and organic extractions, gel permeation on TSK HW 40S, and C<sub>18</sub> Ultrabase separations. Two bovine brains (about 600 g; 35.21 ± 0.62 g of protein equivalent) were thus processed, which led to the recovery of three fractions having precisely the same retention time as rat P<sub>P</sub> and P<sub>2</sub> fractions.

Purification of Bovine P<sub>1</sub> Fraction—P<sub>1</sub> fraction was first loaded on the Sephadex G<sub>25</sub> column. The elution procedure was the same as for rat P fraction. The active fractions were collected onto a C<sub>18</sub> Ultrabase column (250 × 10 mm) equilibrated in a 50 mM ammonium acetate buffer, pH 5. All aliquots (1 ml) were injected into a C<sub>18</sub> Ultrabase reverse phase column (250 × 10 mm), equilibrated in the same buffer. The elution was performed at a flow rate of 4 ml/min with a linear gradient of acetonitrile (0–12%) followed by a 5-min step gradient (50% acetonitrile). The detection wavelength was 240 nm. Thirty fractions of 1 ml were collected. For all the following chromatographic steps, absorbance was measured at 230 nm (except when indicated).
determinations. A coinjection of [3H]5-HT was realized under the same conditions as internal standard (○—○).

buffer, pH 5. Elution was run at 4 ml/min using a linear gradient of acetonitrile (20 min, from 0 to 5%) followed by a 10-min step gradient at 30% acetonitrile. Thirty fractions of 1 min were collected. Two further separations on the same column, using isocratic elutions with a 50 mM ammonium acetate buffer, pH 5, at a flow rate of 0.5 ml/min, were required to achieve the purification of P1 fraction.

Purification of Bovine P1 Fraction—The first separation step was a gel filtration on a Sephadex G25 column. Chromatographic conditions were identical to those used for rat P and bovine P2 fractions. Then active fractions were injected in a C18 Ultrabase column (250 × 10 mm). The mobile phase was a 50 mM ammonium acetate buffer, pH 5. Elution was run at 4 ml/min, and 50 fractions of 1 min were collected. A 60-min linear gradient of acetonitrile was used (0–30%) at a flow rate of 1 ml/min. Sixty fractions of 1 min were collected. Two further separations on the same column, using isocratic elutions with a 50 mM ammonium acetate buffer, pH 5, at a flow rate of 0.5 ml/min, were required to achieve the purification of P1 fraction.

Purification of Bovine P2 Fraction—The first separation step was a gel filtration on a Sephadex G25 column. Chromatographic conditions were identical to those used for rat P and bovine P1 fractions. Then active fractions were injected in a C18 Ultrabase column (250 × 10 mm). The mobile phase was a 50 mM ammonium acetate buffer, pH 5. Elution was run at 4 ml/min, and 50 fractions of 1 min were collected. A 60-min linear gradient of acetonitrile was used (0–30%) at a flow rate of 1 ml/min. Sixty fractions of 1 min were collected. Two further separations on the same column, using isocratic elutions with a 50 mM ammonium acetate buffer, pH 5, at a flow rate of 0.5 ml/min, were required to achieve the purification of P2 fraction.

Amino Acid Analysis—Amino acid contents of bovine P1 and P2 fractions were determined after a 24-h hydrolysis in 6 N HCl at 120 °C. A model 6300 Beckman apparatus was used. Amino acid separation was performed on a C8 reverse phase column after cadmium-ninhydrin reaction. Norleucine was used as an internal standard.

NMR Analysis—The content of the various purified fractions (P, P1, P2) and their chemical structures (P, P1, P2) were determined in D2O and dimethylsulfoxide using a 500-MHz NMR spectrometer (Varian).

Protein Sequencing—The sequence of the purified rat P fraction was determined using a 473 Applied Biosystems protein sequencer using a microcartridge. Chemicals and methods were those recommended by the manufacturer.

Sequence Comparison—Purified peptide sequence was compared with those published in the Swissprot protein bank (Genetic Computer Group, Inc.).

Protein Measurement—The protein equivalents were measured by the Lowry method (19). The standard curve was established with bovine serum albumin.

Pharmacological Studies—Interaction of the purified P fraction (1% of the total preparation) or synthetic LSAL (1 nM or increasing concentrations (10−12 to 10−8 M)) was examined either at various 5-HT receptors or on other neurotransmitter receptors. Experiments were carried out on rat (or bovine when indicated) brain cortical membranes in a total volume of 1 ml.

Rat brain cortices were dissected on ice and rapidly homogenized for 30 s with an Ultraturrax apparatus (Ika-Werke) in a 50 mM Tris-HCl buffer, pH 7.4, containing 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 5 IU/liter aprotinin. Homogenates were then incubated for 10 min at 37 °C to remove endogenous ligands, diluted in 30 volumes (v/v) of the same medium, and centrifuged (17,500 g at 4 °C for 10 min). The pellet was resuspended in 30 volumes of the same buffer and centrifuged as described above. The homogenate was then washed an additional time, and the resulting pellet was resuspended in the appropriate incubation buffer. Incubation buffers and the different specific radiolabeled ligands are described in Table I according to the literature (20–32). Binding assays were performed after 30 min of incubation at 25 °C with 500 μg of protein equivalents/incubate except for the one using [3H]-cyanoindolol, which was incubated for 60 min at 37 °C in the presence of 25 μg of proteins. At the end of the incubation period, the tubes were cooled on ice for 10 min and filtered under vacuum on Whatman GF/B glass fiber filters. Each filter was then washed twice with 5 ml of ice-cold incubation buffer and dried. The radioactivity retained on the filters was then measured either by liquid scintillation counting as described previously (for tritiated radioligands) or by γ-counting (for iodinated radioligand) (Spectrometer Crystal™ multi-detector radioimmuno assay system, Packard).

The uptake of 5-HT, dopamine, noradrenaline, or γ-amino butyric acid, and choline were measured on rat cortical synaptosomes prepared according to the method of Cotman and Matthews (33). Synaptosomes were incubated for 15 min at 37 °C in an oxygenated Krebs-Ringer buffer (20–22). The concentrations of the radioligands were as follows: [3H]5-HT (5 nM), [3H]dopamine (1 nM), and [3H]noradrenaline (1 nM).

The uptake of [3H]5-HT, [3H]dopamine, or [3H]noradrenaline into rat brain synaptosomal membranes was determined at various time intervals after preincubation times of 30 min using 25 μM of 5-HT, dopamine, or noradrenaline, respectively. The uptake of these radioligands was performed under the same conditions as described above (20–22). Each point is the mean ± S.E. of three independent determinations.

A coinjection of [3H]5-HT was realized under the same conditions as internal standard (○—○).

Fig. 1. Size exclusion chromatography of rat brain extract. Rat brain extract, prepared as described under “Experimental Procedures,” was loaded on the top of a TSK HW 40S column (700 × 26 mm; M, separation range: 10,000–1,000). The elution was performed at a flow rate of 2 ml/min with a 50 mM CH3COONH4 buffer, pH 5. Absorbance was observed at 280 nm. All aliquots corresponding to 1% of each fraction were then tested for their abilities to displace [3H]5-HT (5 nM) from its 5-HT1 nonA binding sites on rat brain synaptosomal membranes (●—●). Each binding point is the mean ± S.E. of three independent determinations. A coinjection of [3H]5-HT was realized under the same conditions as internal standard (○—○).

Fig. 2. Reverse phase chromatography of rat P1 fraction. 2 ml of rat P1 fraction were injected in a C18 Ultrabase column (250 × 10 mm). The elution was performed as described under “Experimental Procedures.” 1% aliquots of each fraction were tested for their capacities to displace [3H]5-HT (5 nM) from its 5-HT1 nonA binding sites on rat brain synaptosomal membranes (●—●). Each point is the mean ± S.E. of three independent determinations. A coinjection of [3H]5-HT was realized under the same conditions as internal standard (○—○).
buffer, pH 7.4 (118 mM NaCl, 4.7 mM KCl, 1.17 mM KH₂PO₄, 1.22 mM CaCl₂, 1.25 mM MgSO₄, 25 mM NaHCO₃, and 10 mM glucose) in the presence of 20 nM of the different neurotransmitters with or without aliquots of the purified P fraction (1% of the total preparation) or increasing concentrations of the synthetic LSAL (10⁻² to 10⁻⁵ M). The final incubation volume was 250 µl. Passive uptake was measured at 4°C. Uptake reactions were stopped by the addition of 2 ml of ice-cold incubation buffer (4°C). Incubates were rapidly filtered under vacuum on Whatman GF/B glassfiber filters. Each filter was washed with 15 ml of cold incubation buffer (4°C) and dried. The radioactivity retained on the filters was then measured by liquid scintillation counting.

Stability of LSAL—3 µCi of labeled LSAL were added to the rat cerebral homogenate and extracted as described previously. A blank of extraction was also carried out in the absence of cerebral material. 10% aliquots of the radioactive extracts were then analyzed on a C₁₈ reverse phase column (C₁₈ Ultrabase, 150 x 4 mm) equilibrated with an ammonium acetate buffer (50 mM, pH 5). Elution was run at 1 ml/min with an isocratic step of 3 min followed by a linear gradient of acetonitrile (0–30% in 30 min) and a 5-min step gradient at 50% acetonitrile. Forty fractions of 1 min each were collected, and the radioactivity was measured by liquid scintillation counting.

The retention time of [³H]LSAL under the same experimental conditions was determined by a control injection.

RESULTS

Extractions—Brain extracts were prepared (from about 90 g of tissue) using the usual extraction procedure, i.e. 1 M acetic acid and 75% acetone extractions and ultracentrifugation. The crude homogenate contained an equivalent of 5.3 ± 0.2 g of proteins (mean ± S.E. of three independent determinations). At the various extraction steps, the recovery of the protein equivalent was 1.1 ± 0.1 g in the H₂O supernatant, 0.54 ± 0.03 g in the acidic extract, 0.39 ± 0.01 g in the acetonitrile extract, and 0.049 ± 0.003 g in the final ultracentrifugation supernatant. The resulting crude material obtained is essentially deproteinized and delipidated.

Isolation of the Rat F₁ Fraction—Size exclusion chromatography on a TSK HW 40S column was carried out. This chromatographic step allowed to isolate two biological active fractions able to interact with the binding of [³H]5-HT to 5-HT₁B receptors in rat brain cortical membranes. Each point is the mean ± S.E. of three independent determinations.

Fig. 4. Pattern of activity of the bovine F₁ fraction on reverse phase chromatography. 2 ml of bovine F₁ fraction were injected in a C₁₈ Ultrabase column (250 x 10 mm). The elution was performed as described under “Experimental Procedures.” 1% aliquots of each fraction were tested for their capacities to displace [³H]5-HT (5 nM) from its 5-HT₁B binding sites on rat (solid line) or bovine (dashed line) brain corticale membranes. Each point is the mean ± S.E. of three independent determinations.
was identified as endogenous 5-HT.

Isolation of Rat P, P₁, and P₂ Fractions—The separation of the F₁ fractions on a C₁₈ reverse phase column led to the recovery of an active fraction having a retention time of 21 min and called P fraction. Additional active fractions were also obtained. Their retention times were 3.30 min (NR), 9 min (P₁), and 12 min (P₂), respectively. They were distinct from endogenous 5-HT as controlled by using [³H]5-HT (tᵣ = 17.45 min) (Fig. 2).

The NR fraction was eluted in the void volume of the column. Further attempts to purify it, on reverse or normal phase, on ion exchange, or on hydrophobic columns, as well as on modified reverse phase columns, e.g. -NH₂, -OH, or -CN, did not lead to further separation. Moreover, dialysis of this fraction (membrane cut-off: 1,000) led to the loss of the activity. This result suggests that NR contains a high concentration of salts that interfere with the biological test.

P fraction represented the main activity inducing 83 ± 13% inhibition of the 5-HT₁ nonA binding (eight independent determinations). The two other fractions, P₁ and P₂, tested under the same experimental conditions, were less efficient (60 ± 5% and 30 ± 8% inhibition of the 5-HT₁ nonA binding, respectively, eight independent extracts) (Fig. 2).

Identification of Rat P Fraction—NMR technique demonstrated that the P fraction purified from rat brain extract was homogeneous and only contained a peptide (not shown). It was characterized by amino acid analysis and protein sequencing as LSAL. The amounts of LSAL determined by this analytic process were 500 and 1000 pmol for the two independent batches sequenced. The molecular weight of LSAL being 402.5, it represented a mean of 0.3 μg of the peptide. Thus, the purification index was greater than 10⁵-fold (0.3 μg out of 60 rat brains corresponding to 90 g of wet weight).

The biological activities observed for rat P₁ and P₂ fractions were too low to identify them in further purification steps. Therefore, this attempt was made using bovine brains (600 g of initial material) that were extracted as described above. The extract also contained three active fractions corresponding to the same retention times as those already observed in the rat brain extract (Fig. 4, solid line). When the binding analysis was carried out on bovine brain cortical membranes instead of rat brain membranes, the same pattern of activity was observed (Fig. 4, dashed line). Moreover, the inhibitory effects of the
three fractions were not significantly different from those measured on rat brain cortical membranes. Thus, bovine P₁ and P₂ fractions were purified using essentially the same purification procedure (Figs. 5 and 6).

Amino acid analysis of bovine P₁ and P₂ fractions showed that these compounds contained Leu, Ser, and Ala, Leu, respectively. A ratio of 0.82 (Ser:Leu) and 1.06 (Ala:Leu) between amino acids suggested that P₁ and P₂ fractions corresponded to dipeptidic structures. They were identified as peptide LS for P₁ fraction and peptide AL for P₂ fraction by two-dimensional NMR spectroscopic techniques. Spin systems were identified via through-bond connectivities (TOCSY) and sequential assignment was obtained via through-space connectivities (ROESY) showing unambiguously that the two amino acids were linked (not shown). Using norleucine as internal standard, amino acid analysis showed that P₁ and P₂ fractions represented relatively large amounts (200 μg for each of them) corresponding to a purification index of $4 \times 10^6$.

Stability of LSAL—The stability of LSAL during the different steps of the isolation procedure was tested using a labeled LSAL. Under our experimental conditions, 83 and 81% of the added radioactivities were recovered in the extraction control (blank extract) and the cerebral extract, respectively. The analysis of the content of these extracts on a reverse phase C₁₈ column showed that more than 80% of the recovered radioactivity corresponded to native LSAL ($t_{R} = 20.5$ min) (85.4 and 83% for the blank and cerebral extracts, respectively). Two other minor peaks of radioactivity ($t_{R} = 13$ min and $t_{R} = 26$ min) were detected in both extracts. They represented less than 10% of the total radioactivity. In the brain extract, an additional peak ($t_{R} = 5$ min, 4% of the total radioactivity) was observed (Fig. 7).

Pharmacological Specificity of Rat P Fraction—Aliquots of the purified P fraction (1% of the total fraction) were used to examine its pharmacological properties. This fraction specifically interacted with 5-HT₁ receptors. More precisely, it exhibited a specific interaction with the 5-HT₁B receptor subtypes. Indeed, no significant inhibition was observed on other serotonergic receptor bindings (5-HT₁A, 5-HT₁E/₁F, 5-HT₂A, 5-HT₃) as well as on other neurotransmitter receptor bindings (Table II). Moreover, at the same dose, the P fraction did not show any significant activity either on the uptake of 5-HT or that of other neurotransmitters (or their precursor), i.e., uptake of choline, γ-aminobutyric acid, noradrenaline, and dopamine (Table II).

Regional Distribution of the P Fraction in Rat Brain and Peripheral Tissues—Tissues from 10 rats were extracted and partially purified to the step corresponding to the separation of the F₁ fraction on C₁₈ reverse phase chromatography (as represented in Fig. 2). The resulting P fractions were then tested for their abilities to inhibit 5-HT₁B specific binding. A dose-response curve was established for each P fraction, and the corresponding ID₅₀ was used as a measurement of the amount of LSAL in the fraction and expressed per gram of initial tissue (Table III).

In brain, the hippocampal formation contained the highest amount of inhibitory activity, followed by cortex; intermediate levels were present in the striatum and cerebellum, whereas low levels were detected in the brain stem. The relative quantities per gram of original tissue were 14.28, 6.25, 2.22, 1.82, and 0.70 (arbitrary units), respectively (Table III). In the pe-
Endogenous Peptide Specific for 5-HT<sub>1B</sub> Receptors

**Fig. 7. Stability of LSAL.** 3 μCi of [3H]LSAL were added to the buffer of homogenization (control of extraction or blank extract) or to the rat cerebral homogenate (cerebral extract) and submitted to the extraction process as described under "Experimental Procedures." The extracts were then analyzed on a C<sub>18</sub> reverse phase column (C<sub>18</sub> Ultra-) as described previously. 40 fractions of 1 min were collected, and their radioactivity was counted in a liquid scintillation spectrometer.

**TABLE II**

| Tissues | ID<sub>50</sub> | Relative abundance |
|---------|---------------|-------------------|
| Brain   | g of initial wet weight | 1/ID<sub>50</sub> |
| Hippocampus | 0.07 ± 0.02 | 14.28 ± 4.10 |
| Cortex  | 0.16 ± 0.05 | 6.25 ± 1.95 |
| Striatum | 0.45 ± 0.07 | 2.22 ± 0.35 |
| Cerebellum | 0.55 ± 0.10 | 1.82 ± 0.33 |
| Brain stem | 1.40 ± 0.15 | 0.71 ± 0.08 |
| Periphery |                |                   |
| Heart   | 0.40 ± 0.05 | 2.50 ± 0.31 |
| Kidney  | 0.40 ± 0.03 | 2.50 ± 0.19 |
| Lung    | 0.71 ± 0.10 | 1.41 ± 0.20 |
| Stomach | 2.0 ± 0.11 | 0.50 ± 0.03 |
| Blood   | 7.1 ± 0.5 | 0.14 ± 0.01 |
| Liver   | ND<sup>a</sup> | ND<sup>a</sup> |
| Spleen  | ND<sup>a</sup> | ND<sup>a</sup> |

<sup>a</sup>ND, not determined.

**TABLE III**

Regional distribution of the P fraction in rat brain and periphery. Tissues from 10 rats were extracted and purified as described under "Experimental Procedures." After lyophilization, each P fraction was resuspended in an equal volume of 400 μl. Dose-response curves were determined with the different isolated P fractions on the binding of [3H]5-HT to its 5-HT<sub>1B</sub> binding sites. The corresponding ID<sub>50</sub> values were determined and corrected per g of original tissue wet weight. The relative abundance of P fraction in each tissue was then expressed as the inverse of the calculated ID<sub>50</sub>. Each value is the mean ± S.E. of three independent determinations. This experiment was repeated twice using different extracts.

**Discussion**

The results reported herein describe the isolation and purification of a cerebral factor able to specifically interact with the 5-HT<sub>1B</sub> receptor. The methodology developed to isolate this factor is a classical...
acid and organic procedure followed by HPLC chromatographic techniques. The initial step, including freezing and lyophilization of the brain tissue, was introduced to decrease the endogenous protease activity. The following step, which consisted of an ultracentrifugation (120,000 x g for 60 min at 25 °C) allowed us to separate and to discard endogenous lipids (upper phase). Under these experimental conditions, the resulting aqueous phase contained less than 1% of the original protein equivalent.

The initial size exclusion chromatography led to the separation of four peaks of proteins) in a 50 mM Tris-Cl buffer, pH 7.4, containing 4 mM CaCl₂, 0.1% ascorbic acid, 1 µM pargyline, 0.1 µM 8-OH-DPAT, and 0.1 µM mesulergine (Vₑ = 200 µl). Nonspecific binding was determined in the presence of 0.1 µM 5-CT. Specific binding represented about 50% of the total binding and corresponded typically to 1000 cpm. Each point is the mean ± S.E. of three independent determinations. This experiment was repeated three times. B, Interaction with 5-HT₁D receptors. [³H]5-HT (5 nM) was incubated for 30 min at 25 °C with increasing concentrations of synthetic peptides (LSAL (●—●), LS (○—○), and AL (□—□)) in the presence of bovine brain cortical membranes (100 µg of proteins) in a 50 mM Tris-Cl buffer, pH 7.4, containing 4 mM CaCl₂, 0.1% ascorbic acid, 1 µM pargyline, 0.1 µM 8-OH-DPAT (Vₑ = 200 µl). Nonspecific binding was determined in the presence of 0.1 µM 5-CT. Specific binding represented 50–60% of the total binding and corresponded typically to 1500 cpm. Each point is the mean ± S.E. of three independent determinations. This experiment was repeated twice.

The further C₁₈ reverse phase chromatography carried out to purify the F₁ fraction, which exhibited an inhibitory activity on the binding of [³H]5-HT to 5-HT₁B sites. At that early step of purification, the pharmacological profile of the fraction already exhibited a clear selectivity for 5-HT₁B receptors, since the fraction did not affect the binding of specific radioligands to other neurotransmitter receptors under study (not shown). It was also demonstrated that F₁ fraction did not correspond to endogenous 5-HT₁B as the amine had a different elution time (tₑ = 200 min) for 10 min at 37 °C. Each point is the mean ± S.E. of three independent determinations. This experiment was repeated twice.

The further C₁₈ reverse phase chromatography carried out to purify the F₁ fraction resulted in the separation of four peaks of activity. One of them (NR) was eluted in the void volume of the column and corresponded to a highly polar and dialyzable material (Mᵣ < 1,000). This fraction was analyzed using additional chromatographic systems, i.e. normal phase, hydrophobic column, and ion exchange chromatography. In all of these systems the fraction was retained on the column, suggesting that it mainly consisted of salts. Previously, similar observations were reported (34–39), which did not lead to the identification of any particular compound.

The major activity retained on the column was the P fraction
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(b<sub>R</sub> = 21 min), which inhibited 83 ± 13% of the binding of [3H]5-HT to 5-HT<sub>Tmax</sub>

The latter binding is the specific, high affinity binding of the tritiated amine in the presence of nonradioactive 8-OH-DPAT, which specifically masks the 5-HT<sub>1A</sub> receptors; 10 μM 5-CT is added to the medium to measure the nonspecific binding. Under these conditions, the observed binding essentially represents 5-HT<sub>1B</sub> receptor subtypes in rat. Two other fractions, P<sub>1</sub> and P<sub>2</sub>, having shorter retention times (t<sub>ret</sub> = 9 and 12 min, respectively), also exhibited inhibitory activities.

The P fraction was purified by gel permeation (Sephadex G<sub>25</sub>) and successive reverse phase chromatographies using different matrices (C<sub>18</sub> Ultrabase and Hypercarb columns) and various optimal mobile phases determined after numerous trials. The pharmacological profile of the P fraction was established by examining its effect on the specific binding of various ligands as described under “Experimental Procedures.” These assays were carried out in order to avoid the purification of a fraction that would nonspecifically inhibit the binding of [3H]5-HT. Interestingly enough, at all purification steps, the P fraction exhibited a clear serotonergic specificity since, at the dose that maximally inhibited the 5-HT<sub>Tmax</sub> specific binding (1% of the total purified fraction), it did not significantly interact with the binding of [3H]Imipramine, [3H]prazosin, [3H]dihydralprinol, [3H]Spiroperidol, [3H]Jouinucleidinyl benzylate, [3H]naloxone, and [3H]Lunitrazepam, which label histaminergic, α and β adrenergic, dopaminergic, muscarinic, opiate, and benzodiazepine receptors, respectively. Moreover, it did not affect the binding of [3H]ketanserin (antagonist) and [3H]DIOB (agonist) to 5-HT<sub>2</sub> receptors and that of [3H]BRL 43694 to 5-HT<sub>3</sub> receptors. The effect of the P fraction also appeared restricted to 5-HT<sub>1B</sub> receptors since the transport systems (uptake) of 5-HT itself and that of other neurotransmitters (or their precursors) were not affected (dopamine, noradrenaline, γ-aminobutyric acid, choline). These results indicate that the P fraction is clearly different from those previously reported, which efficiently inhibited the uptake of biogenic amines (34–39). Furthermore, the purified P fraction specifically interacted with a specific 5-HT<sub>1</sub>B receptor subtype, as neither 5-HT<sub>1A</sub> nor 5-HT<sub>1D</sub> receptors (IC<sub>50</sub> = 0.1 nM). At much higher concentrations, LSAL also interacts with the 5-HT<sub>1A</sub> receptors (IC<sub>50</sub> = 1 μM) and is still devoid of any significant activity on the other receptors examined. These results clearly demonstrate that LSAL specifically interacts with 5-HT<sub>1B</sub> Receptor subtype.

As expected, the synthetic peptide exhibited a pharmacological profile very similar to that of the P fraction tested at a dose corresponding to 1% of the total purified fraction. Indeed, it was calculated that this dose corresponded to a peptide concentration of 8.10<sup>-19</sup> M, namely 1% of 0.3 μg of LSAL (molecular weight = 402.5) tested in a volume of 1 ml. Moreover, the synthetic peptide had exactly the same retention time as the purified P fraction on the C<sub>18</sub> Ultrabase reverse phase column (not shown). These results strongly suggest that the active compound contained in the P fraction corresponds to LSAL.

The activities of rat P<sub>1</sub> and P<sub>2</sub> fractions were too low to be traced accurately through the following different chromatographic steps. The extract prepared from bovine brains (600 g of initial weight) also contained three fractions having the same retention times as P<sub>1</sub>, P<sub>2</sub>, and P<sub>3</sub> fractions obtained from rat brain extract. The patterns of inhibitory activity on [3H]5-HT binding were very similar when measured on bovine brain membranes as well as on rat brain membranes. Moreover, the activity of the bovine brain extract closely resembled that of the rat brain extract. These results tend to suggest that the three fractions observed in bovine brain extract are identical to those present in rat. Nevertheless, in bovine brain extract, the inhibitory activity was essentially localized in the P<sub>1</sub> and P<sub>2</sub> fractions, which were identified by amino acid and NMR analysis as AL and LS, respectively. Synthetic peptides, namely LS, AL, and LSAL, coeluted with P<sub>1</sub>, P<sub>2</sub>, and P fractions, respectively, whereas other peptides such as SL, LA, and LASL have different retention times in the same chromatographic system (not shown). These results further support the hypothesis that P fraction in bovine brain corresponds to LSAL and that P<sub>1</sub> and P<sub>2</sub> fractions in rat brain actually are LS and AL, respectively.

LS and AL were poorly active compared with LSAL (100,000 and 10,000 times less efficient, respectively). Accordingly, P<sub>1</sub> and P<sub>2</sub> fractions were poorly active compared with P fraction (using molecular weights of 211 and 213 for P<sub>1</sub> and P<sub>2</sub> fractions, respectively, the 1% dose tested corresponded to an amount close to 0.3 μg/ml of incubate and thus to a final concentration of 14 μM for the two dipeptides). The fact that AL and LS are dipeptides constitutive of LSAL suggests that these dipeptides may originate from the degradation of LSAL. In favor of this hypothesis, it was shown that the extraction procedure applied to the medium in the presence of labeled LSAL and in the absence of tissue does not induce the occurrence of the dipeptides. Moreover, the latter compounds were not found in the extract of a brain homogenate in which [3H]LSAL was added prior to the the extraction process. On the contrary, the major part of [3H]LSAL was found in the extract as the native radioactive compound. This result indicates that the cleavage of the tetrapeptide does not occur during the different steps of extraction and purification. Thus, the presence of the dipeptides observed in the brain extracts suggests that LSAL was degraded in LS and AL prior to the extraction procedure. The fact that P<sub>1</sub> and P<sub>2</sub> fractions are relatively more important than P fraction in bovine brain compared with rat brain extract supports the hypothesis that, during the long post-mortem delay (2-3 h) before processing the bovine brains, LSAL was cleaved in the two corresponding dipeptides. This phenomenon occurred to a lesser extent in rat brain, which could be processed more rapidly. These observations suggest that the cleavage of LSAL in LS and AL may correspond to the inactivation process of this endogenous peptide.

LSAL exhibited similar properties of binding inhibitions in rat brain and in bovine brain cortical membranes, indicating that it also interacted with 5-HT<sub>1D</sub> receptor subtype. It should be emphasized that 5-HT<sub>1D</sub> receptors are in non-rodent species the equivalent of the rodent 5-HT<sub>1B</sub> receptor, as has been shown from their functional properties and the close homology of the genes encoding for the corresponding receptor proteins (40). This observation suggests an important functional role for this peptide since it has been conserved during the evolution. Moreover, its activity is maintained despite the fact that its functional target was modified under the pressure of the evolution leading to different pharmacological properties of 5-HT<sub>1B</sub> vis á vis 5-HT<sub>1D</sub>.

LSAL is an original sequence not represented in any of the
known peptides or peptide precursors (Swissprot protein bank). Moreover, this peptide is not homogeneously distributed within the brain but rather is present in higher amounts in some brain areas (hippocampus, cortex) than in others (brain stem). These brain areas have been shown to contain 5-HT1B receptors (41); however, on the basis of the herein presented results, it is difficult to establish a direct relationship between the distribution of LSAL and that of the 5-HT1B receptors. Autoradiographic studies with the labeled peptide will determine this point. LSAL is also present in peripheral tissues, i.e. in kidneys, which also contain a high density of 5-HT1B receptors (42, 43). The fact that LSAL is not found in significant amounts in liver suggests that it is not the result of the degradative procedure of a circulating protein. These observations support the hypothesis that LSAL may be an endogenous peptide.

Although it is too early to know the origin of this compound and its potential pathophysiological implications, these results demonstrate that LSAL is able to specifically interact with 5-HT1B receptors presumably via a particular binding site; preliminary results indicate that the inhibition corresponds to a noncompetitive interaction, which may suggest an allosteric mechanism. Additional studies are necessary to test this hypothesis and to examine the functional consequences of the existence of such a potential modulator. Nevertheless, the existence of this mechanism of interaction with the 5-HT1B receptor, which controls the serotonergic system activity, may lead to new directions of research in the mechanisms involved in numerous pathophysiological functions implicating the 5-HT1B system. Furthermore, the existence of a direct interaction of an endogenous peptide with a G protein-coupled receptor would result in new concepts in the mechanisms of regulation of the central nervous system.

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