Further Studies on the Endogenous Serotonin-Uptake-Inhibitor-Like Substances in the Human Cerebrospinal Fluid

Shinichiro Goto, Toru Egashira and Yasumitsu Yamanaka

Department of Pharmacology, Oita Medical University, 1-1, Idaigaoka, Hasama-machi, Oita 879-55, Japan

Received September 24, 1992 Accepted October 30, 1992

ABSTRACT—The properties of endogenous substances that inhibit 5HT uptake in human cerebrospinal fluid (CSF) were investigated. Human CSF was loaded onto a Sephadex G-25 column, and each fraction was tested for its ability to inhibit [^{3}H]paroxetine binding in monkey brain preparations. We found four different inhibitory peaks with respective molecular weights (M.W.) of >12400, 2000 and two of <1350. The third and fourth peaks (F-3, F-4: <1350 M.W.) of inhibitory activity were determined to consist of some monoamines (5HT, etc.) or their metabolites (5HIAA, etc.) and other unidentified compounds by using an HPLC-electrochemical detector. The second peak (F-2, M.W. about 2000) displaced [^{3}H]paroxetine binding noncompetitively (decreased B_{max} and did not change K_d) and inhibited [^{3}H]5HT uptake noncompetitively (decreased V_{max} and did not change K_m), but had no effect on either [^{3}H]norepinephrine uptake or [^{3}H]dopamine uptake. These results suggest that the endogenous substances that selectively inhibit 5HT uptake are present in human CSF as low molecular weight compounds.

Keywords: Serotonin uptake, Paroxetine binding, Cerebrospinal fluid (human), Brain (monkey), Antidepressant (endogenous)

The presence of endogenous antidepressant-like substances has been suggested ever since the possible pharmacological relationship between changes in presynaptic sites in imipramine binding and manic depressive disorders was first reported (1). Recently, it has been reported that [^{3}H]imipramine binding sites and [^{3}H]paroxetine binding sites that regulate 5-hydroxytryptamine (5HT) uptake are modulated by unidentified endogenous substance(s) in human plasma (2-5) and in rat brain and plasma (6-9). Abraham et al. (10) demonstrated that α_1-acid glycoprotein, which is present in human plasma, inhibited [^{3}H]imipramine binding, while it enhancd [^{3}H]5HT uptake. In addition, 5-methoxytryptoline and tetrahydro-β-carboline have been shown to be endogenous modulators of [^{3}H]imipramine binding and [^{3}H]5HT uptake (11, 12). Furthermore, we previously reported the possible presence of endogenous substances in human cerebrospinal fluid (CSF) that modulate [^{3}H]imipramine and [^{3}H]paroxetine binding sites and [^{3}H]5HT uptake sites (13, 14). In this study we report more detailed investigations of the properties of the selective 5HT uptake inhibitor-like substances in human CSF.

MATERIALS AND METHODS

Monkeys were anesthetized with ketaral (30 mg/kg, s.c.), and their brains were quickly removed after withdrawing blood. Fifty milliliters of human CSF samples were obtained from 25 patients in the course of routine diagnostic lumbar puncture and were collected in the Central Laboratory of Medicine. The monkey brains and the pooled human CSF were stored at −80℃ until used.

Gel filtration

Five milliliters of evaporated human CSF sample was chromatographed in a Sephadex G-25 column (1.9×87 cm) with 1 mM phosphate buffer, pH 7.4, at a flow rate of 103 ml/hr, and effluent fractions of 5.15 ml/tube were collected. Column effluents were monitored by measuring absorbances at 280 nm. Aliquots (100 μl) of each fraction were assayed for the ability to displace [^{3}H]paroxetine binding. The molecular weights (M.W.s) of endogenous 5HT-uptake-inhibitor-like substances were estimated by chromatography in the same column calibrated with markers of known M.W. The ratios of elution volume to void volume (V_e/V_o) for the markers were as follows: cyanocobalamin (M.W. 1350) 1.96, glucagon (M.W.

3500) 1.56, approtinin (M.W. 6500) 1.12 and cytochrome c (M.W. 12400) 1.02.

Paroxetine binding assay (15)

Monkey frontal cortices were homogenized in 25 vol. of ice-cold 50 mM Tris-HCl buffer (containing 100 mM NaCl and 5 mM KCl, pH 7.4). The P_2 fractions obtained by centrifugation of this homogenate were used as the crude membrane preparations (final concentration of approximately 0.1 mg protein/tube) for the assay. Aliquots of crude membrane suspension were incubated with [^{3}H]paroxetine at 22℃ at a final volume of 250 μl for 180 min. Fluoxetine at 10 μM final concentration was used to determine non-specific binding. The incubation was terminated by rapid filtration of the membrane suspension under reduced pressure through Whatman GF/B glass fiber filters. Each filter was rapidly washed three times with 5 ml of ice-cold 50 mM Tris-HCl buffer, pH 7.4. The filters were then dried and the radioactivities were determined in Triton X-100-toluene scintillation fluid in a liquid scintillation spectrometer.

Monoamine uptake assay

The study of [^{3}H]5HT, [^{3}H]norepinephrine (NE) or [^{3}H]dopamine (DA) uptake into synaptosomes was conducted according to the method of Snyder and Coyle (16), but with minor modifications. The monkey brains (bilateral frontal cortex) were homogenized in 0.32 M sucrose, and the crude synaptosomes were obtained by differential centrifugation. A 100 μl aliquot of crude synaptosomes (final concentration of appoximately 0.2 mg protein/tube) were preincubated at 37℃ for 5 min with Krebs-Henseleit buffer (121 mM NaCl, 25 mM $NaHCO_3$, 11.1 mM glucose, 4.7 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.1 mM ascorbic acid, 130 μM EDTA2Na, and 78.5 μM pargyline, pH 7.4; bubbled with a 95% O_2 / 5% CO_2 gas mixture). Then [^{3}H]-labeled monoamine was added (final concentration: [^{3}H]5HT, 188 nM; [^{3}H]NE, 32 nM; and [^{3}H]DA, 25 nM) and incubated at 37℃ for 2 min (NE uptake) or 10 min (DA uptake and 5HT uptake). Non-specific uptake was calculated from data obtained with incubation at 0℃. The incubation was terminated by rapid filtration of the membrane suspension under reduced pressure through Whatman GF/B glass fiber filters. Each filter was rapidly washed three times with 5 ml of ice-cold saline. The filters were then dried, and the radioactivities were determined by scintillation spectrometry after adding Triton X-100-toluene scintillation fluid.

HPLC-ECD assay

The concentrations of monoamines and their metabolites in CSF were determined by the method of Matsumoto et al. (17), but with minor modifications, using reverse-phase high performance liquid chromatography with electrochemical detection. A 400-μl aliquot of the F-3 or F-4 in human CSF was homogenized in 2 ml of 0.2 M perchloric acid and then centrifuged at 20,000 × g for 15 min. The supernatant was adjusted to pH 3.9 with 1 M CH_3COONa and them assayed on a Eicom HPLC-ECD system. The detector (ECD-100; Eicom Co., Ltd., Kyoto, Japan) was set at a range of 2 mA, and the sample was oxidized with 750 mV potential between the glassy carbon electrode and the Ag/AgCl reference electrode. The filtered and degassed mobile phase consisted of 0.049 M sodium acetate, 0.034 M sodium citrate, 0.074 mM 1-octanesulfonic acid sodium, 0.008 mM EDTA2Na and 10% methanol (v/v) and was adjusted to pH 3.9 with 1 M CH_3COONa. The mobile phase was pumped in at a rate of 1 ml/min.

Protein determination

Protein concentrations were determined by the method of Lowry et al. (18) with bovine serum albumin as a standard.

Chemicals

[Phenyl-6'-^{3}H]-paroxetine (1.11 TBq/mmol), hydroxytryptamine creatinine sulfate 5 [1,2,^{3}H(N)]-(5HT, 1.11 TBq/mmol), norepinephrine, *levo* [Ring 2,5,6,-^{3}H-] (NE, 1.62 TBq/mmol), and dihydroxyphenylethylamine hydrochloride 3,4,-[Ring 2,5,6,-^{3}H-] (DA, 1.3 TBq/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Fluoxetine was donated by Lilly Research Lab. (Indianapolis, IN, U.S.A.). All other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

RESULTS

Gel filtration of human CSF on Sephadex G-25

Human CSF was loaded onto a Sephadex G-25 column (1.9 × 87 cm), and each fraction was tested for its ability to displace [^{3}H]paroxetine binding in monkey brain. Elution profiles from the Sephadex G-25 column are shown in Fig. 1. We found four different inhibitory peaks for [^{3}H]paroxetine binding. The molecular weights (M.W.) of these four substances were estimated by chromatography on a Sephadex G-25 column. The first peak (F-1) was at V_e/V_o of 1.0, with a M.W. greater than 12400. The second peak (F-2) was at V_e/V_o of 1.89, with a M.W. of about 2000. The remaining two peaks (F-3 and F-4) were at V_e/V_o of 2.27 and 3.12, respectively, corresponding to M.W.'s of less than 1350 (Fig. 2). On the other hand, we also found some other peaks which enhanced [^{3}H]paroxetine binding at V_e/V_o of 1.51, 2.02 and

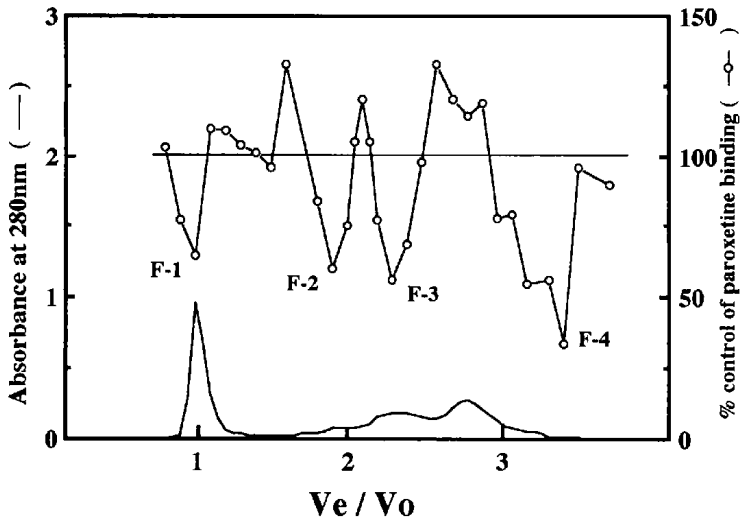

Fig. 1. Gel filtration of human CSF on Sephadex G-25. Human CSF sample was chromatographed in 1 mM phosphate buffer, pH 7.4, at a flow rate of 103 ml/hr, and fractions of 5.15 ml/tube were collected. Column effluents were monitored by measuring the absorbance at 280 nm (——). An aliquot (100 μl) of each fraction was tested for its ability to inhibit [^{3}H]paroxetine binding in monkey brain (—○—).

2.49, corresponding to M.W.'s of about 4000 and two of less than 1350, respectively.

Effects of endogenous substances in human CSF on [^{3}H]-paroxetine binding and [^{3}H]5HT, [^{3}H]DA and [^{3}H]NE uptake in monkey brain synaptosomes

The effects of these four inhibitory peaks in human CSF on [^{3}H]monoamine uptake in monkey brain synaptosomes were tested. All four peaks obtained by gel filtration inhibited [^{3}H]5HT uptake as well as [^{3}H]paroxetine binding. However, they did not affect [^{3}H]DA or [^{3}H]NE uptake (data not shown). Figure 3 shows specifically how F-2 in human CSF markedly inhibited [^{3}H]5HT uptake in a volume dependent manner, but had no effect on the uptake of [^{3}H]DA or [^{3}H]NE.

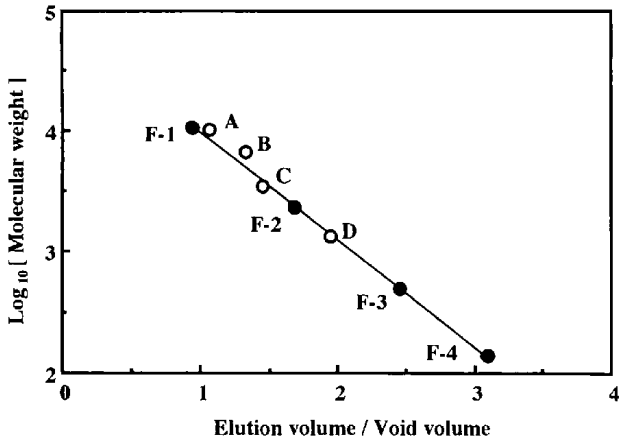

Fig. 2. Estimation of the molecular weight on the endogenous substances in human CSF by Sephadex G-25 gel chromatography. The flow rate was 103 ml/hr in 1 mM phosphate buffer, pH 7.4, and the effluent fractions were monitored at 280 nm. The V_e/V_o ratios were (A) cytochrome c (M.W. 12400) 1.02, (B) approtinin (M.W. 6500) 1.12, (C) glucagon (M.W. 3500) 1.56 and (D) cyanocobalamin (M.W. 1350) 1.96.

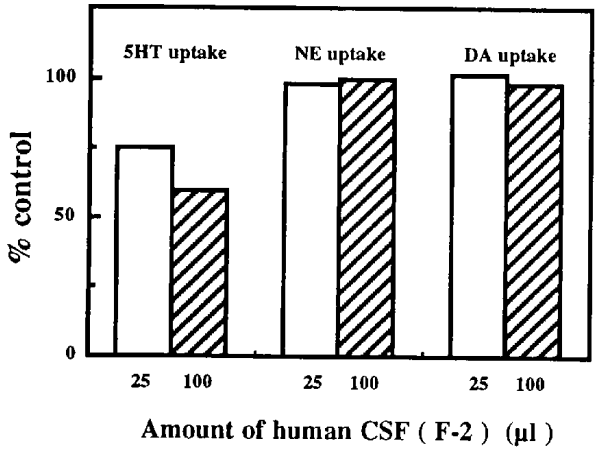

Fig. 3. Effects of the second fraction (F-2) obtained from human CSF by gel chromatography on [^{3}H]5HT, [^{3}H]NE and [^{3}H]DA uptake in synaptosomes. Uptakes of [^{3}H]5HT, [^{3}H]NE and [^{3}H]DA were conducted as described in the text. [^{3}H]5HT uptake (control value is 2.89±0.15 pmol/min/mg protein), [^{3}H]NE uptake (control value is 0.057±0.003 pmol/min/mg protein) and [^{3}H]DA uptake (control value is 0.42±0.04 pmol/min/mg protein).

HPLC-ECD chromatography of F-3 and F-4 obtained from human CSF

The V_e/V_o of F-3 and F-4 obtained from human CSF by gel chromatography was 2.27 and 3.12, repectively. It is believed that F-3 and F-4 are low molecular weight substances such as endogenous ligands. When F-3 and F-4 samples were injected into the HPLC-ECD device, they were found to contain some monoamines (5HT, etc.), their metabolites (5HIAA etc.) and unidentified compounds (data not shown).

Effects of F-2 in human CSF on [^{3}H]paroxetine binding

The non-competitive nature of [^{3}H]paroxetine-binding inhibition by endogenous substances (F-2) is demonstrated in Fig. 4. Scatchard analysis of [^{3}H]paroxetine binding in monkey brain preparations, using [^{3}H]paroxetine concentrations ranging from 0.015 nM to 0.2 nM in the absence and presence of F-2 in human CSF, revealed an apparent decrease in B_{max} (99.7 ± 5.0 fmol/mg protein and 74.7 ± 7.7 fmol/mg protein, in the absence and presence of F-2, respectively) and no change in K_d (131 ± 20 pM and 141 ± 15 pM, in the absence and presence of F-2, respectively).

Lineweaver-Burk analysis of [^{3}H]5HT uptake to monkey brain synaptosomes in the absence and presence of F-2 in human CSF

Kinetic analysis of [^{3}H]5HT uptake to monkey brain synaptosomes and the effect of F-2 in human CSF are shown in Fig. 5. Using [^{3}H]5HT concentrations ranging from 40 nM to 400 nM, a single saturable high affinity uptake site was found. Figure 5 shows that the inhibitory effect of F-2 was non-competitive in nature (V_{max}, 4.81 ± 0.39 pmol/mg protein/min and 3.44 ± 0.35 pmol/mg protein/min and K_m: 0.32 ± 0.03 μM and 0.33 ± 0.02 μM in the absence and presence of F-2, respectively).

DISCUSSION

We previously suggested the existence of the endogenous inhibitor-like substance(s) in the 5HT-uptake system in human CSF, since a small amount of human CSF displaced [^{3}H]imipramine and [^{3}H]paroxetine binding and inhibited [^{3}H]5HT uptake dose-dependently (14). In addition, we also reported that human CSF preferentially inhibited [^{3}H]paroxetine binding rather than [^{3}H]-imipramine binding (13). Furthermore, in this study, we performed more detailed investigations on the relationship between endogenous substances in the human CSF and the 5HT uptake system in the central nervous system. By Sephadex G-25 chromatography, we found four different peaks in human CSF that inhibited [^{3}H]paroxetine binding in the monkey brain preparations. The recognition sites labeled with [^{3}H]imipramine or [^{3}H]paroxetine have been shown to be distinct but allosterically coupled with the 5HT uptake system (19–24). These four fractions also inhibited [^{3}H]5HT uptake. These results suggest that these four fractions contain endogenous 5HT uptake modulator(s). On the other hand, we also found some other peaks that enhanced [^{3}H]paroxetine binding in the same CSF. Substances that act in such a manner have already been reported (10, 25), so in this study, we

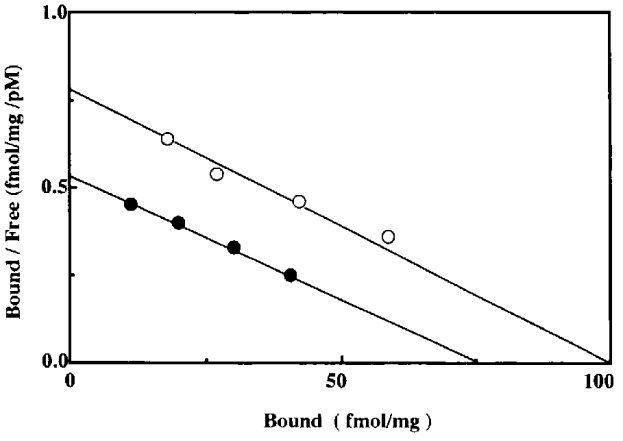

Fig. 4. Scatchard analysis of [^{3}H]paroxetine binding in monkey brain. The specific [^{3}H]paroxetine binding was assayed at different ligand concentrations (20 to 200 pM) in the presence (—●—) and absence (—○—) of F-2 in human CSF (25 μl). Each data point represents the average of quadruplicate determinations in a single experiment.

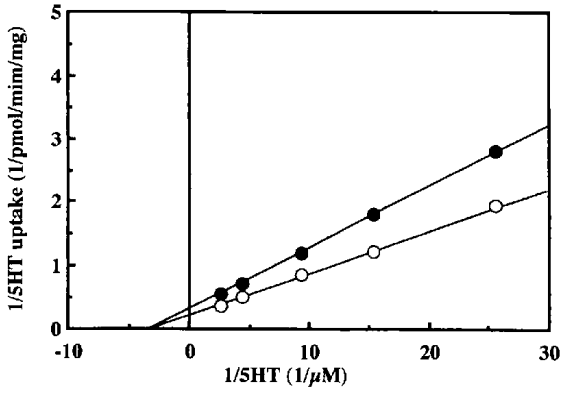

Fig. 5. Lineweaver-Burk analysis of [^{3}H]5HT uptake to monkey brain synaptosomes. [^{3}H]5HT concentrations ranging from 40 nM to 400 nM were used. Specific [^{3}H]5HT uptake was assayed in the presence (—●—) and absence (—○—) of F-2 in human CSF (25 μl). Each data point represents the average of quadruplicate determinations in a single experiment.

did not mention these peaks that enhanced [^{3}H]paroxetine binding and concentrated our efforts to search for endogenous 5HT-uptake-inhibitor-like substances.

The molecular weights of these substances (F-1, F-2, F-3 and F-4) were estimated by chromatography on a Sephadex G-25 column: F-1 at a V_e/V_o of 1.0, with a M.W. greater than 12400; F-2 at a V_e/V_o of 1.89, with a M.W. of about 2000; and F-3 and F-4 at a V_e/V_o of 2.27 and 3.12, respectively, corresponding to M.W.'s less than 1350.

There have been recent reports of endogenous substances such as 5-methoxytryptoline (11), tetrahydro-β-carboline (12) and α_1-acid glycoprotein (10) in human plasma, rat plasma and rat brain that modulate the 5HT-uptake system. Since 5-methoxytryptoline, which appeared to occur endogenously at particularly high levels in the human pineal gland, also inhibited [^{3}H]5HT uptake and [^{3}H]imipramine binding, it should be considered as a putative endogenous ligand modulating 5HT transport (11). Tetrahydro-β-carbolines also represent a family of indoleamine derivatives, some of which are present in the CNS and some peripheral tissues. These compounds have multiple actions including inhibition of [^{3}H]5HT uptake and [^{3}H]imipramine binding in the CNS (12). α_1-Acid glycoprotein (M.W. 45000) was isolated and purified as a potential endogenous 5HT uptake modulator from human plasma, and it inhibited [^{3}H]imipramine binding, but enhanced [^{3}H]5HT uptake (10).

To demonstrate the existence of endogenous substance(s), it is said that known endogenous substances (for example, 5HT, etc.) must be shown to be absent or present in negligible concentrations in an extract fraction (26, 27). Judging from the V_e/V_o ratio in this study, F-3 or F-4 probably represent an endogenous substance of low M.W. Therefore, we analyzed F-3 and F-4 by HPLC-ECD to determine if monoamines are present. Some monoamines (5HT, etc.), their metabolites (5HIAA, etc.) and unidentified compounds were observed. These results indicate that inhibition of [^{3}H]5HT uptake and [^{3}H]paroxetine binding by F-3 and F-4 may be due to monoamines (such as 5HT), their metabolites (such as 5HIAA) and/or unidentified compounds contained in F-3 or F-4.

Based on its V_e/V_o ratio, the first fraction (F-1) may be high molecular weight proteins (M.W. greater than 12400). Abraham et al. (10) reported that an endogenous modulator was present in human plasma (like α_1-acid glycoprotein) and suggested that this modulator acted at the recognition site labeled by [^{3}H]imipramine and enhanced [^{3}H]5HT uptake. It is known that plasma proteins also alter imipramine binding (28–30). These results suggest that high M.W. compounds, such as α_1-acid glycoprotein or albumin, may also regulate the 5HT-uptake system.

Therefore, we further examined the properties of second displacable fraction (F-2, M.W. about 2000). Addition of F-2 displaced [^{3}H]paroxetine binding and inhibited [^{3}H]5HT uptake noncompetitively. However, F-2 had no affect on either [^{3}H]NE uptake or [^{3}H]DA uptake. F-2 may be a low M.W. compound and is likely a selective 5HT-uptake-inhibitor-like substance. We previously reported the existence of MAO-inhibitor-like substances in various animal CSFs as well as in human CSF (25, 31). In addition, a further putative endogenous MAO inhibitor has been isolated from human CSF (13). Moreover, it is well-known that tricyclic antidepressants inhibit MAO activity, imipramine binding and also modulate the 5HT uptake system. From these reports, the materials in human CSF may represent endogenous antidepressant-like substances such as tricyclic antidepressants and may play a possible role in regulating serotonergic activity. To clarify this hypothesis, further purification of F-2 substances and more detailed studies on the changes in the amounts of F-2 substances in pathological models or in depressive model animals are required.

Acknowledgments

This study was supported by a Grant (8-A) from the National Center of Neurology and Psychiatry (NCNP) of the Ministry of Health and Welfare, Japan.

REFERENCES

1 Briley, M.S., Langer, S.Z., Raisman, R. and Sechter, D.: Tritiated imipramine binding sites are decreased in platelets of untreated depressed patients. Science **209**, 303–305 (1980)

2 Angel, I. and Paul, S.M.: Inhibition of synaptosomal 5-[^{3}H]-hydroxytryptamine uptake by endogenous factor(s) in human blood. FEBS Lett. **171**, 280–284 (1984)

3 Brusov, O.S., Femenko, A.M. and Katasonov, A.B.: Human plasma inhibitors of platelet serotonin uptake and imipramine receptor binding: extraction and heterogeneity. Biol. Psychiatry **20**, 235–244 (1985)

4 Barkai, A.I., Baron, M., Kowalik, S. and Cooper, T.B.: Inhibition of ^{3}H-imipramine binding by plasma from depressed and normal subjects. Psychiatry Res. **17**, 261–267 (1986)

5 Takahashi, R., Nankai, M., Ishii, K. and Yoshimoto, S.: Studies on biochemical characteristics of platelets and serum of human patients with depressive disorders as biological markers. *In* Biological Studies on Pathogenesis of Affective Disorders. Annual Report of National Project Team, p. 122, Ministry of Health and Welfare, Tokyo (1988)

6 Barbaccia, M.L., Gandolfi, O., Chuang, D.M. and Costa, E.: Modulation of neuronal serotonin uptake by a putative endogenous ligand of imipramine recognition sites. Proc. Natl. Acad. Sci. U.S.A. **80**, 5134–5138 (1983)

7 Barbaccia, M.L., Melloni, P., Pozzi, O. and Costa, E.: [^{3}H]Imipramine displacement and 5HT uptake inhibition by tryptoline derivatives: In rat brain, 5-methoxytryptoline is not the autacoid for [^{3}H]imipramine recognition site. Eur. J. Pharmacol. **123**, 45–52 (1986)

8 Angel, I., Goldman, M.E., Skolnick, P., Pisano, J.J. and Paul, S.M.: Characterization of endogenous inhibitors of [^{3}H]-imipramine binding and [^{3}H]-serotonin uptake from rat serum. *In* Endocoids, Edited by Lal, H., Labella, F. and Lane, J., pp. 457–464, Alan R. Liss, Inc., New York (1985)

9 Rehavi, M., Ventura, I. and Sarne, Y.: Demonstration of endogenous "imipramine like" material in rat brain. Life Sci. **36**, 687–693 (1985)

10 Abraham, K.I., Ieni, J.R. and Meyerson, L.R.: Purification and properties of a human plasma endogenous modulator for the platelet tricyclic binding/serotonin transport complex. Biochim. Biophys. Acta **923**, 8–21 (1987)

11 Segonzac, A., Schoemaker, H., Tateishi, T. and Langer, S.Z.: 5-Methoxytryptoline, a competitive endocoid acting at [^{3}H]-imipramine recognition sites in human platelets. J. Neurochem. **45**, 249–256 (1985)

12 Langer, S.Z., Raisman, R., Tahraoui, L., Scatton, B., Niddam, R., Lee, C.R. and Claustre, Y.: Substituted tetrahydro-β-carbolines are possible candidates as endogenous ligand of the [^{3}H]imipramine recognition site. Eur. J. Pharmacol. **98**, 153–154 (1984)

13 Egashira, T., Goto, S., Murayama, F. and Yamanaka, Y.: Inhibition of MAO activity, ^{3}H-imipramine binding, ^{3}H-paroxetine binding and ^{3}H-5HT uptake by human cerebrospinal fluid. J. Neural. Transm. Supp. **32**, 447–456 (1990)

14 Goto, S., Egashira, T. and Yamanaka, Y.: Evidence for the existence of serotonin uptake inhibitor-like substances in human cerebrospinal fluid. Japan. J. Pharmacol. **56**, 297–302 (1991)

15 Habert, E., Graham, D., Tahraoui, L., Claustre, Y. and Langer, S.Z.: Characterization of ^{3}H-paroxetine binding to rat cortical membranes. Eur. J. Pharmacol. **118**, 107–114 (1985)

16 Snyder, S.H. and Coyle, J.T.: Regional differences in ^{3}H-norepinephrine and ^{3}H-dopamine uptake into rat brain homogenates. J. Pharmacol. Exp. Ther. **165**, 78–86 (1969)

17 Matsumoto, M., Togashi, H., Yoshioka, M., Hirokami, H., Morii, K. and Saito, H.: Simultaneous high-performance liquid chromatographic determination of norepinephrine, serotonin, acetylcholine and their metabolites in cerebrospinal fluid of anaesthetized normotensive rats. J. Chromatogr. **526**, 1–10 (1990)

18 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265–275 (1951)

19 Briley, M.S., Langer, S.Z. and Sette, M.: Allosteric interaction between the ^{3}H-imipramine binding site and the serotonin uptake mechanism. Br. J. Pharmacol. **74**, 817–818 (1980)

20 Wennogle, L.P. and Meyerson, L.R.: Serotonin uptake inhibitors differentially modulated high affinity imipramine dissociation in human platelet membranes. Life Sci. **36**, 1541–1550 (1985)

21 Meyerson, L.R., Jeni, J.R. and Wennogle, L.P.: Allosteric interaction between the site labelled by ^{3}H-imipramine and the serotonin transporter in human platelets. J. Neurochem. **48**, 560–565 (1987)

22 Backstrom, I., Bergstrom, M. and Marcusson, J.: High affinity [^{3}H]paroxetine binding to serotonin uptake sites in human brain tissue. Brain Res. **486**, 261–268 (1989)

23 D'haenen, H., Waele, M.D. and Leysen, J.E.: Platelet ^{3}H-paroxetine binding in depressed patients. Psychiatry Res. **26**, 11–17 (1988)

24 Graham, D., Esnaud, H., Harbert, E. and Langer, S.Z.: A common binding site for tricyclic and nontricyclic 5-hydroxytryptamine uptake inhibitors at the substrate recognition site of the neuronal sodium dependent 5-hydroxytryptamine transporter. Biochem. Pharmacol. **38**, 3819–3826 (1989)

25 Egashira, T., Takano, R. and Yamanaka, Y.: Modulation of neuronal MAO activity, 5-HT uptake and imipramine binding by endogenous substances in dog cerebrospinal fluid. Biochem. Pharmacol. **36**, 1781–1785 (1987)

26 Lee, C.R., Galzin, A.M., Taranger, M.A. and Langer, S.Z.: Pitfalls in demonstrating an endogenous ligand of imipramine recognition sites. Biochem. Pharmacol. **36**, 945–949 (1987)

27 Artigas, F., Martinez, E. and Adell, A.: Non-specific inhibition of imipramine binding argues against an endogenous ligand. Eur. J. Pharmacol. **181**, 9–15 (1990)

28 Bickel, M.H.: Binding of chlorpromazine and imipramine to red cells, albumin, lipoprotein and other blood components. J. Pharm. Pharmacol. **27**, 733–738 (1975)

29 Dannon, A. and Che, Z.: Binding of imipramine to plasma proteins: Effect of hyperlipoproteinemia. Clin. Pharmacol. Ther. **25**, 316–321 (1979)

30 Weder, H.J. and Bicker, M.H.: Interactions of drugs and proteins: I. Binding of tricyclic thymoleptics to human and bovine plasma proteins. J. Pharmacol. Sci. **59**, 1505–1507 (1970)

31 Egashira, T., Obata, T., Kimba, Y., Takano, R. and Yamanaka, Y.: Endogenous monoamine oxidase inhibitor-like substaces in monkey brain. Biochem. Pharmacol. **38**, 597–602 (1989)