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Augmented brain 5-HT crosses the blood–brain barrier through the 5-HT transporter in rat

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Abstract

The present study re-evaluated an existing notion that serotonin (5-hydroxytryptamine; 5-HT) could not cross the brain to the circulating blood via the blood–brain barrier (BBB). To elevate brain 5-HT alone, 5-hydroxytryptophan (5-HTP; 30–75 mg/kg) was administrated intravenously to anaesthetized rats that had undergone gastrointestinal and kidney resections along with liver inactivation (organs contributing to increasing blood 5-HT after 5-HTP administration). A microdialysis method and HPLC system were used to determine the brain 5-HT levels in samples collected from the frontal cortex. Blood 5-HT levels were determined from whole blood, not platelet-poor plasma, collected from the central vein. We found that blood 5-HT levels showed a significant augmentation whenever brain 5-HT levels were significantly elevated after the administration of 5-HTP in those rats with the abdominal surgical procedures. This elevation was abolished after pretreatment with a selective serotonin reuptake inhibitor (fluoxetine; 10 mg/kg i.v.), although brain 5-HT levels remained augmented. These results indicate that augmented brain 5-HT can cross the BBB through the 5-HT transporter from the brain to the circulating blood.

Introduction

It was previously believed that serotonin (5-hydroxytryptamine; 5-HT) could not cross from the brain to the periphery via the blood–brain barrier (BBB). However, recent *in vitro* studies (Brust *et al*., 2000; Wakayama *et al*., 2002) have revealed the presence of 5-HT transporter mRNA in vascular endothelial cells, indicating that the BBB may act as an efflux transport system for 5-HT. Based on this information, we conducted the present study to re-evaluate the above concept. In other words, we examined the possibility that augmented brain 5-HT may cross the BBB through the 5-HT transporter.

5-HT neurons, which are located in the raphe nuclei of the brainstem, are known to project to broad areas of the whole brain. In addition, 5-HT neuronal activity exhibits a state-dependent change (Jacobs & Azmitia, 1992). These neurons regularly fire during waking, although the firing rate is irregular or silenced during sleep. Therefore, it can be anticipated that there is a steady synaptic release of 5-HT that occurs over a broad area of the brain during waking. In fact, rat brain 5-HT levels revealed state-dependent alterations, i.e. high levels during waking and low levels during sleep (Penalva *et al*., 2003). In the present study, we evaluated the change in extracellular fluid (ECF) 5-HT levels in the brain using a microdialysis method and a high-performance liquid chromatography (HPLC) system.

It has been well established that brain 5-HT is elevated by administration of 5-hydroxytryptophan (5-HTP), i.e. the 5-HT precursor (Okada *et al*., 1972; Löscher *et al*., 1984; Gartside *et al*., 1992). We injected 5-HTP intravenously to rats to elevate brain ECF 5-HT levels in this study. As 5-HTP decarboxylase, which metabolizes 5-HTP to 5-HT, is found not only in the brain but also in the gut, liver and kidneys (West, 1958), a rise in blood 5-HT levels after 5-HTP administration may not be due to the brain alone but also to these other organs. Therefore, to exclude this possibility we surgically removed the potentially complicating organs. We administered 5-HTP to those rats whose gastrointestinal tracts and kidneys had been completely resected and whose livers had been inactivated.

To confirm the role of the BBB 5-HT transporter in the transport of 5-HT from the brain to the periphery, we also examined the effect of selective serotonin reuptake inhibitors (SSRIs) in those rats. Brain ECF 5-HT and blood 5-HT levels were measured after 5-HTP administration in rats with and without SSRI pretreatment.

However, it has been established that blood 5-HT is mainly distributed in platelets and to a lesser degree in the plasma (Artigas *et al*., 1985; Ortiz *et al*., 1988). If brain ECF 5-HT can be transported via the BBB, it would be expected that the augmented 5-HT in the plasma would move quickly into the platelets. Therefore, in this study we evaluated changes in whole blood, not in platelet-poor plasma, to evaluate the BBB efflux transport system for 5-HT from the brain to the circulating blood.

Materials and methods

Animal preparation

All procedures involving animals were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Animal Experimentation Ethics Committee of the Toho University School of Medicine.

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Experiments were performed on 25 male Wistar rats weighing 310–375 (327.6 ± 4.2) g. The rats were adapted to the standard 12 : 12 h light : dark conditions (lights on at 08.00 h) for > 1 week before the experiments. Experiments were then performed during the light cycle. The rats were anaesthetized with 50 mg/kg pentobarbital sodium intraperitoneally. The depth of anaesthesia was continuously controlled by maintaining the absence of nociceptive flexion and corneal reflexes. Supplementary doses (20% of the original dose) were given intravenously when necessary. The anaesthesia was maintained until the end of the experiment, when the animal was killed with an overdose of anaesthetic. All efforts were made to minimize the number of animals used.

Catheters were placed into the central vein near the right atrium for collection of blood samples and for drug injection. To monitor vital signs, we measured the arterial blood pressure by placing a catheter into the femoral artery. Heart rate was calculated from the blood pressure pulse with a tachometer (AT-601G; Nihon Kohden). The animals were fixed in a prone position in a stereotaxic frame. Rectal temperature was maintained at 37 °C with a heating lamp.

Rats used in this study had undergone total resection of the gastrointestinal tract and kidneys, as well as liver inactivation. In these rats, the operative area was shaved and the abdomen opened by a long midline incision with aseptic precautions. Ligatures were placed round the celiac and the superior mesenteric arteries as near to the aorta as possible. To inactive the liver, the portal vein was ligated along with the bile duct. The whole intestine was freed from its ligaments and all vascular attachments were removed. Total resection of the gastroduodenum and the small and large intestines was performed as quickly and carefully as possible to minimize bleeding. After ligation, the kidneys were also removed. The rectum with its vessels was clamped, ligated distally to the clamp, and then cut between the clamp and the point of ligation. Abdominal muscles and skin were then sutured. After the operation, we confirmed that the animal was breathing spontaneously and that the blood pressure was maintained within the normal range (mean arterial pressure 121.45 ± 4.11 mmHg; $n = 20$). The rats were fixed in a prone position in a stereotaxic frame.

Microdialysis procedures and 5-HT measurements

A parietal craniotomy was performed, and the dura was opened to advance the microdialysis probe (0.22 mm diameter; 2 mm exposed membrane; A-I-4–02; Eicom, Japan). Using the atlas of the rat brain (Paxinos & Watson, 1986) as a guide, a probe was slowly and gently inserted into the left frontal cortex (FC), an area 0.5 mm lateral to the midline, 3.2 mm anterior to the bregma and 2.5 mm vertically below the dura. The probe was fixed with dental cement, connected to a microinfusion pump (EP-50; Eicom) and then perfused with Ringer's solution (NaCl, 147 mM; KCl, 4 mM; $CaCl_2$, 1.9 mM) at a flow rate of 1 μL/min.

A stabilization period of at least 1 h was allowed following probe implantation. After the stabilization period, microdialysis sampling from the FC was carried out every 10 min. The perfusate from the FC was injected into a HPLC system (DAM-300 system; Eicom) using an automatic injector (AS-10; Eicom) and immediately analysed for 5-HT. A reverse-phase column (Eicompak PP-ODS, 4.6 mm diameter × 30 mm; Eicom) was used for 5-HT separation. The working electrode was a graphite electrode set at a detector potential of +0.40 V against an $Ag/AgCl_2$ reference electrode. The composition of the mobile phase was 0.1 M phosphoric acid buffer at pH 6.0, containing 1% methanol, 2 mM sodium 1-decanesulphonate (as the ion-pair) and 0.13 mM ethylenediaminetetraacetic acid (EDTA·2Na) at a flow rate of 0.50 mL/min. The column temperature was maintained at 25 °C.

After the stabilization period, three consecutive measurements of 5-HT, which were carried out every 10 min, were done to confirm that the microdialysis sampling from the FC was exhibiting a steady-state baseline. Thereafter, the three different experimental procedures using 5-HTP and/or SSRI were carried out (described below).

Analysis of whole-blood 5-HT levels

Blood (0.5 mL) was obtained in a plastic tube and then 0.5 mL of saline solution was injected back into the animal in order to maintain a stable and constant total blood amount. As per the method of Kremer *et al*. (1990), 0.5 mL of blood was suspended in 2.2 mL of water. Then, 300 μL of the internal standard and 10 μL of a 10% (weight per volume) solution of ascorbic acid in water was added to the suspended blood sample. The sample was then frozen at −20 °C and stored until further analysis of 5-HT.

5-HT analyses of the blood samples were conducted within 1 week after the experiment. Blood samples were thawed, and then 167 μL of methanol was added to 1 mL of blood sample in order to remove the proteins. Blood samples were centrifuged at 4670 ***g*** for 10 min at 4 °C. Five hundred microlitres of the supernatant of the blood sample was suspended in 4.5 mL of the mobile buffer. Twenty microlitres of the blood sample was then injected into the HPLC system. Although we used the same HPLC system to determine blood 5-HT levels, we applied the following different method for blood 5-HT analysis from that for FC 5-HT analysis. 5-HT was separated on a reverse-phase column (Eicompak CA-5ODS; 2.1 mm diameter × 150 mm; Eicom). The mobile phase consisted of a 0.1-M phosphate acid buffer containing 50 mg/L EDTA·2Na and 300 mg/L sodium 1-octanesulphonate (Nacalai Tesque, Japan) as the ion-pair and 20% methanol at pH 6.0. The flow rate was set at 0.22 mL/min and the column temperature was maintained at 35 °C.

Experimental protocols

Experiment 1

The first experiment was performed on five intact rats that did not undergo the abdominal operation. After the stabilization period of 1–2 h, we determined the steady-state level of brain 5-HT by measuring three consecutive microdialysis perfusate samples from the FC at 10-min intervals. Thereafter, 1 mL of 5-HTP (a dose of 75 mg/kg in saline solution) was administered through the venous catheter of the rat.

Microdialysis sampling from the FC was carried out every 10 min throughout the entire experiment. Blood sampling was performed four times as follows. The first blood sample was taken prior to the 5-HTP administration. Following the 5-HTP administration three further blood samples were drawn at 1-h intervals. Note that same amount of saline solution was injected back into the animals in order to maintain a stable and constant total blood amount.

Experiment 2

The second experiment was performed on 10 rats that underwent complete resection of their gastrointestinal tracts and kidneys, along with liver inactivation. One millilitre of 5-HTP (a dose of 75 mg/kg in saline solution) was administered through a venous catheter in six rats, with the same amount of a saline solution injected in four rats that

served as the control animals. Following the injection of 5-HTP or saline solution, samples were obtained from the FC every 10 min for ~2 h.

Blood sampling was performed three times in the six rats given 5-HTP. The first blood sample was taken prior to the injection of 5-HTP (preinjection sample). The second blood sample was obtained when the brain 5-HT level had increased to more than two times higher than the preinjection level, which occurred at 50–70 min after 5-HTP administration. The third blood sample was taken when the brain 5-HT level further increased, which occurred at 10–30 min after the second blood sample was drawn.

In the control experiment, three consecutive blood samples were drawn before the saline solution administration and then every 1 h after saline administration in the four rats that were not given 5-HTP.

Experiment 3

The third experiment was conducted to evaluate the effect of SSRI (fluoxetine hydrochloride; Sigma, USA). The experiment was performed on 10 rats that underwent resection of their gastrointestinal tracts and kidneys along with liver inactivation. Prior to SSRI pretreatment, we determined the steady-state level of the brain 5-HT by measuring three consecutive microdialysis perfusate samples from the FC every 10 min (pretreatment period). Thereafter, 1 mL of SSRI (a dose of 10 mg/kg in saline solution) was administered to rats through a venous catheter. One millilitre of 5-HTP (a dose of 30 mg/kg in saline solution) was administered through the venous catheter at 40 min after the SSRI pretreatment. The dose of 5-HTP given was reduced to approximately half of that given in Experiments 1 and 2. The reason for the reduction in 5-HTP loading was that extremely high levels of brain 5-HT were noted in the first microdialysis perfusate sample from the FC when we administered the same 5-HTP dose (75 mg/kg in saline solution) with SSRI pretreatment in the animals.

Microdialysis sampling from the FC continued every 10 min throughout the experiment, with blood samples drawn three times during the study period. The first blood sample was obtained during the control period, i.e. prior to SSRI pretreatment. The second blood sample was drawn 30 min after SSRI pretreatment, which was 10 min prior to the 5-HTP administration. The third blood sample was drawn at the time when we observed an apparent increase in the FC 5-HT level after the 5-HT administration, which was 30–90 min after 5-HTP administration.

In the control experiment, instead of SSRI pretreatment animals were given 1 mL of saline solution through the venous catheter after the control period, which was described above. One millilitre of 5-HTP was given through the venous catheter at 40 min after the saline pretreatment. Microdialysis sampling from the FC continued every 10 min throughout the experiment. Blood samples were drawn three times during the control experiment. The first sample was drawn during the control period, the second 30 min after saline pretreatment and the third 0–90 min after 5-HTP administration.

Statistical analysis

A one-way ANOVA was used to analyse the data obtained in Experiment 1 while a two-way ANOVA for repeated measures was used for data obtained in Experiments 2 and 3. Significant main effects were further analysed with a Scheffé *post hoc* test. Effects were considered to be statistically significant when P-values were < 0.05. All data are expressed as the mean ± SE.

Results

Experiment 1: effect of 5-HTP administration on the frontal cortex (FC) and blood 5-HT levels in intact rats

The first experiment was performed in intact rats that did not undergo the abdominal operation. Figure 1A shows the statistical data for the time course of the changes in FC 5-HT levels before and after 5-HTP administration. The mean preinjection 5-HT level in FC was 0.39 ± 0.04 pg/10 μL ($n = 5$). When 5-HTP was administered intravenously, there was a gradual increase in FC 5-HT levels. A one-way ANOVA revealed significant changes for the time course of the mean FC 5-HT level after 5-HTP administration ($F_{4,19} = 9.29$, $P < 0.0001$). There was a significant *post hoc* difference after 5-HTP administration between before and 40 min ($P < 0.01$), 50 min ($P < 0.01$), 60 min ($P < 0.001$) and 70 min ($P < 0.01$) after 5-HTP administration. The maximum FC 5-HT level observed at 60 min after the 5-HTP injection was 1.45 ± 0.44 pg/10 μL ($n = 5$), which was approximately three times higher than the mean preinjection level. FC 5-HT levels gradually decreased thereafter. The mean FC 5-HT level at 180 min after 5-HTP administration was 0.70 ± 0.12 pg/10 μL.

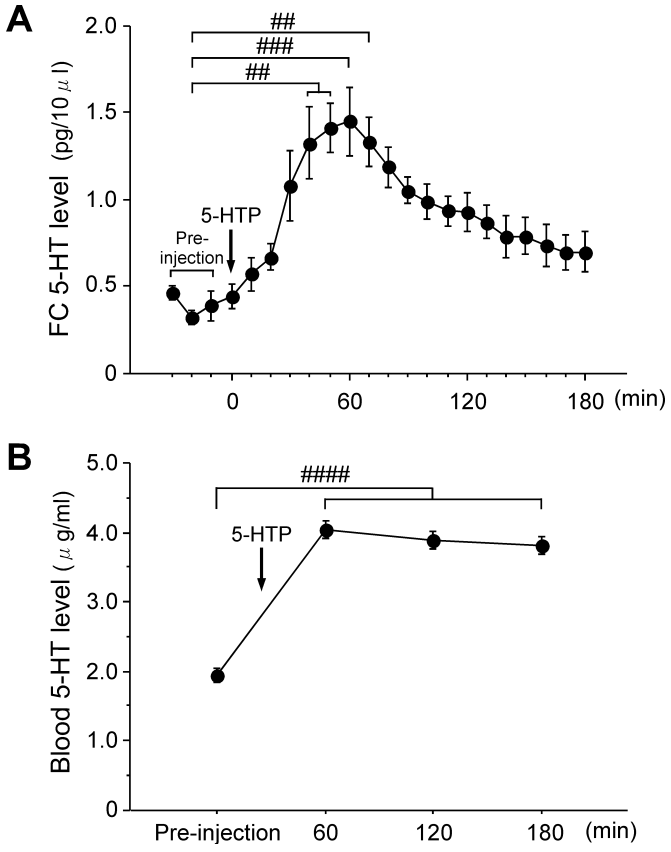

FIG. 1. The effects of 5-HTP administration on (A) the brain and (B) the blood 5-HT levels in intact rats. An arrow indicates the timing of the 5-HTP administration. (A) Time course showing changes in the mean 5-HT level in the FC before and after 5-HTP administration (75 mg/kg, i.v.) in the intact rats. The preinjection level of the FC 5-HT consists of the mean of three successive data points obtained at 10, 20 and 30 min before 5-HTP administration. (B) Time course showing changes in the mean blood 5-HT level before and after 5-HTP administration (75 mg/kg, i.v) in the intact rats. Data are expressed as the mean ± SE ($n = 5$). $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$ vs. preinjection values.

Figure 1B shows the time course for the changes in blood 5-HT levels. The mean blood 5-HT level before 5-HTP administration was 1.95 ± 0.10 μg/mL. A one-way ANOVA revealed significant changes in the time course for the mean blood 5-HT level after 5-HTP administration ($F_{4,3} = 111.5$, $P < 0.0001$). There was a significant *post hoc* difference between before and 60 min ($P < 0.0001$), 120 min ($P < 0.001$) and 180 min ($P < 0.001$) after 5-HTP administration. The maximum blood 5-HT level observed at 60 min after the 5-HTP injection was 4.40 ± 0.14 μg/mL, which was approximately two times higher than the preinjection blood 5-HT level. The blood 5-HT levels continued to remain high until 180 min after 5-HTP administration, although FC 5-HT levels showed an apparent decrease (see Fig. 1A).

Experiment 2: effect of 5-HTP administration on FC 5-HT levels and blood 5-HT levels in rats that had undergone the abdominal operation

The second experiment was performed in 10 rats that had undergone total removal of their gastrointestinal tracts and kidneys, along with liver inactivation. Figure 2 shows the individual time course for the changes in the FC 5-HT levels before and after 5-HTP or saline administration. The mean FC 5-HT level during the preinjection period was 0.89 ± 0.19 pg/10 μL ($n = 6$). After 5-HTP was administered intravenously, there was a gradual increase observed in the FC 5-HT levels, although there were marked differences for the time course and magnitude of the changes in the FC 5-HT level among the six rats examined (Fig. 2A). For example, rat 2 exhibited a relatively rapid and large increase in the FC 5-HT level after 5-HTP administration, with a peak FC 5-HT level of 186.3 pg/10 μL seen at 60 min after 5-HTP administration. On the other hand, rat 4 expressed a slow and small increase in the FC 5-HT level after 5-HTP administration, with a FC 5-HT level of 14.1 pg/10 μL at 60 min and a peak FC 5-HT level of 105.8 pg/10 μL at 120 min after 5-HTP administration. As the time courses for the changes in FC 5-HT levels differed among the six rats after 5-HTP administration, we obtained the first postinjection blood sample at a time when the FC 5-HT level was more than two times higher than the preinjection level. Thus, the first postinjection blood samples (open symbol for each response curve) were drawn between 50 and 70 min after 5-HTP administration in this experiment. The second postinjection samples were taken when the FC 5-HT level further increased, which occurred at 10–30 min after the first postinjection blood samples were drawn.

Figure 2B shows the changes in FC 5-HT levels before and after saline administration in four rats that had total removal of their gastrointestinal tracts and kidneys, along with liver inactivation. The mean FC 5-HT level during the preinjection period was 0.53 ± 0.07 pg/10 μL. Little or no change in the FC 5-HT levels was observed throughout the experiment and up to 120 min after saline administration. Thus for these rats, sequential blood samplings were obtained at 60 and 120 min after saline administration.

Figure 3 shows the statistical data for the time course changes in the blood 5-HT levels after 5-HTP and saline administrations. The mean blood 5-HT level before 5-HTP administration was 2.14 ± 0.11 μg/mL ($n = 6$). There was a gradual increase in the mean blood 5-HT level after 5-HTP administration, whereas there was little change noted for the mean blood 5-HT level after saline administration. A two-way ANOVA revealed significant changes in the time course for the mean blood 5-HT level after 5-HTP administration. The interaction effect on the time course of the mean blood 5-HT level was significant for 5-HTP administration × saline administration ($F_{1,2} = 43.65$, $P < 0.001$). In addition, individual ANOVA revealed a significant change in the time course for the mean blood 5-HT level after 5-HTP administration ($F_{3,2} = 50.41$, $P < 0.001$), which was not seen after saline administration ($F_{3,2} = 0.77$, $P = 0.50$). With regard to the mean blood 5-HT level after 5-HTP administration, there was a

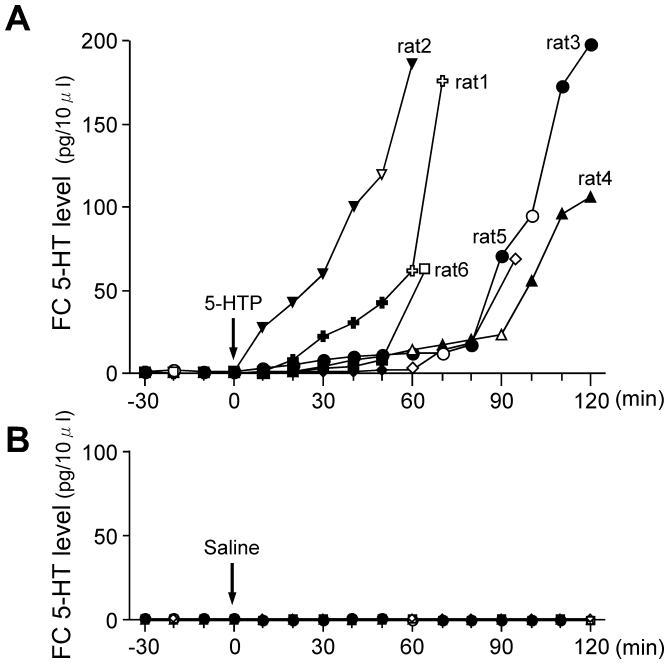

FIG. 2. The effects of (A) 5-HTP and (B) saline administration on FC 5-HT level in rats that had undergone total removal of their gastrointestinal tracts and kidneys, along with liver inactivation. (A) Time courses showing changes in individual FC 5-HT levels before and after 5-HTP administration in six rats that had undergone the abdominal operation. The arrow indicates the timing of 5-HTP administration (75 mg/kg, i.v). (B) Time courses showing changes in individual FC 5-HT levels before and after saline administration (arrow) in four rats that had undergone the abdominal operation. Open symbols indicate the times when blood samples were drawn.

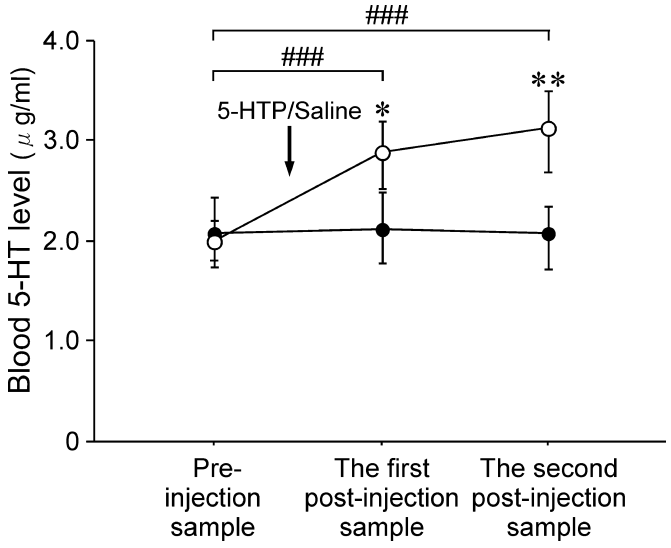

FIG. 3. Time courses showing changes in the mean blood 5-HT levels before and after 5-HTP (○, $n = 6$) and saline (●, $n = 4$) administrations in rats that had undergone total removal of their gastrointestinal tracts and kidneys, along with liver inactivation. The arrow indicates the time of the 5-HTP or saline administration. Data are expressed as the mean ± SE. $^{###}P < 0.001$ vs. preinjection values. $^{*}P < 0.05$, $^{**}P < 0.01$, 5-HTP vs. Saline.

significant *post hoc* difference observed between the preinjection sample and the first ($P < 0.001$) and second ($P < 0.001$) postinjection samples. There was also a significant *post hoc* difference between 5-HTP administration and saline administration for both the first ($P < 0.05$) and second ($P < 0.01$) postinjection samples.

Experiment 3: combined effects of SSRI and 5-HTP administrations on FC 5-HT levels and blood 5-HT levels in rats that had undergone the abdominal operation

The third experiment was also performed in 10 rats that had undergone total removal of their gastrointestinal tracts and kidneys, along with liver inactivation. Figure 4A shows individual time courses for the changes in FC 5-HT levels before and after 5-HTP administration in rats pretreated with SSRI. SSRI administration was performed after the control period and at the point where steady-state FC 5-HT levels could be obtained (Fig. 4B). There was a small nonsignificant increase in FC 5-HT levels after SSRI administration. The mean FC 5-HT level at 30 min after SSRI administration was 1.03 ± 0.20 pg/10 μL, while the mean FC 5-HT level during the control period was 0.46 ± 0.11 pg/10 μL. The first blood sample was obtained during the control period while the second blood sample was drawn at 30 min after SSRI pretreatment. When 5-HTP (30 mg/kg in saline solution) was given intravenously to SSRI-pretreated rats, a more rapid increase in FC 5-HT levels was observed when compared to the corresponding data for rats without SSRI pretreatment (Fig. 2A). The third blood sample was obtained at 30–60 min after 5-HTP administration.

Figure 4B shows individual time courses for the changes in FC 5-HT levels after 5-HTP administration in rats without SSRI pretreatment. Saline solution was administered in the same manner as for the SSRI-pretreated rats. When 5-HTP (30 mg/kg in saline solution) was administered intravenously, we observed differences for both the time course and the magnitude of the changes of the FC 5-HT levels among the five rats studied, which was similar to our findings in Experiment 2. For example, rats 6 and 7 showed rapid and large increases in the FC 5-HT levels after 5-HTP administration. Thus, in these cases blood sampling was performed at 40 min after 5-HTP administration. In the remaining three rats, blood sampling was not performed until FC 5-HT levels clearly increased. In rat 10, a blood sample was obtained at 60 min after 5-HTP administration, and in rats 8 and 9 samples were drawn at 90 min after 5-HTP administration.

Figure 5A shows statistical data for changes in the FC 5-HT levels after 5-HTP administration in rats with and without SSRI pretreatment. The mean FC 5-HT level at the 5-HTP postinjection (Fig. 5A) represents the FC 5-HT level obtained from the last microdialysis sampling point after 5-HTP administration. The mean FC 5-HT levels after 5-HTP administration in rats with and without SSRI pretreatment were 45.40 ± 12.75 pg/10 μL ($n = 5$) and 42.35 ± 12.55 pg/10 μL ($n = 5$), respectively. One-way ANOVA revealed significant changes in the mean FC 5-HT levels after 5-HTP administration in both the rats with SSRI pretreatment ($F_{4,2} = 12.34$, $P < 0.01$) and those without SSRI pretreatment ($F_{4,2} = 10.93$, $P < 0.01$).

Figure 5B shows the statistical data for the time courses of the changes in blood 5-HT levels after 5-HTP administration in rats with and without SSRI pretreatment. The mean blood 5-HT level before SSRI pretreatment (control, Fig. 5B) was 2.02 ± 0.07 μg/mL ($n = 5$). There was a small decrease in the mean blood 5-HT level after SSRI pretreatment (SSRI pretreatment; Fig. 5B), whereas there was little change observed for the mean blood 5-HT level after 5-HTP administration (5-HTP postinjection; Fig. 5B). On the other hand, the mean blood 5-HT level after 5-HTP administration in rats without SSRI pretreatment (Fig. 5B) showed a marked increase after 5-HTP

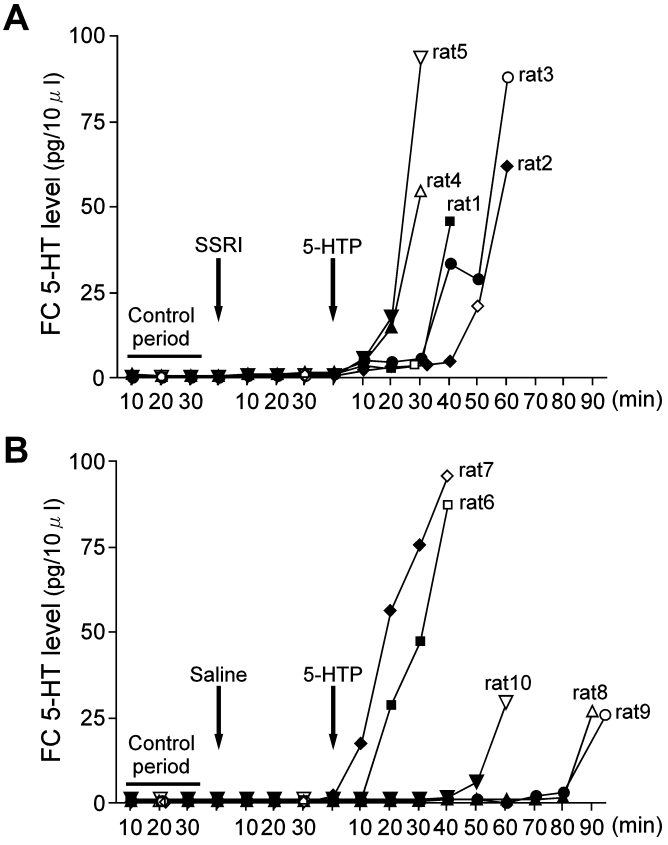

FIG. 4. The effects of 5-HTP administration on individual FC 5-HT levels in rats (A) with or (B) without SSRI pretreatment. Note that this experiment was performed in rats that had undergone total removal of their gastrointestinal tracts and kidneys, along with liver inactivation. (A) Time courses showing changes for the individual FC 5-HT levels before and after 5-HTP administration in five SSRI pretreated rats. The arrow labelled SSRI indicates the time of pretreatment with SSRI (10 mg/kg i.v.). The arrow labelled 5-HTP indicates the time of 5-HTP administration (30 mg/kg, i.v.). Open symbols indicate the time when blood samples for 5-HT analysis were drawn. Note that blood samples obtained during the control period (first blood sample) were drawn 20 min before SSRI pretreatment. The second blood sample was obtained at 30 min after SSRI pretreatment, corresponding to 10 min before 5-HTP administration in all five rats. The third blood sample was drawn at the point where we observed an apparent increase in the FC 5-HT level after 5-HTP administration in each rat. See text for details. (B) Time courses showing changes in the individual FC 5-HT levels before and after 5-HTP administration in the five rats that did not receive any SSRI pretreatment (saline administration). The arrows labelled with saline or 5-HTP indicate the times when saline or 5-HTP were administered. Open symbols indicate the times when blood samples for 5-HT analysis were obtained. The times when the first, second and third blood samples were drawn are the same as those described in A.

administration, which was in contrast to the very small change noted after the saline pretreatment (saline pretreatment, Fig. 5B). Two-way ANOVA revealed significant changes in the time course after 5-HTP administration for both SSRI and saline pretreatment. As illustrated in Fig. 5B, the interaction effect for (SSRI + 5-HTP) administration × (saline + 5-HTP) administration was found to be significant ($F_{1,2} = 55.12$, $P < 0.01$). Individual ANOVA revealed a significant change in the time courses of the mean blood 5-HT level after (SSRI + 5-HTP) administration ($F_{4,2} = 8.34$, $P < 0.05$) and (saline + 5-HTP) administration ($F_{4,2} = 69.38$, $P < 0.0001$). With regard to the mean blood 5-HT level after SSRI or saline pretreatment, there was a significant *post hoc* difference noted between the control and the SSRI/saline pretreatments ($P < 0.05$) only in the SSRI-pretreated rats. As for the mean blood 5-HT level after 5-HTP administration,

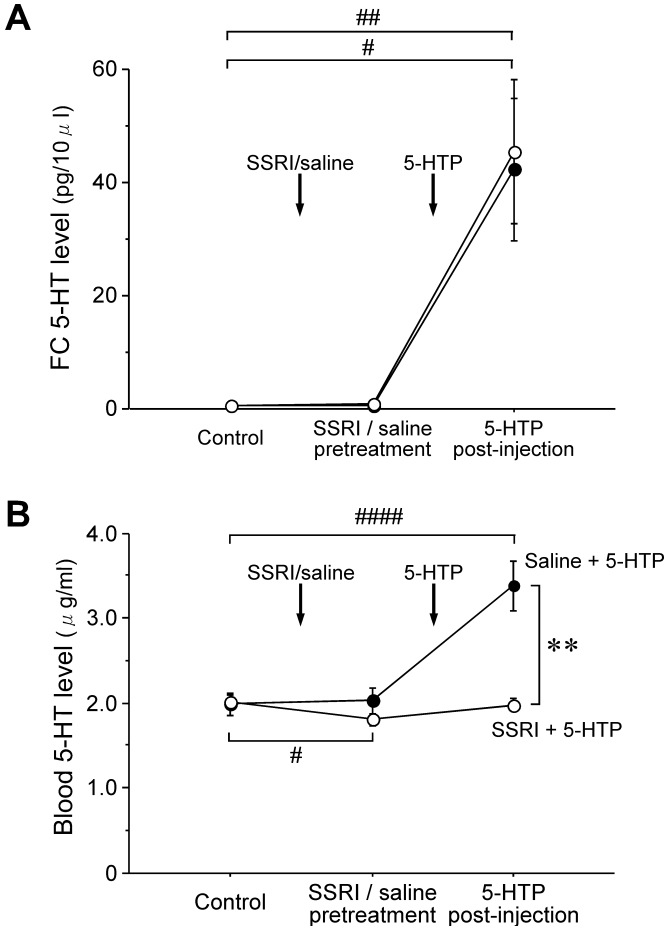

FIG. 5. The effects of 5-HTP administration on (A) FC 5-HT levels and (B) blood 5-HT levels in rats with SSRI or saline pretreatment. All rats underwent a surgical procedure to totally remove their gastrointestinal tracts and kidneys, along with liver inactivation. Arrows labelled SSRI or saline indicate the times of the SSRI (10 mg/kg i.v) or saline pretreatments. Arrows labelled 5-HTP indicate the times of the 5-HTP (30 mg/kg i.v) administrations. ○, Data obtained in rats with SSRI pretreatment ($n = 5$); ●, data points from rats without SSRI pretreatment (saline administration, $n = 5$). Data are expressed as the mean ± SE. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#\#}P < 0.0001$ vs. control. $^{**}P < 0.01$, SSRI + 5-HTP vs. Saline + 5-HTP.

there was a significant *post hoc* difference observed between the control and the 5-HTP postinjection ($P < 0.0001$) in the rats that were not pretreated with SSRI.

There was also a significant *post hoc* difference noted in the blood 5-HT level for the 5-HTP postinjection between the SSRI- and saline-pretreated rats ($P < 0.01$).

Discussion

The present study revealed that whole-blood 5-HT levels exhibited significant augmentation when brain 5-HT levels were elevated after 5-HTP administration in rats that had undergone total removal of their gastrointestinal tracts and kidneys, along with liver inactivation.

This result implies that 5-HT may cross the brain into the circulating blood via the BBB. However, there are two major reasons that may be cited as evidence as to why 5-HT cannot possibly cross from the brain into the circulating blood via the BBB.

First, the BBB is formed by tight junctions of the brain capillary endothelial cells. The role of these junctions is to prevent neurotransmitters including 5-HT from crossing the junction and thus ensuring that all neurotransmitters are retained in the brain. However, recent *in vitro* studies (Brust *et al*., 2000; Wakayama *et al*., 2002) have revealed the presence of 5-HT transporter mRNA in vascular endothelial cells. This indicates that the BBB may act as an efflux transport system for 5-HT. Based on this new data, we conducted the present *in vivo* functional study. As a result, we found that indeed 5-HT can cross from the brain into the circulating blood via the BBB.

The second reason that is cited as to why 5-HT cannot cross the BBB is related to the 5-HT content that is found within the organs. It has been reported that >90% of the total 5-HT content in the whole body is distributed within the gastrointestinal tract (West, 1958; Gaginella, 1995), and that only a very small percentage of the total 5-HT content is found within the brain. Therefore, it has been believed that the augmented ECF 5-HT in the brain does not contribute to any significant changes in the 5-HT levels within the circulating blood. Therefore, we administered 5-HTP intravenously in rats that had undergone the abdominal operation in an attempt to elevate brain 5-HT alone. As a result, we found that whole-blood 5-HT levels significantly increased whenever brain ECF 5-HT levels were elevated by the 5-HTP administration in the rats undergoing the abdominal operation. Therefore, it is reasonable to hypothesize that the augmented brain ECF 5-HT does contribute to a significant change in 5-HT levels within the circulating blood. In other words, augmented brain ECF 5-HT can translocate from the brain into the blood via the BBB.

This hypothesis may be further supported by the data concerning regional differences of the 5-HTP decarboxylase, which is the enzyme responsible for metabolizing 5-HTP to 5-HT. A study by West (1958) demonstrated that there was high 5-HTP decarboxylase activity in the kidneys (188 μg/tissue) and the liver (125 μg/tissue), with only a very low activity noted in the gastrointestinal tract (1–2 μg/tissue). The brain exhibited moderate activity for 5-HTP decarboxylase (32 μg/tissue). In addition, 5-HT that is produced by the gastrointestinal tract is thought to be metabolized by monoamine oxidase in the liver, as it circulates through the portal vein (Gillis, 1985). Therefore, it is unlikely that the gastrointestinal tract would make any significant contribution towards increasing blood 5-HT when 5-HTP is administered to the intact rat.

With regard to intrarenal formation of 5-HT by renal decarboxylase, Stier & Itskovitz, 1985) demonstrated that administration of 5-HTP resulted in an increase in urinary 5-HT without a concomitant increase in plasma 5-HT in the rat. Based on this result, it is reasonable to speculate that intrarenal formation of 5-HT may not contribute to an increase in 5-HT in whole blood after 5-HTP administration.

Based on data by West (1958), the whole organs that exhibit 5-HTP decarboxylase activity include the kidneys, the liver, the gastrointestinal tract, the brain and the skin. After 5-HTP administration in rats that underwent resection of their gastrointestinal tracts and kidneys along with liver inactivation, the skin in addition to the brain were the two organs found to be capable of augmenting the whole-blood 5-HT levels. However, skin has been demonstrated to show the lowest amount of enzyme activity (1 μg/tissue). Thus, it is less likely that 5-HT in the skin contributes to any significant augmentation of 5-HT in the whole blood after 5-HTP administration, even though we can not completely rule out this possibility.

The other new finding of the present study is that SSRI pretreatment in rats lacking functional kidneys, gastrointestinal tract and liver abolished elevation of whole-blood 5-HT levels that was induced by 5-HTP administration, even though the brain 5-HT levels remained increased. These results suggest that the 5-HT transporters that are located on the brain endothelial cells play the inevitable role associated with crossing from the brain via the BBB into the

circulating blood. As discussed earlier, the *in vitro* study by Brust *et al*. (2000) revealed the presence of a 5-HT transporter mRNA in the brain endothelium, indicating that cerebral endothelial cells are able to actively participate in the removal of the released 5-HT within the brain. This possibility was functionally proven in the present *in vivo* physiological study that used the SSRI. Thus, we hypothesized that the 5-HT transporters located on the brain endothelial cells may act as the efflux transport system for the 5-HT that crosses from the brain into the circulating blood.

What physiological role does the 5-HT transporter located on the brain endothelium actually play? It has been established that the 5-HT transporter is present not only in the synaptic terminals of 5-HT neurons but also in the brain endothelial cells (Brust *et al*., 2000; Wakayama *et al*., 2002). Therefore, we can hypothesize that both 5-HT transporters play a significant role in 5-HT homeostasis within the brain. There is no doubt that 5-HT transporters located on the terminals of 5-HT neurons play an inevitable role in 5-HT homeostasis within the synaptic cleft. On the other hand, it has been reported that 5-HT's role is more in line with volume transmission than classical neurotransmission (Fuxe & Agnati, 1991; Hornung, 2003; Fuxe *et al*., 2007). 5-HT released from the axonal terminals and varicosities diffuses over a long distance to act on other neurons. In this particular case, the released 5-HT could be detected as a change in ECF 5-HT in the brain. Augmented ECF 5-HT within the brain may be drained away via the endothelial 5-HT transporter that is located in the nearby endothelial cells, thereby maintaining 5-HT homeostasis in the brain. In other words, the physiological role of the BBB is not only to act as a barrier but also to play a role as a regulatory interface for brain ECF 5-HT.

In summary, increased brain ECF 5-HT is removed from the brain into the circulating blood via the 5-HT transporter system located on the brain endothelial cells.

Abbreviations

5-HT, 5-hydroxytryptamine, serotonin; 5-HTP, 5-hydroxytryptophan; BBB, blood–brain barrier; ECF, extracellular fluid; FC, frontal cortex; HPLC, high-performance liquid chromatography; SSRI, selective serotonin reuptake inhibitor.

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