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Effects of conditioned fear stress on serotonin neurotransmission and freezing behavior in rats

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Abstract

In an attempt to clarify the role of the brain serotonergic system in the psychopathology of anxiety, we examined the effect of a psychological stress, conditioned fear stress, on extracellular serotonin (5-hydroxytryptamine, 5-HT) concentrations in the rat medial prefrontal cortex using the method of in vivo microdialysis, while simultaneously observing conditioned fear stress-induced freezing behavior, an index of anxiety. Conditioned fear stress increased extracellular 5-HT levels in the medial prefrontal cortex, and this 5-HT level increase was followed by a resolution of the freezing behavior. A dose of 10 mg/kg of a selective 5-HT reuptake inhibitor, citalopram, administered 60 min before exposure to conditioned fear stress increased extracellular 5-HT concentrations immediately and potently, reducing freezing behavior. These findings strongly suggest that facilitation of brain 5-HT neurotransmission decreases anxiety, which is in agreement with the clinical reports that selective 5-HT reuptake inhibitors are effective in the treatment of anxiety disorders.

Keywords: 5-HT (hydroxytryptamine, serotonin); Conditioned fear stress; Freezing behavior; Microdialysis, in vivo ; Medial prefrontal cortex; Citalopram

1. Introduction

Recent clinical studies have indicated that various serotonin (5-hydroxytryptamine, 5-HT)-related agents, such as selective 5-HT reuptake inhibitors (Den Boer and Westenberg, 1988; Eriksson and Humble, 1990; Van der Kolk et al., 1994; Van Vliet et al., 1994; Boyer, 1995; Oehrberg et al., 1995; Gorman, 1997; Gunasekara et al., 1998), 5-HT_{1A} receptor agonists (Kahn et al., 1988; Dubovsky, 1990; Boyer and Feighner, 1993; Cutler et al., 1993), 5-HT_2 receptor antagonists (Kahn et al., 1988; Stefanski and Goldberg, 1997), monoamine oxidase inhibitors (Eriksson and Humble, 1990; Lott et al., 1997; Tiller et al., 1997; Schneier et al., 1998) and 5-HT-precursor 5-hydroxy-L-tryptophan (Kahn et al., 1988; Eriksson and Humble, 1990), are effective in the treatment of anxiety disorders. These findings support the hypothesis that a defect in the brain 5-HT neuron system is closely related to anxiety disorders. However, there is continuing controversy as to whether facilitation of brain 5-HT neurotransmission decreases (Eriksson and Humble, 1990; Handley and Mc Blane, 1993) or increases anxiety (Wise et al., 1972; Iversen, 1984).

In rodents, exposure to an environment paired with previously inescapable electric foot shock, reliably elicits a response characterized by a period of crouching and complete immobility (Blanchard and Blanchard, 1969; Bolles, 1970; Bolles and Collier, 1976; Bouton and Bolles, 1980). This behavior is termed conditioned fear stress-induced freezing behavior. Conditioned fear stress is regarded as a psychological stress without physical stimuli, and it has been proposed that freezing behavior could be used as a model of anxiety (Fanselow and Helmstetter, 1988; Kalin et al., 1988).

We have reported that conditioned fear stress-induced freezing behavior in rats was attenuated by selective 5-HT reuptake inhibitors, 5-HT_{1A} receptor agonists and 5-hydroxyl-L-tryptophan (Hashimoto et al., 1996; Inoue et al., 1996). Furthermore, it has been shown that conditioned fear stress selectively increased 5-HT metabolism in the medial prefrontal cortex (Inoue et al., 1993, 1994). These findings also suggest that the brain, especially the medial prefrontal cortex serotonergic system, is deeply involved in the psychopathology of anxiety.

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In an attempt to clarify the role of the brain serotonergic system in the psychopathology of anxiety, we examined the effect of conditioned fear stress on extracellular 5-HT concentrations in the medial prefrontal cortex using in vivo microdialysis while simultaneously observing the conditioned fear stress-induced freezing behavior. Moreover, we also studied the effects of a selective 5-HT reuptake inhibitor, citalopram, on extracellular 5-HT concentrations in the medial prefrontal cortex and on freezing behavior under conditioned fear stress.

2. Materials and methods

2.1. Animals and drug

Male Sprague–Dawley rats were obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan). They weighed between 230 and 270 g, were housed in groups of four and maintained in a 12 L:12 D (lights on at 0700), temperature-controlled environment (22 ± 1°C) with free access to food and water. Experiments began after a 2-week acclimatization period. Rats were tested between 0830 and 1530.

Citalopram hydrobromide (H. Lundbeck, Denmark) was dissolved in 0.9% sterile saline and injected subcutaneously. The drug was administered in a volume of 1 ml/kg.

2.2. Conditioned fear stress-induced freezing

The duration of the conditioning sessions was one 5-min session on 1 day, or two 30-min sessions conducted over 2 consecutive days. Rats were subjected to inescapable electric foot shock for a total of 2.5 min (2.5 mA scrambled shock, 10 ms shock every 100 ms; shock duration of 30 s × 5; and an intershock interval of 30 s), or 15 min (1.0 mA scrambled shock, 10 ms shock every 100 ms; shock duration of 30 s × 30; an intershock interval of 30 s, on 2 consecutive days), in a chamber with a grid floor (30 × 24 × 30 cm, Med Associates, USA). Electric shock was provided via an ENV-410 shock generator (Med Associates). This generator provides a high-voltage, high-resistance circuit with resistance controlled by dial settings calibrated by the manufacturer in a short circuit current. At a setting of 2.5 mA, the generator actually gives a shock intensity of 0.2 mA to rats. About 28 h after the electric foot shock, the rats were again placed and observed for 30 min in the shock chamber, but no current was applied to the floor of the chamber. During the observation period (three 10-min blocks; blocks 1–3), the duration of the freezing behavior was recorded using a time-sampling procedure as previously described (Hashimoto et al., 1996, 1997). Freezing was defined as the absence of all observable movement of the skeleton and the vibrissae, except those related to respiration. All other behavior was scored as activity. The animal was classified as either freezing or active according to its behavior throughout the entire 10-s period. The percentage score represented the number of 10-s periods during which the animal froze for the entire 10 s. Behavior was recorded on videotape and scored by two independent observers (one of whom did not know the experimental grouping of the animals). Inter-scorer reliability by this method was very high (more than 0.95).

2.3. Microdialysis

Rats were implanted stereotaxically under pentobarbital anesthesia (30 mg/kg, i.p.) with AG-4 guide cannulae (Eicom, Japan) leading to the surface of the medial prefrontal cortex at the following coordinates relative to the bregma: A + 3.2, ML + 0.8, DV + 1.0 mm (Paxinos and Watson, 1986). Then, dialysis probes with an outer diameter of 0.105 mm (A-I-4-03; Eicom) were inserted into the guide cannulae so that 3.0 mm of the probe was exposed to the tissue of the medial prefrontal cortex. Rats were housed individually after these operations.

On the following day, perfusion was started in the home cage using artificial cerebrospinal fluid (145 mM NaCl, 3.0 mM KCl, 1.3 mM $CaCl_2$, 1.0 mM $MgCl_2$) at a flow rate of 2 μl/min. Dialysis samples were collected in sample vials containing 10 μl of 0.2 M acetic acid with 20 mg/l cystein. Following initial perfusion for 3 h, dialysate samples were collected every 10 min for 200 min. Twenty microliters of the dialysate was injected into a high-performance liquid chromatography (HPLC) system to determine the extracellular levels of 5-HT. Values were expressed as a percentage of the average level of the baseline samples.

2.4. HPLC

The HPLC system consisted of an EP-300 liquid chromatograph pump (Eicom), a DGU-4A degasser (Shimadzu, Japan), a reversed phase ODS column, Eicompak CA-5ODS 150 × 2.1 mm (Eicom), an ECD-300 electrochemical detector (Eicom) and a C-R4A chromatopac (Shimadzu). The mobile phase was a 0.1-M phosphate buffer (pH 6.0) containing 20% (v/v) methanol, 50 mg/l Na_2EDTA and 500 mg/kg 1-octanesulfonate. The buffer was filtered before use. Separations were conducted at 25°C with a flow rate of 0.23 ml/min. The electrochemical detector was set at a potential of 450 mV. A standard solution containing authentic 5-HT was injected every working day, and the amount of 5-HT was determined by comparison with the peak height of the standard.

2.5. Experimental design

Experiment I was designed to study the effects of conditioned fear stress on 5-HT neurotransmission and freezing behavior. Rats were subjected to inescapable electric foot shock (1: 2.5 mA, 30 s × 5, intershock interval of

30 s; or 2: 1.0 mA, 30 s × 30, intershock interval of 30 s, on 2 consecutive days) in a chamber with a grid floor. Control rats (non-conditioned fear stress) were placed in the shock chamber, but no current was applied to the floor of the chamber. Subsequently, rats were implanted stereotaxically with guide cannulae leading to the surface of medial prefrontal cortex, and dialysis probes were inserted into the guide cannulae. Perfusion was started about 20 h later using artificial cerebrospinal fluid. One hundred minutes after the start of the collection of dialysate samples, the rats were again placed for 30 min in the shock chamber, but no current was applied to the floor of the chamber. After exposure to conditioned fear stress, rats were returned to the home cages.

Experiment II was designed to study the effects of citalopram on 5-HT neurotransmission and on freezing behavior under conditioned fear stress. Rats were subjected to inescapable electric foot shock (2.5 mA, 30 s × 5, intershock interval of 30 s) in a chamber with a grid floor. Subsequently, rats were implanted stereotaxically with guide cannulae leading to the surface of medial prefrontal cortex, and dialysis probes were inserted into the guide cannulae. Perfusion was started about 20 h later using artificial cerebrospinal fluid. One hundred minutes after the start of collection of dialysate samples, the rats were again placed for 30 min in the shock chamber, but no current was applied to the floor of the chamber. Citalopram (10 mg/kg) or saline was administered 60 min before exposure to conditioned fear stress. After exposure to conditioned fear stress, rats were returned to the home cages.

These procedures were approved by the Hokkaido University School of Medicine Animal Care and Use Committee and were in compliance with the Guide for the Care and Use of Laboratory Animals, Hokkaido University School of Medicine.

2.6. Data analysis

All the data are presented as the means ± S.E.M. of individual values of the rats from each group. 5-HT baselines were calculated for each rat from the mean of dialysate 5-HT samples collected before exposure to the stressor (Experiment I) or administration of the drug (Experiment II). The 5-HT content of dialysate samples were expressed as a percent change from baseline. The effect of conditioned fear stress (100–200 min), or drug (50–200 min), on 5-HT levels was analyzed by a two-way analysis of variance (ANOVA) with repeated measures, using treatment as a between-subjects variable and time as a repeated-measures variable. The effects of conditioned fear stress, or the drug, at individual time points were compared with corresponding control group values using Dunnett's test. Behavioral analysis was performed using Student's *t*-test.

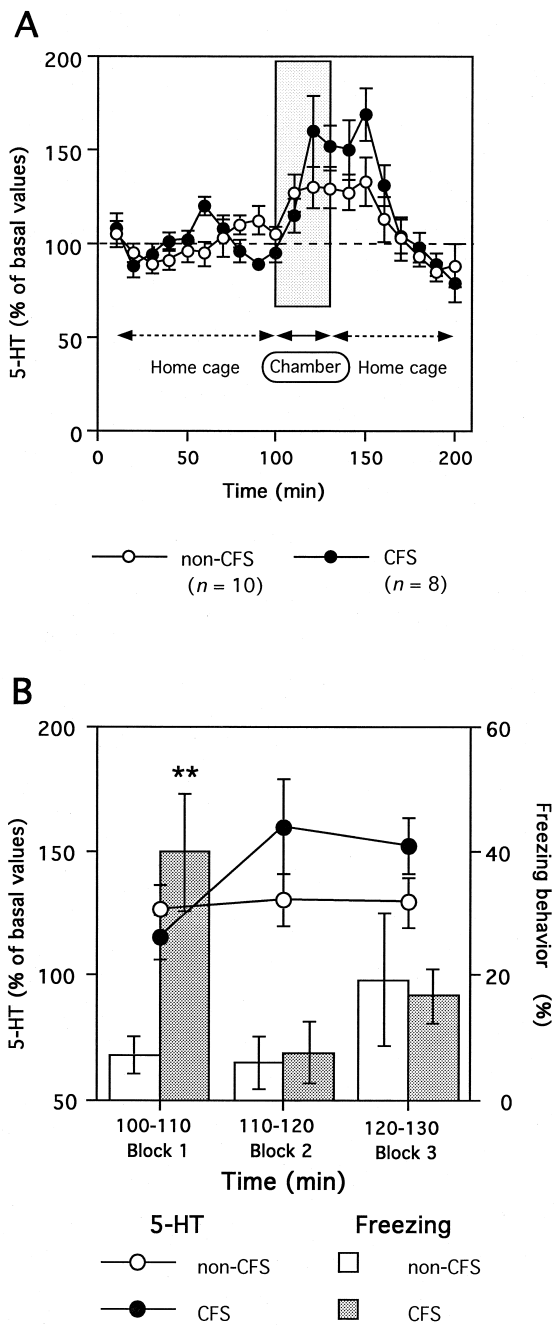

Fig. 1. Effects of conditioned fear stress on extracellular 5-HT concentrations in the medial prefrontal cortex (A) and extracellular 5-HT concentrations and freezing behavior (B). Rats were subjected to inescapable electric foot shock (2.5 mA, 30 s × 5, intershock interval of 30 s) in a chamber with a grid floor. Subsequently, rats were implanted stereotaxically with guide cannulae leading to the surface of the medial prefrontal cortex, and dialysis probes were inserted into the guide cannulae. Perfusion was started about 20 h later using artificial cerebrospinal fluid. The rats were again placed for 30 min in the shock chamber, but no current was applied to the floor of the chamber. Values represent the mean ± S.E.M. Conditioned fear stress (CFS) group showed a significant increase in 5-HT concentrations compared with non-CFS group (two-way ANOVA, $P < 0.05$, 100–200 min). $^{**}P < 0.01$ significant difference from the corresponding non-CFS group.

3. Results

3.1. Effects of conditioned fear stress on extracellular 5-HT levels and freezing behavior

Fig. 1A and B show the results of Experiment I-1 (rats were conditioned by the electric foot shock of 2.5 mA, 30 s × 5). Fig. 1A illustrates the time-course effect of conditioned fear stress on extracellular 5-HT levels in the medial prefrontal cortex. The absolute values of 5-HT per dialysis sample of control and treated animals were 0.19 ± 0.02 and 0.34 ± 0.14 pg/20 μl, respectively. Two-way ANOVA indicated a significant effect of treatment ($F(1,155) = 6.621$, $P < 0.05$) and a significant effect of time ($F(9,155) = 10.214$, $P < 0.01$), but not interaction between treatment and time ($F(9,155) = 1.150$, n.s.). Group values for the effect of conditioned fear stress did not differ between the conditioned fear stress group and the non-conditioned fear stress group. Fig. 1B indicates the time-course effects of conditioned fear stress on freezing behavior and on 5-HT levels while the animals were in the chamber. Conditioned fear stress-induced freezing behavior was observed immediately after transfer to the chamber (0–10 min, block 1 in Fig. 1B), and the freezing duration of the conditioned fear stress group was significantly increased compared to that of the non-conditioned fear stress group ($P < 0.01$, Student's *t*-test). At 10–20 min and 20–30 min after the transfer (blocks 2 and 3 in Fig. 1B, respectively), no significant difference was observed in freezing between the two groups. It was found that the 5-HT levels of the conditioned fear stress group did not increase while the animals were freezing, and that there was a tendency for 5-HT levels to increase when freezing was resolved.

Fig. 2A and B show the results of Experiment I-2 (rats were conditioned by the electric foot shock of 1.0 mA, 30 s × 30 × 2 days). Fig. 2A illustrates the time-course effect of conditioned fear stress on extracellular 5-HT levels in the medial prefrontal cortex. The absolute values of 5-HT per dialysis sample of control and treated animals were 0.18 ± 0.03 and 0.13 ± 0.02 pg/20 μl, respectively. Two-way ANOVA indicated a significant effect of treatment ($F(1,136) = 18.077$, $P < 0.01$) and a significant effect of time ($F(9,136) = 5.820$, $P < 0.01$), but not interaction between treatment and time ($F(9,136) = 0.926$, n.s.). Group values for the effect of conditioned fear stress differed between the conditioned fear stress group and the non-conditioned fear stress group at 120 min ($P < 0.05$, Dunnett's test) and 130 min ($P < 0.01$, Dunnett's test). Fig. 2B indicates the time-course effects of conditioned fear stress on freezing behavior and on 5-HT levels while the animals were in the chamber. Conditioned fear stress-induced stubborn freezing behavior was observed immediately after transfer to the chamber (0–10 min, block 1 in Fig. 2B), and the freezing duration of the conditioned fear stress group was significantly increased compared to that of the

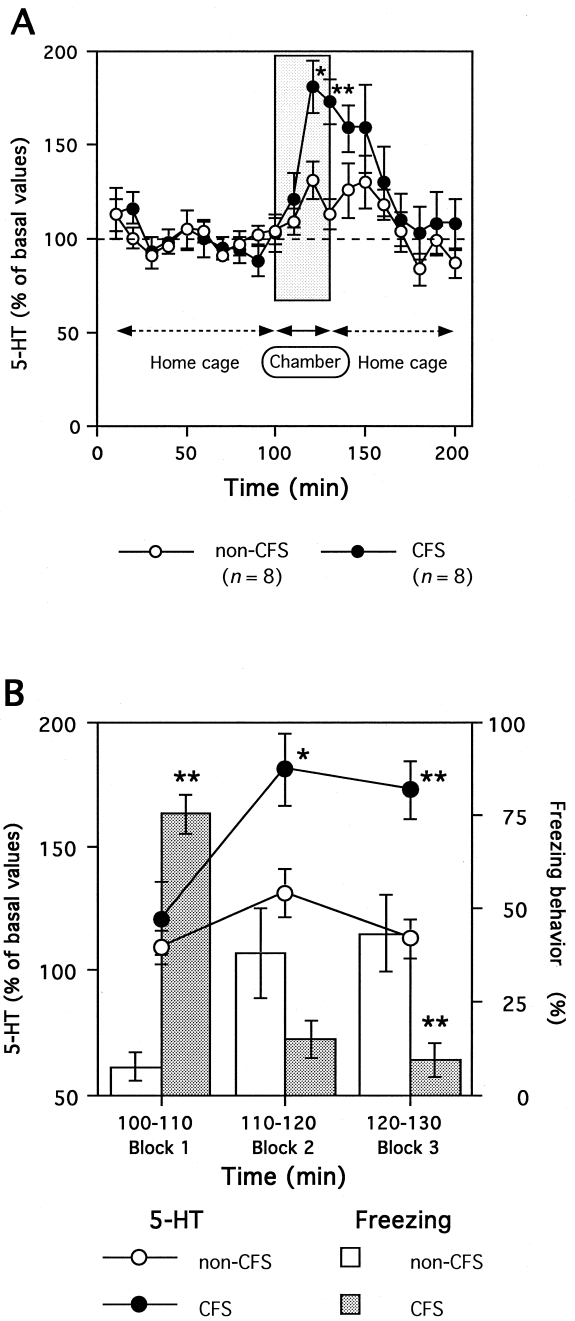

Fig. 2. Effects of conditioned fear stress on extracellular 5-HT concentrations in the medial prefrontal cortex (A) and extracellular 5-HT concentrations and freezing behavior (B). Rats were subjected to inescapable electric foot shock (1.0 mA, 30 s × 30, intershock interval of 30 s, on 2 consecutive days) in a chamber with a grid floor. Subsequently, rats were implanted stereotaxically with guide cannulae leading to the surface of the medial prefrontal cortex, and dialysis probes were inserted into the guide cannulae. Perfusion was started about 20 h later using artificial cerebrospinal fluid. The rats were again placed for 30 min in the shock chamber, but no current was applied to the floor of the chamber. Values represent the mean ± S.E.M. Conditioned fear stress (CFS) group showed a significant increase in 5-HT concentrations compared with non-CFS group (two-way ANOVA, $P < 0.01$, 100–200 min). $^{*}P < 0.05$, $^{**}P < 0.01$ significant difference from the corresponding non-CFS group.

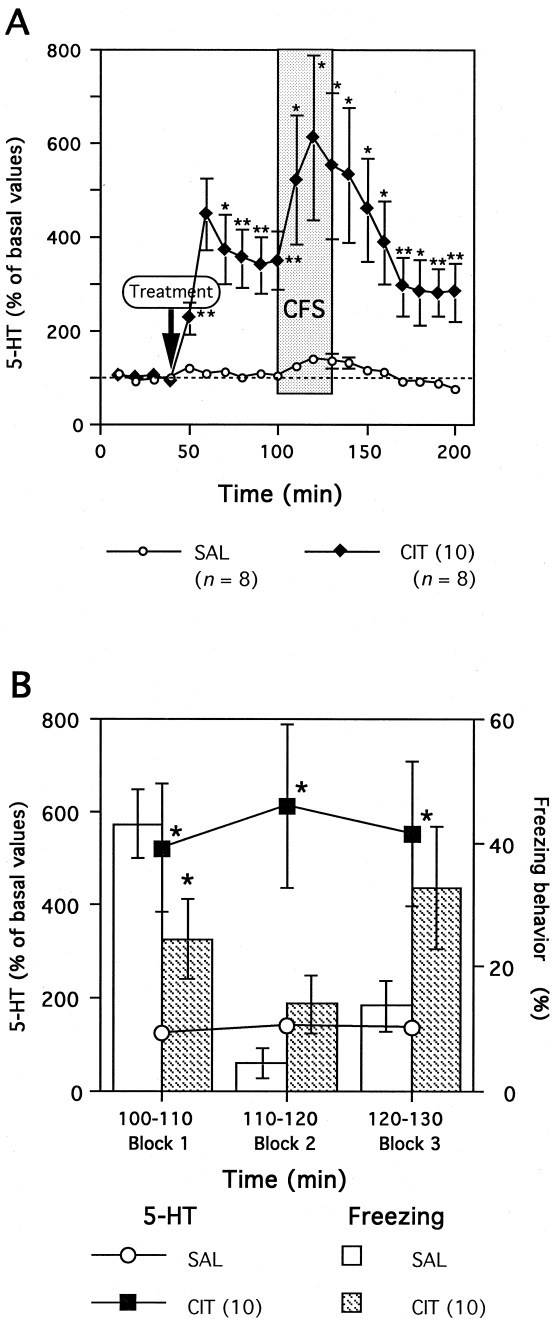

Fig. 3. Effects of citalopram on extracellular 5-HT concentrations in the medial prefrontal cortex (A) and extracellular 5-HT concentrations and freezing behavior (B) under conditioned fear stress. Rats were subjected to inescapable electric foot shock (2.5 mA, 30 s×5, intershock interval of 30 s) in a chamber with a grid floor. Subsequently, rats were implanted stereotaxically with guide cannulae leading to the surface of the medial prefrontal cortex, and dialysis probes were inserted into the guide cannulae. Perfusion was started about 20 h later using artificial cerebrospinal fluid. The rats were again placed for 30 min in the shock chamber, but no current was applied to the floor of the chamber. Citalopram (CIT, 10 mg/kg) or saline (SAL) was administered subcutaneously 60 min before exposure to conditioned fear stress (CFS). Values represent the mean ± S.E.M. CIT group showed a significant increase in 5-HT concentrations compared with SAL group (two-way ANOVA, $P < 0.01$, 50–200 min). $^{*}P < 0.05$, $^{**}P < 0.01$ significant difference from the corresponding SAL group.

non-conditioned fear stress group ($P < 0.01$, Student's *t*-test). At 10–20 min and 20–30 min after the transfer (blocks 2 and 3 in Fig. 2B, respectively), freezing behavior of the conditioned fear stress group was resolved. It was found that the 5-HT levels of the conditioned fear stress group did not increase while the animals were freezing, and that their 5-HT levels were significantly increased compared to that of the non-conditioned fear stress group when freezing was resolved. During blocks 2 and 3 of the non-conditioned fear stress group, freezing behavior was spuriously observed (Fig. 2B).

3.2. Effects of citalopram on extracellular 5-HT levels and freezing behavior under conditioned fear stress

Fig. 3A and B show the results of Experiment II. Fig. 3A illustrates the time-course effect of citalopram on extracellular 5-HT levels in the medial prefrontal cortex. The absolute values of 5-HT per dialysis sample of control and treated animals were 0.28 ± 0.06 and 0.34 ± 0.10 pg/20 μl, respectively. Citalopram increased extracellular 5-HT concentrations immediately and strongly. Two-way ANOVA indicated a significant effect of treatment ($F(1,213) = 101.975$, $P < 0.01$), but not an effect of time ($F(15,213) = 1.608$, n.s.) nor an interaction between treatment and time ($F(15,213) = 0.880$, n.s.). Group values for the effect of citalopram differed between the groups treated with citalopram and saline at 50 min and from 70 to 200 min (Dunnett's test). Fig. 3B indicates the time-course effects of citalopram on freezing behavior and on 5-HT levels while the animals were in the chamber. At 0 to 10 min after the transfer (block 1 in Fig. 3B), while freezing behavior was observed in the saline group, a dose of 10 mg/kg of citalopram significantly reduced freezing behavior ($P < 0.05$, Student's *t*-test), and the 5-HT levels of citalopram group were significantly increased compared to that of the saline group ($P < 0.05$, Dunnett's test). At 10 to 20 min after the transfer (block 2 in Fig. 3B), 5-HT levels of both groups peaked, and the freezing behavior of the saline group was resolved. At 20 to 30 min after the transfer (block 3 in Fig. 3B), freezing behavior was spuriously observed in the citalopram group.

4. Discussion

In the present study, we have established a system which can simultaneously evaluate the dynamic changes of behavior and 5-HT release induced by the psychological stress of conditioned fear stress, using in vivo microdialysis. This study has shown three important features. The first is that conditioned fear stress induced freezing behavior, an index of anxiety, and increased extracellular 5-HT levels in the medial prefrontal cortex. The second is that the 5-HT levels of the conditioned fear stress group did not increase while the animals were freezing, and their 5-HT

levels increased when freezing was resolved. The third is that the increase of 5-HT levels in the medial prefrontal cortex induced by a selective 5-HT reuptake inhibitor reduced freezing behavior. These findings strongly suggest that facilitation of brain 5-HT neurotransmission decreases anxiety, and this is in agreement with the clinical reports that selective 5-HT reuptake inhibitors are effective in the treatment of anxiety disorders (Den Boer and Westenberg, 1988; Eriksson and Humble, 1990; Van der Kolk et al., 1994; Van Vliet et al., 1994; Boyer, 1995; Gorman, 1997; Gunasekara et al., 1998).

In a preliminary examination, we found that the rats conditioned by the electric foot shock of 1.0 mA, 30 s $\times$ 30 $\times$ 2 days exhibited stubborner freezing behavior than those conditioned by the electric foot shock of 2.5 mA, 30 s $\times$ 5. Accordingly, Experiment I was designed to find out whether the changes of 5-HT release were anxiety-dependent. In Experiment I, conditioned fear stress induced freezing behavior as well as increased extracellular 5-HT concentrations in the medial prefrontal cortex. These responses were anxiety-dependent because when the maximal effects of conditioned fear stress on the duration of the freezing behavior per 10 min block were, respectively, 39.8 and 75.4%, the maximal effects of conditioned fear stress on 5-HT levels were 159.7 and 181.0%, respectively. It has been established that the spontaneous output of 5-HT measured in brain dialysates originates predominantly from 5-HT neurons and changes in accordance with their electric activity (Sharp et al., 1989). From these findings, it has become evident that brain 5-HT neurons were activated, and 5-HT releases were increased at the terminal under anxiety conditions. The data obtained in the present study are in agreement with the previously reported findings that conditioned fear stress increased 5-HT metabolism only in the medial prefrontal cortex (Inoue et al., 1993; Inoue et al., 1994) and that aversive conditions, i.e., the elevated plus maze, increased cortical 5-HT release up to about 160% of baselines in guinea-pigs (Rex et al., 1993).

Citalopram is a potent and selective inhibitor of 5-HT reuptake. In addition to its lack of effect on other monoamine reuptake mechanisms, citalopram has a low affinity for the receptors of a variety of neurotransmitters (Hyttel, 1982). We have reported that citalopram reduced conditioned fear stress-induced freezing behavior without affecting motor activity under the conditions of Experiment II (Hashimoto et al., 1996). The inhibitory effects of citalopram on freezing behavior have been reproduced in the present study. Since citalopram (10 mg/kg) increased extracellular 5-HT concentrations in the medial prefrontal cortex immediately and strongly in this study and the preliminary study (350% of baselines), it seems reasonable to suppose that the increase of 5-HT levels in the serotonergic nerve terminals induced by citalopram reduced freezing behavior, or in other words, produced an anxiolytic effect.

The excess extracellular 5-HT produced by 5-HT reuptake inhibitors in the cell body region (raphe nuclei) activates presynaptic 5-HT_{1A} autoreceptors and slows down the firing rate of 5-HT neurons and their terminal release (Artigas, 1993). The possibility exists that citalopram, by its presynaptic indirect action, attenuated freezing behavior. Citalopram, however, strongly increased 5-HT levels in the medial prefrontal cortex in this study. It is obvious that the mechanism of the selective 5-HT reuptake inhibitor-induced antifreezing action is the facilitation of brain 5-HT neurotransmission, and not the suppression of the firing rate of 5-HT neurons and their terminal release. In fact, recent studies have shown that co-administration of 5-HT_{1A} autoreceptor antagonist with a selective 5-HT reuptake inhibitor allows the selective 5-HT reuptake inhibitor to induce an immediate increase in terminal 5-HT release (Hjorth, 1993, 1996; Hjorth and Auerbach, 1994), and a 5-HT_{1A} receptor antagonist enhanced the antifreezing effect of a selective 5-HT reuptake inhibitor by blocking the autoreceptor-mediated negative feedback mechanisms of 5-HT neurons, but did not antagonize the effect of a selective 5-HT reuptake inhibitor by blocking its presynaptic indirect action (Hashimoto et al., 1997).

While selective 5-HT reuptake inhibitors exhibit a clinical anxiolytic effect only after several weeks treatment (Oehrberg et al., 1995), acute challenge with citalopram reduced conditioned fear stress-induced freezing behavior in this study. It is likely that initiation of the anxiolytic response is associated with the 5-HT levels at the nerve terminal. A low dose (1 mg/kg) of citalopram did not reduce freezing behavior (Hashimoto et al., 1996), and did not affect extracellular 5-HT concentrations in the frontal cortex of rats repeatedly given saline, but the same dose level did markedly raise extracellular 5-HT in rats previously injected with citalopram (10 mg/kg) for 14 days (Invernizzi et al., 1994). A low dose of selective 5-HT reuptake inhibitors increases terminal 5-HT levels when presynaptic 5-HT_{1A} receptors were desensitized after long-term treatment (Artigas, 1993). This study, on the other hand, revealed that a high dose (10 mg/kg) of citalopram markedly increased extracellular 5-HT in the medial prefrontal cortex. It seems reasonable to suppose that if acute challenge with a selective 5-HT reuptake inhibitor adequately increases 5-HT levels in the nerve terminal, the increased 5-HT produces an anxiolytic effect immediately.

The 5-HT levels of the conditioned fear stress group did not increase while the animals were freezing, and their 5-HT levels were increased when freezing was resolved in Experiment I. Namely, the maximum of the extracellular 5-HT concentrations was detected during block 2 but not during block 1, while freezing behavior was observed during block 1 and reduced during block 2. It is possible that the increase of 5-HT levels during block 2 is associated with the resolution of freezing behavior. Since we calculated the volume of the tube from the dialysis probe

to the sample vial, the time lag before recovery to the sample vial is negligible. In fact, transfer to the chamber induced a mild increase in 5-HT levels during block 1 in the non-conditioned fear stress group (Experiment I-1), and citalopram immediately increased extracellular 5-HT concentrations (Experiment II).

In Experiment II, the maximal 5-HT level of the saline group (140.0%) was revealed during block 2 in the same way that the 5-HT levels of the conditioned fear stress group did not increase while the animals were freezing, and their 5-HT levels increased when freezing was resolved in Experiment I. While citalopram significantly reduced freezing behavior during block 1, the 5-HT level of the citalopram group during block 1 was 521.9% and separate from the 5-HT level of the saline group when freezing was resolved during block 2. The expression of conditioned fear stress-induced anxiety is attenuated by 5-HT. The process of resolution in conditioned fear stress-induced anxiety, on the other hand, may be associated with various neurotransmitters, including 5-HT.

During blocks 2 and 3 of the non-conditioned fear stress group in Experiment I-2 and during block 3 of the citalopram group in Experiment II, freezing behavior was spuriously observed. Rats were, however, obviously sleeping in these periods. Spurious freezing behavior was sedation induced by adaptation and not by a relapse of anxiety.

We have attempted to clarify the role of the brain serotonergic system in the psychopathology of anxiety. In conclusion, (1) brain 5-HT neurons are activated and 5-HT releases are increased at the serotonergic nerve terminal under anxiety conditions; (2) the increase of 5-HT levels in the terminal produces an anxiolytic action and is closely related to the pharmacological effects of the selective 5-HT reuptake inhibitor-class of anxiolytics. It should be, however, taken into consideration that various neurotransmitter systems, including 5-HT, are related to the psychopathology of anxiety. There are needs for further investigation and accumulation of clinical evidence.

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References

Artigas, F., 1993. 5-HT and antidepressants: new views from microdialysis studies. Trends Pharmacol. Sci. 14, 262.

Blanchard, R.J., Blanchard, D.C., 1969. Crouching as an index of fear. J. Comp. Physiol. Psychol. 67, 370–375.

Bolles, R.C., 1970. Species-specific defense reactions and avoidance learning. Psychol. Rev. 77, 32–48.

Bolles, R.C., Collier, A.C., 1976. The effect of predictive cues on freezing in rats. Anim. Learn. Behav. 4, 6–8.

Bouton, M.E., Bolles, R.C., 1980. Conditioned fear assessed by freezing and by the suppression of three different baselines. Anim. Learn. Behav. 8, 429–434.

Boyer, W.F., 1995. Serotonin uptake inhibitors are superior to imipramine and alprazolam in alleviating panic attacks: a meta analysis. Int. Clin. Psychopharmacol. 10, 45–49.

Boyer, W.F., Feighner, J.P., 1993. A placebo-controlled double-blind multicenter trial of two doses of ipsapirone versus diazepam in generalized anxiety disorder. Int. Clin. Psychopharmacol. 8, 173–176.

Cutler, N.R., Sramek, J.J., Wardle, T.S., Hesselink, J.M., Roeschen, J.K., 1993. The safety and efficacy of ipsapirone vs. lorazepam in outpatients with generalized anxiety disorder (GAD): single site findings from a multicenter trial. Psychopharmacol. Bull. 29, 303–308.

Den Boer, J.A., Westenberg, H.G.M., 1988. Effect of serotonin and noradrenaline uptake inhibitor in panic disorder: a double-blind comparative study with fluvoxamine and maprotiline. Int. Clin. Psychopharmacol. 3, 59–74.

Dubovsky, S.L., 1990. Generalized anxiety disorder: new concepts and psychopharmacologic therapies. J. Clin. Psychiatry 51, 3–10, Suppl.

Eriksson, E., Humble, M., 1990. Serotonin in psychiatric pathophysiology: a review of data from experimental and clinical research. In: Pohl, R., Gershon, S. (Eds.), Progress in Basic Clinical Pharmacology, Vol. 3. The Biological Basis of Psychiatric Treatment. Karger, Basel, pp. 66–119.

Fanselow, M.S., Helmstetter, F.J., 1988. Conditional analgesia, defensive freezing, and benzodiazepines. Behav. Neurosci. 102, 233–243.

Gorman, J.M., 1997. The use of newer antidepressants for panic disorder. J. Clin. Psychiatry 58, 54–58, Suppl. 14.

Gunasekara, N.S., Noble, S., Benfield, P., 1998. Paroxetine. An update of its pharmacology and therapeutic use in depression and a review of its use in other disorders. Drugs 55, 85–120.

Handley, S.L., Mc Blane, J.W., 1993. 5HT drugs in animal models of anxiety. Psychopharmacology 112, 13–20.

Hashimoto, S., Inoue, T., Koyama, T., 1996. Serotonin reuptake inhibitors reduce conditioned fear stress-induced freezing behavior in rats. Psychopharmacology 123, 182–186.

Hashimoto, S., Inoue, T., Koyama, T., 1997. Effects of the co-administration of 5-HT_{1A} receptor antagonists with an SSRI in conditioned fear stress-induced freezing behavior. Pharmacol. Biochem. Behav. 58, 471–475.

Hjorth, S., 1993. Serotonin 5-HT_{1A} autoreceptor blockade potentiates the ability of the 5-HT reuptake inhibitor citalopram to increase nerve terminal output of 5-HT in vivo: a microdialysis study. J. Neurochem. 60, 776–779.

Hjorth, S., 1996. (−)Pindolol, but not buspirone, potentiates the citalopram-induced rise in extracellular 5-hydroxytryptamine. Eur. J. Pharmacol. 303, 183–186.

Hjorth, S., Auerbach, S.B., 1994. Further evidence for the importance of 5-HT_{1A} autoreceptors in the action of selective serotonin reuptake inhibitors. Eur. J. Pharmacol. 260, 251–255.

Hyttel, J., 1982. Citalopram: pharmacological profile of a specific serotonin uptake inhibitor with antidepressant activity. Prog. Neuropsychopharmacol. Biol. Psychiatry 6, 277–295.

Inoue, T., Koyama, T., Yamashita, I., 1993. Effect of conditioned fear stress on serotonin metabolism in the rat brain. Pharmacol. Biochem. Behav. 44, 371–374.

Inoue, T., Tsuchiya, K., Koyama, T., 1994. Regional changes in dopamine and serotonin activation with various intensity of physical and psychological stress in the rat brain. Pharmacol. Biochem. Behav. 49, 911–920.

Inoue, T., Tsuchiya, K., Koyama, T., 1996. Serotonergic activation reduced defensive freezing in the conditioned fear paradigm. Pharmacol. Biochem. Behav. 53, 825–831.

Invernizzi, R., Bramante, M., Samanin, R., 1994. Chronic treatment with citalopram facilitates the effect of a challenge dose on cortical sero-

tonin output: role of presynaptic 5-HT_{1A} receptors. Eur. J. Pharmacol. 260, 243–246.

Iversen, S.D., 1984. 5-HT and anxiety. Neuropharmacology 23, 1553–1560.

Kahn, R.S., van Praag, H.M., Wetzler, S., Asnis, G.M., Barr, G., 1988. Serotonin and anxiety revisited. Biol. Psychiatry 23, 189–208.

Kalin, N.H., Sherman, J.E., Takahashi, L.K., 1988. Antagonism of endogenous CRH systems attenuates stress-induced freezing behavior in rats. Brain Res. 457, 130–135.

Lott, M., Greist, J.H., Jefferson, J.W., Kobak, K.A., Katzelnick, D.J., Katz, R.J., Schaettle, S.C., 1997. Brofaromine for social phobia: a multicenter, placebo-controlled, double-blind study. J. Clin. Psychopharmacol. 17, 255–260.

Oehrberg, S., Christiansen, P.E., Behnke, K., Borup, A.L., Severin, B., Soegaard, J., Calberg, H., Judge, R., Ohrstrom, J.K., Manniche, P.M., 1995. Paroxetine in the treatment of panic disorder. A randomised, double-blind, placebo-controlled study. Br. J. Psychiatry 167, 374–379.

Paxinos, G., Watson, C., 1986. The Rat Brain in Stereotaxic Coordinates. Academic Press, New York, NY.

Rex, A., Marsden, C.A., Fink, H., 1993. Effect of diazepam on cortical 5-HT release and behavior in the guinea-pig on exposure to the elevated plus maze. Psychopharmacology 110, 490–496.

Schneier, F.R., Goetz, D., Campeas, R., Fallon, B., Marshall, R., Liebowitz, M.T., 1998. Placebo-controlled trial of moclobemide in social phobia. Br. J. Psychiatry 172, 70–77.

Sharp, T., Bramwell, S.R., Clark, D., Grahame-Smith, D.G., 1989. In vivo measurement of extracellular 5-hydroxytryptamine in hippocampus of the anaesthetized rat using microdialysis: change in relation to 5-hydroxytryptaminergic neuronal activity. J. Neurochem. 53, 234–240.

Stefanski, R., Goldberg, S.R., 1997. Serotonin 5-HT2 receptor antagonist: potential in the treatment of psychiatric disorders. CNS Drugs 7, 388–409.

Tiller, J.W., Bouwer, C., Behnke, K., 1997. Moclobemide for anxiety disorders: a focus on moclobemide for panic disorder. Int. Clin. Psychopharmacol. 12, S27–30, Suppl. 6.

Van der Kolk, B.A., Dreyfuss, D., Michaels, M., Shera, D., Berkowitz, R., Fisler, R., Saxe, G., 1994. Fluoxetine in posttraumatic stress disorder. J. Clin. Psychiatry 55, 517–522.

Van Vliet, I.M., Den Boer, J.A., Westenberg, H.G., 1994. Psychopharmacological treatment of social phobia: a double blind placebo controlled study with fluvoxamine. Psychopharmacology 115, 128–134.

Wise, C.D., Berger, B.D., Stein, L., 1972. Anxiety-reducing activity by reduction of serotonin turnover in the brain. Science 177, 180–183.