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Selective serotonin reuptake inhibitor reduces conditioned fear through its effect in the amygdala

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Abstract

Selective serotonin reuptake inhibitors are first-line treatment for most anxiety disorders, but their mechanism of anxiolytic action has not been clarified. Selective serotonin reuptake inhibitors are anxiolytic in conditioned fear stress (re-exposure to an environment paired previously with inescapable electric footshocks). To clarify the brain regions where selective serotonin reuptake inhibitors act, we examined the effect of microinjection of the selective serotonin reuptake inhibitor, citalopram, into the amygdala, medial prefrontal cortex and mediodorsal nucleus of the thalamus on freezing behavior, an index of fear, induced by conditioned fear stress. Bilateral injection of citalopram into the amygdala before testing reduced freezing significantly, while bilateral injection into the medial prefrontal cortex or mediodorsal nucleus of the thalamus did not. These results suggest that the anxiolytic effect of a selective serotonin reuptake inhibitor in conditioned fear is mediated by its effect in the amygdala, and support the hypothesis of serotonin function in anxiety by which facilitation of serotonin neurotransmission decreases anxiety.

Keywords: Conditioned fear; Selective serotonin reuptake inhibitor; Amygdala; Serotonin; Microinjection; Anxiety

1. Introduction

Selective serotonin reuptake inhibitors have been widely used to treat most anxiety disorders in addition to depressive disorders, and their efficacy has been established in several randomized placebo-controlled studies (Zohar and Westenberg, 2000). As in vivo microdialysis studies have shown that selective serotonin reuptake inhibitors increase extracellular serotonin concentrations in various brain regions that receive serotonergic innervation (Fuller, 1994), the mechanism of anxiolytic action of selective serotonin reuptake inhibitors should show that selective serotonin reuptake inhibitors facilitate serotonin neurotransmission by increasing extracellular serotonin concentrations, but this hypothesis has not been proven. Furthermore, traditional animal models of anxiety have failed to show the anxiolytic effect of selective serotonin reuptake inhibitors (Borsini et al., 2002), which makes it difficult to study the mechanism of anxiolytic action of selective serotonin reuptake inhibitors.

We found that conditioned fear stress is a useful model of anxiety to detect the anxiolytic effect of selective serotonin reuptake inhibitors in animals (Hashimoto et al., 1996; Inoue et al., 1996). Acute systemic administration of selective serotonin reuptake inhibitors before re-exposure to a shock chamber reduces conditioned freezing behavior, an index of fear or anxiety, to a contextual stimulus, while noradrenaline or dopamine reuptake inhibitors fail to reduce it (Hashimoto et al., 1996). Contextual stimuli are often involved in the psychophathological processes of anxiety disorders, especially anticipatory anxiety. This model has contributed to the understanding of the mechanism of action of selective serotonin reuptake inhibitors (Inoue et al., 2000). On the basis of these studies, selective serotonin reuptake inhibitors are thought to alleviate anxiety by increasing extracellular concentrations of serotonin as a result of blocking the

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serotonin transporter, i.e., the facilitation of serotonin neurotransmission (Hashimoto et al., 1999). However, little is known about the sites of anxiolytic action of selective serotonin reuptake inhibitors in the brain.

The amygdala has an essential role in the development and expression of conditioned fear to both an elementary stimulus and a contextual stimulus (LeDoux, 2000), and its damage produces deficits in fear conditioning in rodents (LeDoux, 2000) and humans (Bechara et al., 1995; LeDoux, 2000). Other brain areas, such as the hippocampus, medial prefrontal cortex and mediodorsal nucleus of the thalamus, are also associated with fear conditioning: lesions of the medial prefrontal cortex enhanced fear conditioning (Morgan and LeDoux, 1995), whereas lesions of the mediodorsal nucleus of the thalamus inhibit fear conditioning (Li, 2001). Both the medial prefrontal cortex and the mediodorsal nucleus of the thalamus have dense reciprocal connections with the amygdala (Krettek and Price, 1977a,b; McDonald, 1991; McDonald et al., 1996). These brain regions may be candidate sites of anxiolytic action of selective serotonin reuptake inhibitors.

The aim of this study is to clarify the brain regions where selective serotonin reuptake inhibitors act as anxiolytics in conditioned fear. In this study, we examined the effects of intracranial injections of citalopram, a selective serotonin reuptake inhibitor (Hyttel and Larsen, 1985), administered directly into the amygdala, medial prefrontal cortex or mediodorsal nucleus of the thalamus on the expression of contextual conditioned fear by using freezing as an index of fear.

2. Materials and methods

2.1. Subjects

Male Sprague–Dawley rats obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan), weighing 230–250 g at time of the conditioned fear test, were housed in groups of four and were maintained in a 12:12-h light/dark (light phase; 0630–1830 h), temperature-controlled environment (22±1 °C) with free access to food and water. Conditioning began after a 2-week period of acclimatization. Rats were tested between 0800 and 1300 h. All 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.

2.2. Surgery

Surgery was performed under sodium pentobarbital (40 mg/kg, intraperitoneally) anesthesia using aseptic conditions. The head position was adjusted to place the bregma and lambda in the same horizontal plane in a stereotaxic frame. Rats were stereotaxically implanted with bilateral 26-gauge stainless steel guide cannulae directed toward the amygdala (immediately dorsal to the basolateral nucleus of the amygdala), mediodorsal nucleus of the thalamus or medial prefrontal cortex [coordinates relative to bregma: AP −2.8, ML ±5.0, V 7.6 for the amygdala; AP −2.3, ML ±0.8, V 4.6 for the mediodorsal nucleus of the thalamus; AP +3.2, ML ±0.5, V 3.0 for the medial prefrontal cortex taken from the stereotaxic atlas of Paxinos and Watson (1997)]. After surgery, rats were housed individually. When not used for injection, the guide cannulae were occluded with obturators made of 33-gauge stainless steel wire.

2.3. Drug

Bilateral infusions were given with 33-gauge injector cannulae connected by polyethylene tubing to motor-driven microsyringes. The exact placement of injector cannula tips was verified at the end of the experiments by standard histological methods. Citalopram hydrobromide (0.3 or 3 μg) (H. Lundbeck, Copenhagen) dissolved in 0.5 μl saline was infused through each injector at a rate of 0.5 μl/min. The injectors were left in place for 30 s after the infusion. Saline alone was administered as a control.

2.4. Apparatus and conditioning

For contextual fear conditioning, rats were individually subjected to inescapable electric foot shocks for a total of 2.5 min in a shock chamber with a grid floor (19×22×20 cm, Medical Agent, Japan) (Hashimoto et al., 1996). Electric shocks were applied by a Model SGS-02D Shock Generator (Medical Agent). Five foot shocks (2.5 mA scrambled shock, 30 s duration) were delivered at intershock intervals of 35–85 s (mean 60 s). At the setting of 2.5 mA, this generator gave a shock intensity of 0.2 mA to the rats.

2.5. Behavioral procedures

After a recovery period of at least 10 days, the rats (7–8 rats/group) were contextually conditioned to a shock chamber. Twenty-four hours after fear conditioning, bilateral infusions were given simultaneously by using 33-gauge injector cannulae projecting 1.0 mm beyond the tips of guide cannulae. Ten minutes after the infusion, the rats were again placed in the shock chamber and were observed for 5 min without shocks. Conditioned fear, as measured by freezing, develops from the contextual stimuli of the conditioned chamber (Fanselow, 1980). During the observation, the duration of freezing behavior was recorded by using a time-sampling procedure (Fanselow, 1980) modified as previously described (Hashimoto et al., 1996). Freezing was defined as the absence of all observable movement of the skeleton and the vibrissae, except movements related to respiration. The rats were classified as either freezing or active according to their behavior during a 10-s period. The

percentage score of freezing was the number of 10-s periods during which the rats froze for 10 s.

2.6. Motor activity

To exclude the possibility that citalopram administration reduced freezing nonspecifically by increasing spontaneous activity, motor activity was measured after infusion of a representative dose of citalopram that significantly inhibited freezing. The rats (9 rats/group) were implanted with bilateral cannulae aimed at the amygdala, and, after a recovery period of at least 10 days, were infused with 3 μg citalopram or saline 10 min before testing. Their motor activity in Plexiglas boxes (38×33×17 cm) was recorded as described by Ohmori et al. (1994) automatically for 5 min by electronic digital counters with infrared cell sensors between 0800 and 1300 h. Horizontal movement was digitized and fed into a computer. Locomotion contributed predominantly to the count, but other body movements also contributed to the count when these movements contained substantial horizontal components.

2.7. Data analysis

All the data are the means±S.E.M. of the individual values of the rats from each group. Statistical analysis of the data was conducted by using nonparametric Mann–Whitney *U*-tests. The statistical significance was set at $P<0.05$.

2.8. Histological verification

After the behavioral experiment, the brains of rats were removed and stored at −80 °C, and then were sectioned at 40-μm thickness in a cryostat at −10 °C. Coronal slices of each rat brain were stained with toluidine blue. Cannula placement was determined under a light microscope histologically. Only the data obtained from rats with verified cannula tip locations were used.

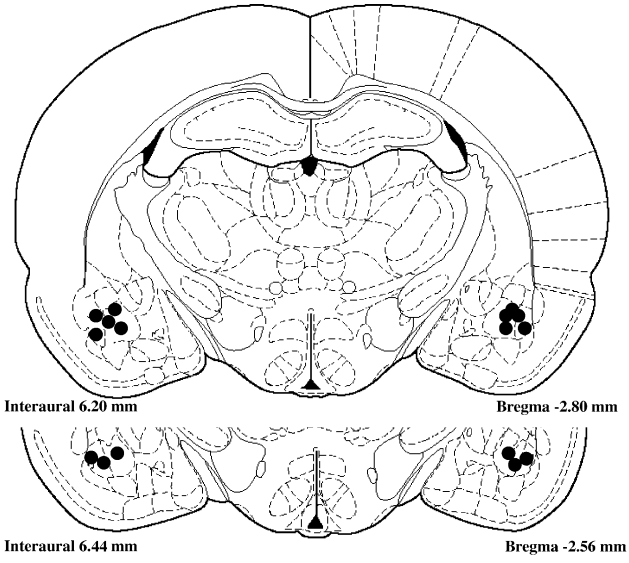

Fig. 1. Coronal sections through the amygdala from the atlas of Paxinos and Watson (1997; 2.56–2.8 mm posterior to bregma). Solid circles represent location of bilateral injection cannula tips for the rats of the citalopram 3 μg group.

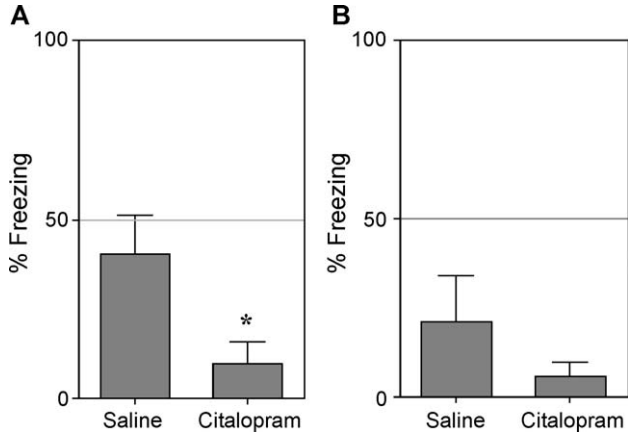

Fig. 2. Effect of bilateral citalopram microinjections at doses of 3 μg/site (A) and 0.3 μg/site (B) into the amygdala on freezing induced by conditioned fear. Citalopram was administered 24 h after footshock and 10 min before conditioned fear stress. The mean percentages±S.E.M. for freezing scored during a 5-min observation period are given. Behavior was sampled at 10-s intervals. (A) $P=0.0333$, $N=7$, $^*P<0.05$; (B) $P=0.7629$, $N=8$.

3. Results

3.1. Histology

Tissue damage was not apparent in either of the drug groups or the saline groups. Fig. 1 schematically depicts all the accurate placements in the amygdala of the citalopram 3 μg group. The histological results were plotted on repre-

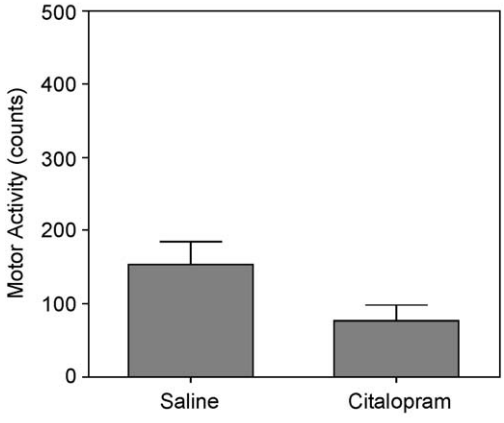

Fig. 3. Effect of bilateral citalopram microinjections (3 μg/site) into the amygdala on motor activity (counts) for 5 min in unshocked rats. Motor activity was counted 10 min after citalopram microinjection. Represented are the mean counts±S.E.M. of motor activity. $P=0.0701$, $N=9$.

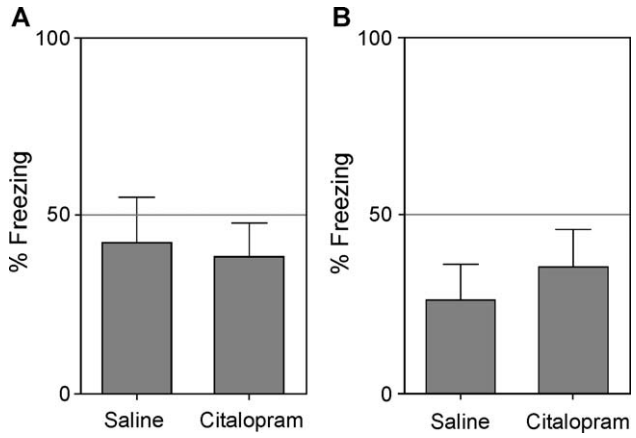

Fig. 4. Effect of bilateral citalopram microinjections (3 μg/site) into the mediodorsal nucleus of the thalamus (A) and the medial prefrontal cortex (B) on freezing induced by conditioned fear. Citalopram was administered 24 h after footshock and 10 min before conditioned fear stress. The mean percentages±S.E.M. for freezing scored during a 5-min observation period are given. Behavior was sampled at 10-s intervals. (A) P=0.6849, N=7–8; (B) P=0.488, N=8.

sentative sections taken from the rat brain atlas of Paxinos and Watson (1997).

3.2. Effect of citalopram injection into the amygdala on conditioned freezing

Consistent with the results of our previous studies (Hashimoto et al., 1996; Inoue et al., 1996), which showed that subcutaneous citalopram administration decreased conditioned freezing, bilateral citalopram injection at a dose of 3 μg/site into the amygdala given 10 min before testing reduced conditioned freezing significantly (Fig. 2A). A lower dose of citalopram (0.3 μg/site) into the amygdala tended to reduce conditioned freezing, but this effect was not statistically significant (Fig. 2B). Bilateral citalopram injection (3 μg/site) into the amygdala of unshocked rats showed a tendency to decrease spontaneous motor activity in their home cages, but this effect was not statistically significant (Fig. 3).

3.3. Effect of citalopram injection into the mediodorsal nucleus of the thalamus and medial prefrontal cortex on conditioned freezing

Bilateral injection of 3 μg citalopram into the mediodorsal nucleus of the thalamus or the medial prefrontal cortex given 10 min before testing did not change conditioned freezing (Fig. 4).

4. Discussion

The reduction of conditioned freezing by the selective serotonin reuptake inhibitor citalopram injected into the amygdala of rats that had been shocked, and the lack of spontaneous motor activity in unshocked rats, suggests that reduced conditioned freezing after intra-amygdala citalopram injection is due to decreased fear, but is not due to increased spontaneous motor activity induced by citalopram, which may affect freezing nonspecifically. Because the local administration of citalopram into the medial prefrontal cortex or mediodorsal nucleus of the thalamus did not affect conditioned freezing, the effect of citalopram injection in reducing conditioned fear is specific to the amygdala, although the effect of citalopram on other brain regions needs to be examined. This study suggests that the anxiolytic action of selective serotonin reuptake inhibitors, which have been first-line treatment for various anxiety disorders in recent decades (Zohar and Westenberg, 2000), is produced through their effect on the amygdala.

The results of this study agree with our previous study that showed that systemic administration of citalopram reduces conditioned freezing without causing spontaneous motor activity (Hashimoto et al., 1996). In conditioned fear, selective serotonin reuptake inhibitors and selective 5-HT_{1A} receptor agonists exert anxiolytic effects that are suggested to be mediated by stimulation of post-synaptic serotonin receptors (especially 5-HT_{1A} receptor) in the nerve terminal areas but not in the raphe nucleus (Inoue et al., 1996). This hypothesis is confirmed by the finding that intra-amygdala citalopram injection reduced conditioned fear.

Before the introduction of selective serotonin reuptake inhibitors to treat anxiety disorders, a reduction in serotonin neurotransmission was thought to alleviate anxiety and fear, e.g., 5-HT_{1A} receptor agonists, such as buspirone, exert anxiolytic effects by stimulating the 5-HT_{1A} autoreceptor in the raphe nucleus, leading to inhibition of the firing of serotonergic neurons (Traber and Glaser, 1987). However, this classical hypothesis of serotonin function in anxiety does not agree with more recent clinical data showing that facilitation of serotonin neurotransmission prevents anxiety (Erikkson and Humble, 1990). Several clinical placebo-controlled studies have consistently shown that drugs assumed to facilitate serotonin neurotransmission, such as selective serotonin reuptake inhibitors, monoamine oxidase inhibitors and serotonin precursors, are effective in treating anxiety disorders (Erikkson and Humble, 1990; Zohar and Westenberg, 2000). The results of this study on conditioned fear support the hypothesis of Erikkson and Humble (1990).

Recent in vivo microdialysis studies examined the effect of conditioned fear on extracellular serotonin concentrations in some brain areas. Exposure to contexts paired with foot shocks, i.e., conditioned fear, increased extracellular serotonin concentrations in the amygdala (Kawahara et al., 1995) and medial prefrontal cortex (Yoshioka et al., 1995; Hashimoto et al., 1999). Simultaneous observation of extracellular serotonin concentrations and freezing indicated that serotonin levels do not increase while the rats freeze, and that the serotonin levels increase after the rats recover from freezing (Hashimoto et al., 1999). Serotonergic activation occurs after the expression of conditioned fear

and does not induce fear. Thus, increases in extracellular serotonin concentrations after conditioned fear appear to reflect coping with stress rather than the emotional reaction to stress. Selective serotonin reuptake inhibitors may enhance a physiological and adaptive reaction in the brain to anxiety or fear induced by aversive stimuli.

The amygdala has a crucial role in fear conditioning (LeDoux, 2000). Among subnuclei of the amygdala, the basolateral nucleus of the amygdala receives input of contextual information in the acquisition of conditioned fear (Maren and Fanselow, 1995; LeDoux, 2000), and bilateral excitotoxic lesions of the basolateral nucleus of the amygdala after fear conditioning abolishes the expression of conditioned fear to both contextual and acoustic conditioned stimuli (Maren et al., 1996). The amygdala, including the basolateral nucleus of the amygdala, receives a dense serotonergic innervation from the dorsal raphe nucleus (Fallon and Ciofi, 1992) and includes several subtypes of serotonin receptors (Radja et al., 1991; Wright et al., 1995). Whereas 5-HT_2 and 5-HT_3 agonists significantly increased the neuronal discharge rate in nearly all subdivisions of the amygdala, including the basolateral nucleus of the amygdala, a 5-HT_{1A} agonist significantly inhibited the firing rate (Stein et al., 2000). As lesion of the basolateral nucleus of the amygdala inhibits the expression of contextual conditioned fear (Maren et al., 1996), the inhibitory action induced by 5-HT_{1A} receptor activation may evoke a serotonin-induced reduction in conditioned freezing. Although the injection cannula was inserted into the basolateral nucleus of the amygdala in this study, it is possible that citalopram diffused into the basolateral nucleus of the amygdala as well as the lateral and central nuclei, as Wise and Hoffman (1992) suggested that intracerebral drug injection in a 0.5-μl volume can diffuse one or more millimeters from the infusion site. Accordingly, the results of this study do not permit a definitive conclusion about the site of selective serotonin reuptake inhibitor action within the amygdala. Furthermore, the serotonin receptor subtypes that mediate the anxiolytic action of selective serotonin reuptake inhibitors need further clarification.

Although selective serotonin reuptake inhibitors reduced contextual conditioned fear stress in our previous studies (Hashimoto et al., 1996; Inoue et al., 1996) and this study, the effects of selective serotonin reuptake inhibitors on fear or anxiety were variable in other conventional animal models of anxiety used to screen the anxiolytic effects of benzodiazepines, such as conflict models and the elevated plus-maze (for review, see Borsini et al., 2002). In conflict models, these drugs acutely produced anxiolytic effects that disappeared after chronic administration, but not in another study (Borsini et al., 2002; Handley and McBlane, 1993). In the elevated plus-maze test, acute and chronic effects of selective serotonin reuptake inhibitors were anxiogenic in the half of studies and produced no effect in the other half of studies, but an anxiolytic action was not reported in any of the studies (Borsini et al., 2002). As Borsini et al. (2002) indicated, the different responses to selective serotonin reuptake inhibitors in various animal models of anxiety are interesting, because these models, which have some construct validity, might be useful to understand different aspects of anxiety-related behaviors. Furthermore, as File et al. (2000) hypothesized, serotonin in the dorsal hippocampus causes anxiety in the elevated plus-maze test and social interaction test. Thus, serotonin in different brain regions, such as the amygdala and hippocampus, may play different roles in various animal models of anxiety.

In this study, we showed that intra-amygdala injections of the selective serotonin reuptake inhibitor citalopram reduced the expression of conditioned fear to a contextual stimulus, and that increased concentrations of serotonin in the amygdala, which induce facilitation of serotonergic neurotransmission, decreased conditioned fear. Our findings strongly support the view that an increase in extracellular serotonin after selective serotonin reuptake inhibitor administration is the mechanism of anxiolytic action of selective serotonin reuptake inhibitors. The conditioned fear model is useful to clarify why selective serotonin reuptake inhibitors are effective in the treatment of most anxiety disorders, and provides new insights into our understanding of the pathogenesis and pathophysiology of anxiety disorders.

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