Effects of Serotonergic Anxiolytics on the Freezing Behavior in the Elevated Open-Platform Test in Mice

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Received February 6, 2007; Accepted September 21, 2007

Abstract. Freezing behavior is thought to be a sign of fear in animals. We examined whether the freezing behavior during the elevated open-platform stress, which is a psychological stressor without painful stimulus, is modulated by serotonergic neurotransmission and would be a useful marker for screening anxiolytic and/or antidepressant. Male ICR mice (6–8-week-old) were individually placed on an elevated open-platform and the duration of freezing behavior of mouse was measured for 10 min. Fluoxetine and citalopram, selective serotonin (5-HT) reuptake inhibitors, markedly decreased the duration of freezing. Fenfluramine, a 5-HT releaser, and 8-OH-DPAT, a potent 5-HT₁A-receptor agonist, also significantly decreased the duration of freezing. In contrast, the 5-HT-synthesis inhibitor p-chlorophenylalanine significantly increased the duration of freezing. Diazepam, a benzodiazepine anxiolytic, had no effect on the duration of freezing at doses having no effect on locomotor activity. Imipramine and clomipramine, tricyclic antidepressants, also did not affect the duration of freezing. Reboxetine, a selective noradrenaline reuptake inhibitor, significantly increased the duration of freezing. These results indicate that the activation of serotonergic neurotransmission attenuates the fear-related behavior in the elevated open-platform test, while the activation of noradrenergic neurotransmission increases the fear-related behavior. In addition, this test is convenient for assaying anxiolytic drugs that affect serotonergic neurotransmission.

Keywords: screening method, serotonin (5-HT), anxiolytic, selective 5-HT reuptake inhibitor (SSRI), antidepressant

Introduction

Various drugs affecting serotonin (5-HT) neurotransmission, such as selective 5-HT reuptake inhibitors (SSRIs) and 5-HT₁A-receptor agonists, are effective in the treatment of anxiety disorders. In animal studies, researchers have, however, reported conflicting observations: that is, serotonergic agents induce anxiolytic-like or anxiogenic-like responses. One potential interpretation of these findings is that the nature of stress in different animal models differs qualitatively (1, 2). Methods that are used to assess natural aversion in animals, such as the elevated plus-maze test, are widely used to assess the anxiety state of animals and to screen the anxiolytic effect of drugs. Although the anxiolytic-like effect of drugs that act on γ-aminobutyric acid (GABA)/benzodiazepine neurotransmission is clearly demonstrated in the elevated plus-maze test, the drugs that affect serotonergic neurotransmission vary greatly depending on the report (1–7).

The response to punishment, which is defined as the presentation of a noxious stimulus that results in the suppression of behavior, is also used as an index of the anxiety/fear state of animals (8). The four-plate test, shock-probe burying test and conditioned fear test measure the punished responses of animals. These tests are sensitive to the anxiolytic effects of serotonergic agents such as SSRIs and 5-HT₁A-receptor agonists (1, 9–11). However, painful electric shocks are generally required to elicit the punished responses and ‘pain’ is
one of the problems in using these tests as a screening method. For example, if the novel drug has an anti-nociceptive effect, further investigations would be required to negate the possibility that the drug-induced anti-punishment effects were due to changes in pain sensitivity. Therefore, investigators need a new method that is simple and highly sensitive to the effects of serotonergic anxiolytics, but does not require a painful stimulus.

Elevated open-platform stress is often used as a psychological stressor without a painful stimulus (12, 13). Rodents display characteristic behavioral responses that consist of stress-related behaviors (freezing behavior, urination, defecation) along with exploratory behaviors (walking, rearing, sniffing, head-dipping) on the elevated open-platform. Freezing behavior is characterized by a crouching posture with no movement except for respiratory movement and uses it to cope with a fearful stimulus (14). Therefore, we hypothesized that elevated open-platform stress-induced freezing behavior might be a useful index of the anxiety/fear state in animals. In addition, since conditioned fear stress-induced freezing behavior is suppressed by serotonergic anxiolytics (11), we speculated that freezing behavior during elevated open-platform stress might also be suppressed by serotonergic anxiolytics.

To clarify these hypotheses, we examined the effects of several anxiolytics and antidepressants on the duration of freezing in the elevated open-platform test. In addition, we also investigated the effect of 5-HT depletion on the duration of freezing.

Materials and Methods

This study was carried out in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology of Japan. Every effort was made to minimize the number of animals used in the following experiments.

Animals

Male ICR mice (Tokyo Laboratory Animals Science Co., Ltd., Tokyo), 6–8 weeks of age, were used. They were housed 5 per cage and had free access to food and water. The animal room was maintained at 24 ± 1°C and 55 ± 5% humidity with a 12-h light-dark cycle (light on at 8:00, light off at 20:00). The experiments were performed between 11:00 and 17:00 each day. Each animal was used only once.

Drugs

The drugs used in this study were fluoxetine hydrochloride (LKT Laboratories, Inc., St. Paul, MN, USA); dl-p-chlorophenylalanine methyl ester hydrochloride (PCPA), citalopram hydrobromide, clomipramine hydrochloride, (-)-8-hydroxy-2-(di-n-propylamino) tetralin hydrobromide (8-OH-DPAT), and imipramine hydrochloride (Sigma Chemical Co., St. Louis, MO, USA); diazepam and (-)-fenfluramine hydrochloride (Wako Pure Chemical Industries, Ltd., Osaka); and reboxetine mesylate (Tocris Cookson, Ltd., Bristol, UK). Other chemicals were purchased from Sigma and Wako. Fluoxetine and citalopram are SSRIs. Fenfluramine is a 5-HT releaser. 8-OH-DPAT is a potent 5-HT1A receptor agonist. PCPA is a synthesis inhibitor of 5-HT. Diazepam is a benzodiazepine-receptor agonist. Imipramine and clomipramine are tricyclic antidepressants. Reboxetine is a selective noradrenaline-reuptake inhibitor. PCPA was dissolved in distilled water. Diazepam was dissolved in ethanol and then diluted in saline with one drop of Tween-80 (final ethanol concentration is 0.12%). The other drugs were dissolved in saline. Each drug was administered at a volume of 0.1 mL/10 g of body weight. The dose ranges of drugs were based on our pilot study and the previous reports (15–22). All drugs except for PCPA were injected 30 min before testing. PCPA was injected i.p. twice daily for 3 days, and the experiment was conducted 15 h after the last injection.

Elevated open-platform test

The experiment was conducted in a room illuminated by white fluorescent light (300 lux). The transparent acrylic cylinder (11.4-cm diameter, 18-cm-high) was placed upside-down and mice were placed individually on the top (open-platform) for 10 min. When the mouse slipped off the platform, it was immediately replaced on the open-platform and the measurement was continued. The behavior of mice was recorded by a video camera and an observer blindly measured the duration of freezing. Freezing behavior was defined as the absence of movement excluding respiration. The duration of freezing was the total amount of time that the animal showed freezing. PCPA-treated mice and control mice were decapitated immediately after the behavioral experiment, and the whole brain was removed and stored at −80°C for measurement of brain monoamine contents.

Locomotor activity

The locomotor activity of mice was measured by a digital counter with an infrared sensor (NS-AS01; Neuroscience, Inc., Tokyo). The apparatus detects the movement of animals based on the release of infrared
rays associated with their temperature and records a
digital count. A mouse was placed in a transparent
plastic cage (27 × 17 × 13 cm), a transparent plastic
ceiling was installed, and an infrared sensor was placed
at the center of the ceiling. Mice were placed in the
measurement cage and then recording was started. Total
activity counts were recorded automatically for 10 min,
which was the same duration as the measurement period
in the elevated open-platform test.

Monoamine contents
The contents of 5-HT and its metabolite 5-hydroxy-
indoleacetic acid (5-HIAA), noradrenaline and its
metabolite 3-methoxy-4-hydroxyphenylethanol (MHPG),
and dopamine and its metabolites 3,4-
dihydroxyphenylethanol (DOPAC) and 3-methoxy-
4-hydroxyphenylethanol (HVA) were determined by
HPLC-ECD equipped with an SC-5ODS column
(Eicom, Co., Kyoto). Whole brains were homogenized
in 2.5 ml of solution containing 0.2 M perchloric acid
with 100 µM EDTA (2Na) and 1 mg/l isoproterenol as
an internal standard. The homogenates were centrifuged
at 20,000 × g for 15 min at 4°C, and the supernatants
were obtained and maintained at pH 3.0 using 1 M
sodium acetate. The samples of 40 µl were analyzed.
The mobile phase consisted of sodium acetate
(0.1 M)/citric acid (0.1 M) buffer, pH 3.5, containing
15% (v/v) methanol, sodium 1-octanesulfonate, and
EDTA (2Na). The flow rate was set to 0.5 ml/min with
a column temperature of 25°C.

Statistics
The data are expressed as means ± S.E.M. Significant
differences were determined by one-way analysis of
variance (ANOVA) for factorial comparisons and the
Dunnett test for multiple comparisons. Aspin-Welch’s
t-test was used to evaluate the significance of differences
between groups. Probability values of less than 0.05
(P<0.05) were considered statistically significant.

Results
Fluoxetine (0.1–10 mg/kg, i.p.) and citalopram
(0.1–10 mg/kg, i.p.) dose-dependently and significantly
decreased the duration of freezing in mice (Fig. 1A).
Fluoxetine (0.1–10 mg/kg, i.p.) also dose-dependently and significantly decreased the duration of freezing (Fig. 1A). Furthermore, 8-OH-DPAT (3–30 µg /kg, i.p.) dose-dependently and significantly decreased the duration of freezing (Fig. 1A). Fluoxetine, citalopram, fenfluramine, or 8-OH-DPAT had no significant effect on locomotor activity in mice at the doses examined (Fig. 1B).

PCPA (250 mg/kg, i.p. twice daily for 3 days) significantly increased the duration of freezing in mice (Fig. 2A). PCPA (250 mg/kg, i.p. twice daily for 3 days), however, did not affect locomotor activity in mice (Fig. 2B). The 5-HT and 5-HIAA contents were significantly decreased in PCPA-treated mice without changing the contents of noradrenaline, dopamine, or their respective metabolites (Table 1).

Diazepam (0.03 – 1 mg/kg, i.p.) had no significant
effect on the duration of freezing in the elevated open-
platform test (Fig. 3A) or locomotor activity in mice
(Fig. 3B). However, i.p. treatment with diazepam at
3 mg/kg significantly increased the duration of freezing in the elevated open-platform test (Fig. 3A). Diazepam at 3 mg/kg significantly decreased locomotor activity in mice (Fig. 3B).

Imipramine (0.1 – 10 mg/kg, i.p.) and clomipramine
(0.1 – 10 mg/kg, i.p.) did not affect the duration of freezing in the elevated open-platform test (Fig. 4A).
Reboxetine (0.1 – 1 mg/kg, i.p.) dose-dependently and significantly increased the duration of freezing (Fig. 4A). Imipramine (0.1 – 10 mg/kg, i.p.), clomipramine (0.1 – 10 mg/kg, i.p.), or reboxetine (0.1 – 1 mg/kg, i.p.) had no effect on locomotor activity (Fig. 4B).

Table 1. Effects of PCPA on the contents of 5-HT, noradrenaline (NA), dopamine (DA), and their major metabolites in the whole brain

<table>
<thead>
<tr>
<th>Contents (ng/mg tissue)</th>
<th>Saline</th>
<th>PCPA</th>
</tr>
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<tbody>
<tr>
<td>5-HT</td>
<td>0.2806 ± 0.0064</td>
<td>0.0803 ± 0.0056***</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>0.1395 ± 0.0030</td>
<td>0.0372 ± 0.0015***</td>
</tr>
<tr>
<td>NA</td>
<td>0.1193 ± 0.0066</td>
<td>0.1160 ± 0.0032</td>
</tr>
<tr>
<td>MHPG</td>
<td>0.2812 ± 0.0103</td>
<td>0.2747 ± 0.0107</td>
</tr>
<tr>
<td>DA</td>
<td>0.6811 ± 0.0131</td>
<td>0.6827 ± 0.0209</td>
</tr>
<tr>
<td>DOPAC</td>
<td>0.0559 ± 0.0014</td>
<td>0.0541 ± 0.0025</td>
</tr>
<tr>
<td>HVA</td>
<td>0.0797 ± 0.0013</td>
<td>0.0760 ± 0.0033</td>
</tr>
</tbody>
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Data represent the mean with S.E.M. of 10 mice. ***P<0.001 vs saline-treated mice.
Fluoxetine, citalopram, fenfluramine, and 8-OH-DPAT markedly decreased the duration of freezing in the elevated open-platform test without affecting locomotor activity. In addition, PCPA-induced depletion of 5-HT significantly increased the duration of freezing. These results indicate that the freezing behavior in the elevated open-platform test is modulated by the serotonergic activity in the brain. In addition, the activation of 5-HT\textsubscript{1A} receptors produces the anti-freezing effect in the elevated open-platform test. Reboxetine significantly increased the duration of freezing without affecting locomotor activity. Reboxetine selectively inhibits the reuptake of synaptic noradrenaline without any marked influence on other neurotransmissions. Several lines of evidence indicate that noradrenergic and serotonergic neurotransmissions may play opposite roles in the mechanisms of fear learning and fear expression. Noradrenergic neurotransmission elicits the fear-related behavior in animals while serotonergic neurotransmission suppresses the fear-related behavior. Therefore, the reboxetine-induced activation of noradrenergic neurotransmission may cause the prolonged duration of freezing in the elevated open-platform test. Imipramine and clomipramine did not affect the duration of freezing in the elevated open-platform test.

Diazepam failed to affect the duration of freezing in the elevated open-platform test at the doses that did not significantly affect locomotor activity in mice. Significant prolongation of duration of freezing in diazepam (3 mg/kg, i.p.)-treated mice is likely to be reflected in the hypolocomotor activity. We previously reported that diazepam at 0.3 mg/kg exerted the significant anxiolytic-like effect in the mouse hole-board test. Therefore, the freezing behavior in the elevated open-platform test may not be affected by diazepam at the dose having no effect on locomotor activity. The elevated plus-maze test is a popular method for screening anxiolytics and examining their mechanisms of action. This test is reported to be suitable for detecting anxiolytics acting on GABAergic systems but not serotonergic ones. On the other hand, the elevated open-platform test detects the anxiolytics that affects serotonergic neurotransmission. Therefore, the elevated open-platform test is more sensitive than the elevated plus-maze test to screen serotonergic anxiolytics and to study the serotonergic function in the brain, although it needs to be accompanied by the measurement of locomotor activity as an additional behavior test. In the elevated plus-maze test, increase of 5-HT levels in the hippocampus induces the anxiety-like behavior in rats possibly mediated by the activation of 5-HT\textsubscript{2C} receptors. In contrast, the increase of 5-HT levels by SSRI exerts the anti-freezing effect in the elevated open-platform test. Since the activation of 5-HT\textsubscript{1A} receptors decreased the duration of freezing, the anti-freezing effect of SSRIs would be mediated by the activation of 5-HT\textsubscript{1A} receptors. Therefore, it is possible that 5-HT receptor subtypes associating with the regulation of mouse behavior are different between the elevated plus-maze test and the elevated open-platform test.
microdialysis studies, imipramine and clomipramine increased equally the extracellular levels of both 5-HT and noradrenaline (28, 29). Therefore, the absence of anti-freezing effects for tricyclic antidepressants may be due to their dual effects on the serotonergic and noradrenergic neurotransmissions.

The cliff-avoidance reaction on the elevated open-platform is known to be an index of behavioral teratology in rodents, which can be impaired by motor, arousal, or cognitive dysfunction (30, 31). Impairment of the cliff-avoidance reaction is defined as falling (or jumping) from the edge of the open-platform. In the present study, we did not observe disturbance of either the cliff-avoidance reaction (data not shown) or locomotor activity in mice. Therefore, the anti-freezing effects of serotonergic agents are not related to motor or cognitive impairment.

In conclusion, the present findings indicate that the freezing behavior in the elevated open-platform test is modulated oppositely by serotonergic and noradrenergic neurotransmissions. In addition, anxiolytics that affect serotonergic neurotransmission can be assayed by the elevated open-platform test.

Acknowledgements

We thank Mr. N. Kaneda and Ms. A. Kurihara for their excellent technical assistances.

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