

Anxiety-Like Behavior in Elevated Plus-Maze Tests in Repeatedly Cold-Stressed Mice

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ABSTRACT—To clarify the relationship between SART (specific alternation of rhythm in temperature) stress (repeated cold stress) and anxiety, the effects of various types of stress on the behavior of mice were studied in elevated plus-maze tests and then the effects of anxiolytics were evaluated. The percentage of time spent in the open arms of the plus-maze apparatus decreased in mice subjected to SART stress without change in the total number of arm entries. No change was noted in mice subjected to other stresses, such as 1-h, 2-day and 5-day cold stress and 1-h, 15-h and 5 × 15-h restraint stress. The reduction in the percentage of time spent in the open arms caused by SART stress was inhibited by single and repeated administrations of diazepam and alprazolam and by a single administration of buspirone, which have no influence on the percentage of time spent in the open arms in nonstressed mice, but not by flumazenil, WAY-100635 and chronic treatment with buspirone. The effects of diazepam and buspirone were antagonized by flumazenil and WAY-100635, respectively. The behavior of SART-stressed mice in the plus-maze would thus appear to arise from anxiety, to which benzodiazepine and serotonin receptors are related, but the diazepam binding inhibitor, an endogenous anxiogenic protein, is not. Thus SART-stressed animals may be useful for investigating the psychopharmacological and neuropharmacological basis of anxiety.

Keywords: Elevated plus-maze, Stress, SART stress, Anxiety, Repeated cold stress

The relationship between anxiety and stress is a point of much interest. Chronic stress induces mood disorder-like behavior in mammals including humans, and it may be a main factor in the development of anxiety (1, 2). Exposure to various types of stress results in anxiogenic behavior in tests for anxiety in animals. Social stress (3, 4), inescapable electric foot-shocks (5), immersion in water (6) and exposure to unpleasant smells such as cat odor (7) reduce the exploration of open spaces in an elevated plus-maze and cause reduction in social interactions in mice (7, 8).

The behavioral characteristics of animals exposed to SART (specific alternation of rhythm in temperature) stress (9–13) were studied. SART stress (14) is stimulation by repeated and sudden changes in environmental temperature from room temperature to cold temperature, an event that may be encountered by humans in daily life such as in early spring or autumn or when leaving an air-conditioned room in summer or a heated room in winter. Animals exposed to SART stress are a model of autonomic imbalance (15) and show adverse biological events (16–19) and physiological

abnormalities (14, 20–23). Abnormal behavior is shown in open-field (9), step-down (11) and forced swimming tests (12, 13). This abnormal behavior is normalized by antianxiety agents (9, 12, 13). Changes in neurotransmitters related to abnormal behavior have been noted (24–26). Certain aspects of these abnormalities are thought to be related to anxiety.

To clarify the relationship between SART stress and anxiety, the present study examined the effects of SART stress on the behavior of mice in an elevated plus-maze, a model of anxiety for rodents and used to find anxiolytic agents. Attention was directed to the effects of various types of stress in order to compare these with the effects of SART stress and to find therapeutically effective drugs for treating anxiety relative to the performance of mice in an elevated plus-maze. For this purpose, the effects of benzodiazepines (diazepam and alprazolam) and 5-HT_{1A} serotonin receptor agonist (buspirone) were studied on the behavior of SART-stressed mice in the elevated plus-maze. In addition, flumazenil, a benzodiazepine receptor antagonist (27) and WAY-100635, a 5-HT_{1A} serotonin receptor antagonist (28), were also investigated.

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MATERIALS AND METHODS

Animals

Male ddY mice (Japan SLC, Hamamatsu), weighing 23–28 g at the start of the study, were used in accordance with ethical procedures approved for the care and use of laboratory animals by The Japanese Pharmacological Society. They were housed in groups of 8–10 in plastic cages (21.6 × 31.6 × 13.0 cm) and allowed standard laboratory diet (MF; Oriental Yeast, Tokyo) and tap water ad libitum before the experiments. Temperature was maintained at 23–25°C, with a 12-h light-dark cycle (lights on at 07:00 h, off at 19:00 h).

Exposure of animals to stress

SART stress: Mice were stressed essentially as previously reported (29). Plastic cages (21.6 × 31.6 × 13.0 cm) for stress exposure were prepared in a room maintained at 24°C and others in a room at 4°C. Mice in groups of 8–10 were alternately transferred at 1-h intervals to cages placed in the two rooms between 09:00 and 16:00 h and housed in cages in the latter room (4°C) between 16:00 and 09:00 h overnight. These procedures were repeated for 6–8 days and thereafter terminated at 11:00 h on the final day of stress. The stressed mice were used a half-hour or later after being taken out of the cold room (4°C).

Cold stress: The mice were kept at 4°C for 1 h, 2 days or 5 days. Mice that were cold-stressed for 1 h or 2 days were subjected to the test immediately after stress loading. In the 5-day cold-stressed mice, tests were carried out 1 h after stress loading.

Restraint stress: Four types of restraint stress were applied in the same manner as previously reported (13). 1) 1-h restraint stress: Mice were restrained in wire cylinders for 1 h from 13:00 to 14:00 h in the room maintained at 24°C, 2) 15-h restraint stress: Mice were restrained in wire cylinders between 18:00 and 09:00 h overnight in the room maintained at 24°C, 3) and 4) Repeated restraint stress: Mice received 1-h or 15-h restraint stress every day for 5 days repeatedly. Mice subjected to acute stress 1 and 2 were examined immediately after stress loading and those subjected to chronic stress 3 and 4 were studied 1-h after the final stress loading.

Fasting groups were given no food or water in the same schedule as for 3 and 4 restraint stress.

Elevated plus-maze test

Elevated plus-maze tests were conducted as previously (30) with modification. The apparatus consisted of two open arms (5 × 30 cm) and two closed arms (5 × 30 × 15 cm) radiating from a central platform (5 × 5 cm) to form a plus-sign figure. The apparatus was situated 40 cm from the floor. The open arm edges were 0.5 cm in height to keep the

mice from falling, and the closed arm edges were 15 cm in height. The mice were individually examined in 5-min sessions in this apparatus. Each mouse was placed in the central platform facing one open arm. The numbers of entries into open and closed arms and the time spent in the respective arms were recorded for a 5-min period. The percentage of time spent in the open arms ((open / (open + closed)) × 100) was calculated for each mouse.

Drugs

Diazepam (Wako Pure Chemical Industries, Osaka) and alprazolam (Sigma Chemical Co., St. Louis, MO, USA) were suspended in 0.5% carboxymethylcellulose sodium salt solution and orally administered to the mice. These drugs were administered only once 60 min before the test, or once daily for 7 days of stress loading, 7 times in all. Flumazenil (Biomol Research Laboratories, Inc., Plymouth Meeting, PA, USA) was suspended in physiological saline to which Tween 80 (2 drops/10 ml) had been added and given i.p. to mice 30 min before the test. Buspirone hydrochloride (Research Biochemicals International, Natick, MA, USA) and WAY-100635 (*N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinyl-cyclohexane-carboxamide maleate) (Sigma) were dissolved in physiological saline and given i.p. to mice 60 and 30 min before the test, respectively. In control experiments, the respective vehicles were administered in the same manner. In the repeated-treatment experiments, the elevated plus-maze test was conducted on the day following the final dose. Nonstressed mice were treated with drugs following the same schedule. The doses of flumazenil and WAY-100635 in this experiment were chosen in accordance with previous reports in this research area (31, 32).

Statistical analyses

The results were expressed as means with S.E.M. The statistical significance of differences between or among groups was analyzed by the Student's *t*-test, Tukey's multiple comparison test or Fisher's exact probability test; and *P* < 0.05 indicated a significant difference.

RESULTS

SART stress-induced anxiogenic-like behavior in mice

The results of the elevated plus-maze test in SART-stressed mice are shown in Fig. 1 and compared with those for nonstressed mice. SART stress significantly decreased the time spent in the open arms. The times spent by nonstressed and SART-stressed mice were 49.1 ± 2.3 and 19.1 ± 2.0 s, respectively. SART stress did not change the numbers of entries into the open and closed arms. The percentage of time spent in the open arms was significantly reduced due to SART stress. In the plus-maze apparatus,

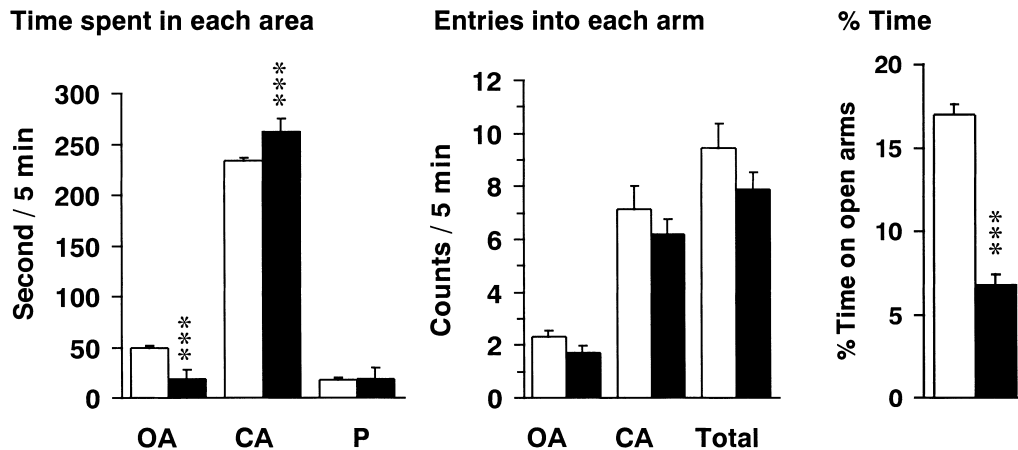


Fig. 1. Behavioral characteristics of SART-stressed mice in elevated plus-maze tests. Data represent means \pm S.E.M. of 20 mice. □, No stress; ■, SART stress. OA, open arms; CA, closed arms; P, platform. *** $P < 0.001$ vs nonstressed group (Student's t -test).

SART stress stimulated defecation. Five of 20 SART-stressed mice defecated, although no nonstressed mice did so, in significant contrast ($P = 0.024$, Fisher's exact probability test).

Effects of stress type on percentage of time spent in open arms

The results of the elevated plus-maze test for mice exposed to various types of stress are shown in Table 1. Cold stress for 1 h, 2 days and 5 days did not induce significant changes in the percentage of time in the open arms in comparison with the nonstressed group. Restraint stress of 1 h,

15 h or 5×15 h or fasting, which was a control for restraint stress, also did not show significant changes.

Effects of antianxiety drugs on SART stress-induced anxiogenic-like behavior

Single administrations of diazepam increased the percentage of time spent in the open arms, which had been noted to have decreased due to SART stress. The decrease in the percentage of time caused by SART stress was significantly reversed by daily doses of diazepam of 2 mg/kg per day $\times 7$ (Fig. 2).

As shown in Fig. 3, single doses of alprazolam led to greater percentages of time in the open arms in the SART-stressed mice. Repeated doses of alprazolam of 0.2 mg/kg per day $\times 7$ increased the percentage of time spent in the open arms.

As shown in Fig. 4A, single doses of flumazenil were shown to have no significant effects on the percentage of time spent in the open arms in both nonstressed and SART-stressed mice as assessed by Tukey's test. Although the percentage of time in the open arms in nonstressed mice tended to increase, this increase was not statistically significant. As shown in Fig. 4B, the diazepam-induced enhancement of the percentage of time spent in the open arms in SART-stressed mice was attenuated by pretreatment with flumazenil, a benzodiazepine receptor antagonist, to the same level in the group treated only with flumazenil.

The effects of buspirone on the SART stress-induced decrease in the percentage of time spent in the open arms are indicated in Fig. 5. Single doses of buspirone dose-dependently increased and normalized the percentage of time spent in the open arms of SART-stressed mice, but had no effect on nonstressed mice. On the other hand, repeated doses of buspirone failed to produce a significant effect

Table 1. Effects of various types of stress on the time spent in open arms of the elevated plus-maze in mice

Treatment	%Time in open arms
No stress	17.3 \pm 1.8
Cold stress	
1 h	13.0 \pm 3.2
2 days	12.4 \pm 2.9
5 days	15.4 \pm 1.8
No stress	15.6 \pm 0.9
Restraint stress	
1 h	14.7 \pm 1.4
15 h \times 1	15.0 \pm 1.0
15 h \times 5	16.4 \pm 1.8
Fasting	
15 h \times 1	18.0 \pm 2.9
15 h \times 5	18.1 \pm 1.1

Data represent means \pm S.E.M. of 5–7 stressed mice or 10 nonstressed mice. None of the data is significantly different from the respective nonstressed control groups.

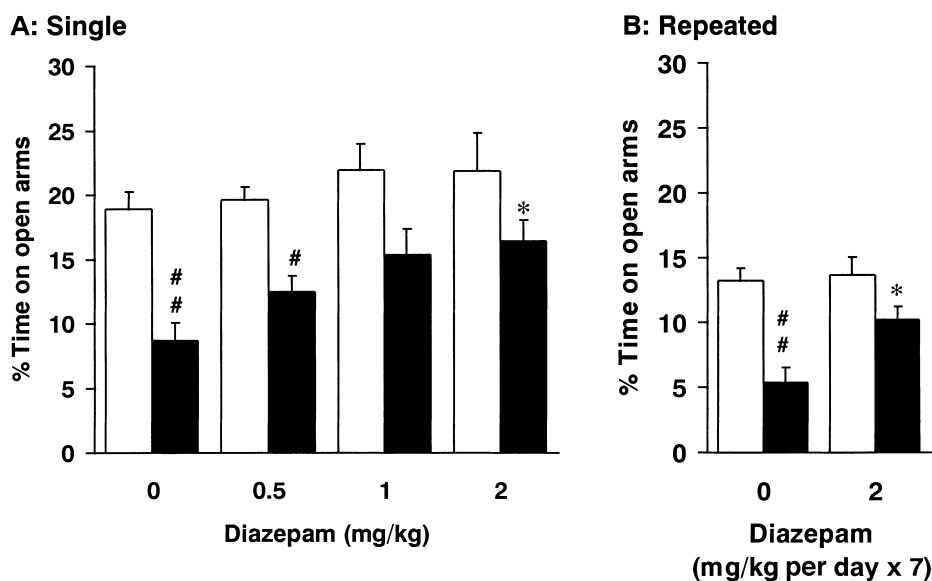


Fig. 2. Effects of diazepam on SART stress-induced reduction of percentage of time spent in open arms of elevated plus-maze. Data represent means \pm S.E.M. of 6 – 10 mice. \square , No stress; \blacksquare , SART stress. [#] $P < 0.05$ and ^{##} $P < 0.01$ vs respective nonstressed control group (vehicle-treated group) and ^{*} $P < 0.05$ vs respective SART-stressed control group (vehicle-treated group) (Tukey's test).

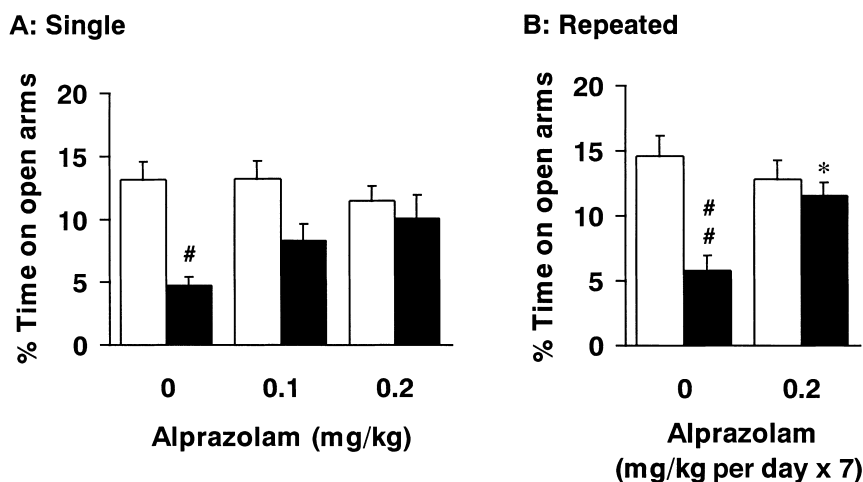


Fig. 3. Effects of alprazolam on SART stress-induced reduction of percentage of time spent in open arms of elevated plus-maze. Data represent means \pm S.E.M. of 5 – 10 mice. \square , No stress; \blacksquare , SART stress. [#] $P < 0.05$ and ^{##} $P < 0.01$ vs respective nonstressed control group (vehicle-treated group) and ^{*} $P < 0.05$ vs respective SART-stressed control group (vehicle-treated group) (Tukey's test).

(Fig. 5B). However, when the stressed mice chronically treated with buspirone (10 mg/kg per day for 7 days) during stress loading were tested 60 min after the last drug, they showed an increased percentage of time spent in the open arms (6.10 ± 2.35) compared to that of the vehicle-treated group (3.08 ± 0.93).

The effect of WAY-100635, a 5-HT_{1A} serotonin receptor antagonist, on the buspirone-induced enhancement of the percentage of time spent in the open arms in SART-stressed mice is shown in Fig. 6. WAY-100635 (1 mg/kg) did not

have any influence on the percentage of time spent in the open arms in SART-stressed mice. However, the same dose of WAY-100635 significantly attenuated the buspirone-induced enhancement of the percentage of time spent in the open arms.

DISCUSSION

Behavior in the elevated plus-maze is a model of anxiety for rodents and may serve as a new basis for developing

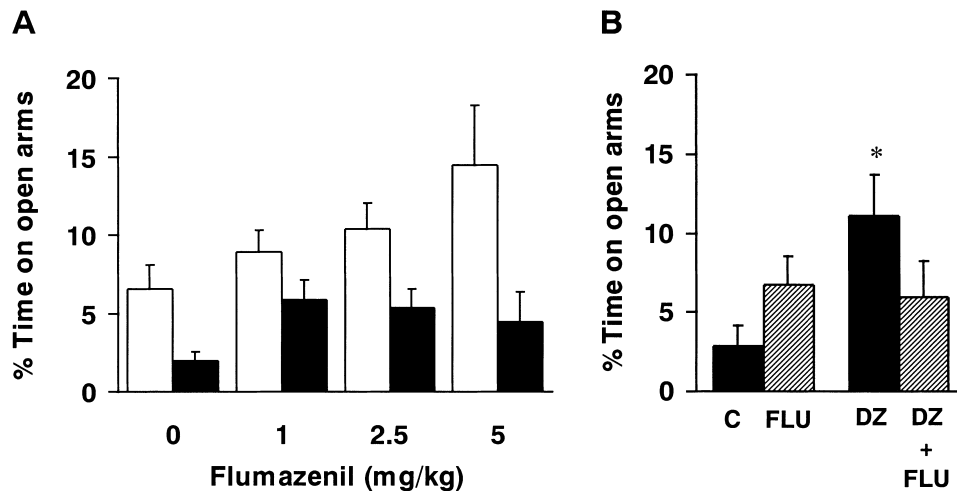


Fig. 4. Effects of flumazenil on percentage of time spent in open arms in SART-stressed mice with and without diazepam treatment. A) Results of single doses of flumazenil injected i.p. 30 min before the test. Data represent means \pm S.E.M. of 10 – 12 mice. \square , No stress; \blacksquare , SART stress. No significant differences were observed statistically. B) Antagonistic effect of flumazenil on anxiolytic effect of diazepam in SART-stressed mice. Data represent means \pm S.E.M. of 6 or 7 mice. C: SART-stressed control mice; Flu: stressed mice treated with 2 mg/kg of flumazenil (i.p.), 30 min before the test; DZ: stressed mice treated with 2 mg/kg of diazepam (p.o.) 60 min before the test; DZ+Flu: stressed mice treated with diazepam (2 mg/kg) 60 min before and flumazenil (2 mg/kg) 30 min before the test. * $P < 0.05$ vs control group, C (group without treatment) (Tukey's test).

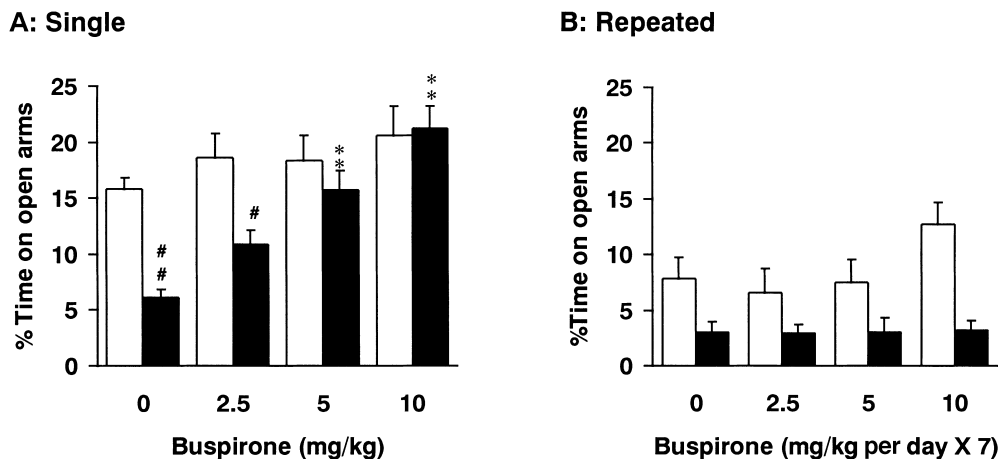


Fig. 5. Effects of buspirone on SART stress-induced reduction of percentage of time spent in open arms of elevated plus-maze. Data represent means \pm S.E.M. of 6 – 12 mice. \square , No stress; \blacksquare , SART stress. * $P < 0.05$ and # $P < 0.01$ vs nonstressed control group (vehicle-treated group) and ** $P < 0.01$ vs SART-stressed control group (vehicle-treated group) (Tukey's test).

anxiolytic agents and investigating psychological and neurochemical factors of anxiety. Rats spent less time exploring the open arms than the closed arms of the novel Y-shaped elevated maze. The elevated plus-maze is a modification of the elevated Y-maze (33). Rats were allowed to freely explore two elevated open and two elevated closed arms of the elevated plus-maze apparatus that has been confirmed applicable to rats and mice (30). This test is widely used for its capacity to detect the effects of most anxiolytics including new anxiolytic agents such as buspirone (34), although a few studies have shown opposite

results (35).

The behavior of animals on an elevated plus-maze is influenced by such stressors as electric shock (5), forced swim (6), surgical stress and even saline injection (36). Immobilization (37, 38), social defeat (3) and exposure to smells unpleasant to rats such as cat odor (7) reduce exploration of the open arms in the elevated-plus maze. In this study, SART stress significantly reduced the time spent in the open arms but not the numbers of entries into the open and closed arms. Thus, SART-stressed mice exhibited anxiety-like behavior in the elevated plus-maze test. Cold

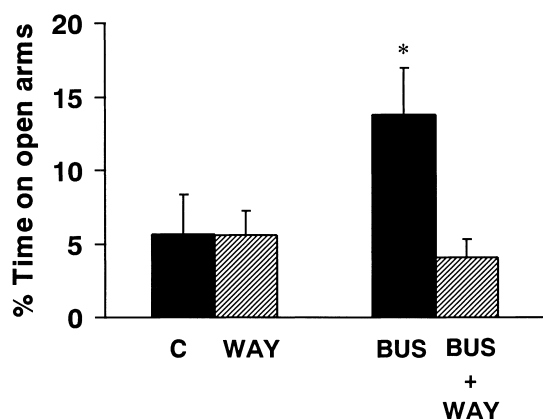


Fig. 6. Antagonistic effect of WAY-100635 on anxiolytic effect of buspirone in plus-maze test in SART-stressed mice. C: SART-stressed control mice; WAY: stressed mice treated with 1 mg/kg of WAY-100635, i.p. 30 min before the test; BUS: stressed mice treated with 10 mg/kg of buspirone p.o. 60 min before the test; BUS + WAY: stressed mice treated with buspirone 60 min before and WAY-100635 30 min before the test at same doses. Data represent means \pm S.E.M. of 7 or 8 mice. * $P < 0.05$ vs control group, C (Tukey's test).

stress of 1 h, 2 days and 5 days and restraint stress of 1 h, 15 h and 5×15 h stress did not alter the percentage of time spent in the open arms. These conflicting results are probably due to different types of stress, schedules of stress loading and/or timing of tests, but the present results provide no adequate explanation.

The abnormal behavior, anxiety-like behavior in SART-stressed mice in the elevated plus-maze test, was improved by anxiolytics such as diazepam, alprazolam and buspirone without any influence on nonstressed mice. The diazepam-induced enhancement of the percentage of time spent in the open arms by SART-stressed mice was antagonized by flumazenil, a benzodiazepine-receptor antagonist. Consequently, the diazepam- and alprazolam-induced enhancement of the percentage of time spent in the open arms by SART-stressed mice may be mediated through activation of benzodiazepine receptors. SART-stressed animals exhibit abnormal behavior in open-field (9), step-down (11) and forced swimming tests (12, 13). This abnormal open-field behavior, the increases in locomotor activity, rearing and defecation and the decrease in grooming observed in SART-stressed rats was partially inhibited by diazepam and alprazolam (9). The abnormal behavior, the shortened immobility time in the forced swimming test in SART-stressed mice, was improved by diazepam and alprazolam (12). The effects of SART stress, such as prolongation of the QRS interval in the electrocardiogram (ECG) (22) and electroencephalogram (EEG) alterations (23), were normalized by diazepam and alprazolam. These and previous data suggest that SART-stressed animals are very likely in

a state of anxiety.

Baldwin and File (27) observed that flumazenil reversed the anxiety observed after withdrawal of chlordiazepoxide in rats following chronic treatment with chlordiazepoxide and suggested that the antianxiety action of flumazenil depends on blockade of the action of the endogenous benzodiazepine inverse agonist, diazepam binding inhibitor (DBI), which is released under stressful situations. DBI, an endogenous negative modulator of the benzodiazepine receptor complex, appears to exhibit pharmacological effects similar to those of anxiogenic β -carboline esters (39). DBI reduced the amplitude of outward chloride current induced by GABA in patch-clamp experiments using whole cells (40). Intracerebroventricular injection of DBI elicited several behavioral changes resembling anxiety in rats (41). The release of endogenous DBI has been linked to stress such as exposure to acute loud noise (42). DBI thus appears linked to anxiety. In the present study, flumazenil showed no significant effect on the percentage of time spent in the open arms by SART-stressed mice, suggesting that DBI is not linked to anxiety-like behavior of SART-stressed mice.

Brain serotonin is involved in mood disorders such as depression and anxiety (43) as well as in nociception and thermoregulation. 5-HT_{1A} receptor agonists and antagonists modify the anxiety level of human subjects. 5-HT_{1A} agonists such as buspirone (44) are generally anxiolytics and antagonists show anxiogenic effects (45). 5-HT_{1A} receptors are involved in the regulation of synaptic transmission, as are presynaptic autoreceptors in midbrain serotonergic nuclei, or postsynaptic receptors in the hippocampus. In both areas, activation of 5-HT_{1A} receptor results in the opening of K⁺ channels that produce both hyperpolarization and decrease in membrane resistance. The stimulation of presynaptic receptors by agonists induces inhibition of the firing rate of serotonergic neurons and suppresses 5-HT synthesis, as well as the reduction in 5-HT turnover and release (46, 47). The hyperactive serotonergic system causes anxiety-like behavior in rodents and humans (45, 48). The activation of the serotonergic system may thus contribute to the development of anxiety-related disorders. The anxiety-like behavior observed in SART-stressed mice in the elevated plus-maze was improved more by buspirone than by diazepam or alprazolam. The abnormal behavior of SART-stressed mice may thus be related to the serotonergic system. SART stress was shown to decrease serotonin in various brain areas of rats such as the cerebral cortex, hypothalamus, thalamus and midbrain, but the ratio, 5-HIAA/5-HT, did not change in these areas (26). Accordingly, serotonergic neuron activity in the brain of SART-stressed animals is considered to decrease. These changes in serotonergic neuron activity in SART-stressed animals conflict with the generally accepted relationship between anxiety and the serotonergic system in the brain. The following explanation

may explain why SART-stressed mice exhibit anxiety-like behavior in the elevated plus-maze test: Serotonergic neuron activity in the brain of SART-stressed animals decreases chronically to induce upregulation of 5-HT receptors involving 5-HT_{1A}. Thus SART-stressed mice respond strongly even to a slight 5-HT release in response to such novel environments as the plus-maze and stressful situations. The hypersensitivity of 5-HT receptors is thought to greatly enhance the anxiolytic effect of buspirone on SART-stressed mice when compared to nonstressed mice. It is certain that the anxiolytic effect of buspirone was mediated by 5-HT_{1A} receptors because it was counteracted by WAY-100635, a highly selective 5-HT_{1A} serotonin receptor antagonist (28).

The anxiety-like behavior of SART-stressed mice thus appears to be more closely related to an abnormality of the serotonin receptors, and abnormal behaviors in SART-stressed animals may partially arise from anxiety related to serotonin.

Chronic diazepam administered during stress loading prolonged the decreased percentage of time spent in the open arms caused by SART stress. The decreased percentage of time was also reversed by chronic alprazolam treatment. In contrast, chronic buspirone treatment did not influence the percentage of time spent in the open arms of SART-stressed mice, which were tested 24 h after administration of the last drug. However, when the stressed mice that had been chronically treated with buspirone during stress loading were tested 60 min after the last drug, they showed an increased percentage of time in the open arms when compared to that of the vehicle-treated group. That is, buspirone could show acute effects but not chronic ones on SART stress-induced anxiety, and in this study, no tolerance developed to buspirone's anxiolytic effects even 7 days after its continuous administration. From these results, the serotonergic nervous system essentially has no important implications in the development of anxiety-like behavior of SART-stressed mice, although serotonin receptors including 5-HT_{1A} are thought to be changed in SART-stressed mice.

In conclusion, the behavior of SART-stressed mice in the elevated plus-maze is thought to be due to anxiety. Benzodiazepine and serotonin receptors are closely related to anxiety in SART-stressed mice, and the sensitivity of 5-HT_{1A} receptors may increase in SART-stressed mice. SART stress may be useful for investigating the psychological and neuropharmacological basis of anxiety, although additional studies should be conducted in this regard.

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