□原著論文□

Repeated treatment with yokukansan, a traditional Japanese herbal medicine, suppresses the increase in the conditioned fear response induced by sigma$_1$ (σ_1) receptor agonist in mice

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Abstract

Yokukansan is a traditional Japanese herbal medicine that has been reported to improve stress-related psychiatric symptoms such as anxiety. Recently, we found that repeated treatment with yokukansan synergistically enhances the anxiolytic-like effect of fluvoxamine, an antidepressant that preferentially activates brain 5-HT neurotransmission, in the contextual fear conditioning paradigm in mice. A growing body of evidence indicates that fluvoxamine has affinity for and acts as an agonist for sigma$_1$ (σ_1) receptor. Therefore, the present study examined whether σ_1 receptor plays a role in the enhancing effect of yokukansan on the anxiolytic-like effect of fluvoxamine in mice, as estimated by the contextual fear conditioning paradigm. A single administration of SA4503 (1 mg/kg, i.p.), a selective σ_1 receptor agonist, before the test session significantly enhanced freezing behavior in mice. In a combination study, the enhancing effect of SA4503 (1 mg/kg, i.p.) on freezing behavior was significantly attenuated in mice that had been repeatedly pretreated with yokukansan (1 g/kg, p.o.) once a day for 6 days after the conditioning session. These results indicate that chronic treatment with yokukansan downregulates σ_1 receptor-mediated signaling, which positively modulates the conditioned fear response, and this may be related, at least in part, to the mechanism of the synergistic interaction between yokukansan and fluvoxamine regarding anxiety.

Keywords：yokukansan, fluvoxamine, contextual fear conditioning, anxiolytic effect, σ_1 receptor, mouse

抑肝散の慢性投与はシグマ$_1$（σ_1）受容体作動薬による恐怖条件付けストレス反応の増強を抑制する

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抄　録

抑肝散は，不安などのストレス性精神症状を改善することが報告されている漢方薬である．最近，著者らは，セロトニン神経伝達を促進させる抗うつ薬であるフルボキサミンの抗不安様効果を，抑肝散が相乗的に増強することを見出した．一方，フルボキサミンは，シグマ$_1$（σ_1）受容体に対してアゴニストとして作用することが報告されている．そこで，本研究では，フルボキサミンの抗不安様効果に対する抑肝散の増強効果におけるσ_1受容体の役割について検討した．選択的σ_1受容体作動薬であるSA4503（1 mg/kg, i.p.）の単回投与により，文脈的恐怖条件付け試験におけるマウスのすくみ行動が増強した．一方，このSA4503の効果は，抑肝散（1 g/kg, p.o.）を1日1回6日間，慢性的に前投与することにより減弱した．以上の結果より，恐怖条件付けストレス反応を促

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進的に制御しているσ_1受容体のダウンレギュレーションが，不安に対する抑肝散とフルボキサミンの相乗的な抑制効果に関与している可能性が示唆された.

キーワード：抑肝散，フルボキサミン，文脈的恐怖条件付け，抗不安効果，σ_1受容体，マウス

Ⅰ. Introduction

Yokukansan is a traditional Japanese herbal medicine that has been approved in Japan as a remedy for neurosis, insomnia, and irritability and night crying in children. Recently, an *in vitro* binding study demonstrated that yokukansan binds to 5-HT_{1A} receptors and acts as a partial agonist[1]. Moreover, repeated administration of yokukansan increases and decreases 5-HT_{1A} and 5-HT_{2A} receptor function in the prefrontal cortex, respectively[2–4]. Both agonism of 5-HT_{1A} receptor and antagonism of 5-HT_{2A} receptor have been shown to have anxiolytic-like effects in animal models of anxiety[5–7]. Therefore, yokukansan may have a beneficial effect in patients suffering from anxiety disorders. Indeed, several studies have reported that anxiety-like behaviors induced by both innate fear and memory-dependent fear are suppressed by treatment with yokukansan[8–10].

Selective 5-HT reuptake inhibitors (SSRIs), antidepressants that preferentially activate brain 5-HT neurotransmission, are clinically used for the treatment of anxiety disorders, including obsessive-compulsive disorder (OCD) and post-traumatic stress disorder (PTSD)[11,12]. This clinical evidence indicates that SSRIs have both antidepressant and anxiolytic properties. Recently, to evaluate the usefulness of co-treatment with both an antidepressant and an herbal medicine in psychiatric patients, we investigated the effect of yokukansan on the anxiolytic-like effect of fluvoxamine as estimated by the contextual fear conditioning paradigm in mice[13]. The findings indicated that repeated treatment with yokukansan synergistically enhances the anxiolytic-like effect of fluvoxamine in conjunction with a decrease in cortical 5-HT_{2A} receptor expression, suggesting that augmentation therapy with a combination of both drugs may be useful for the treatment of anxiety disorders.

The σ_1 receptor is an intracellular chaperone protein that originates from endoplasmic reticulum membranes, but is also localized on nuclear, mitochondrial, and plasma membranes[14,15]. Accumulating evidence suggests that σ_1 receptors are particularly concentrated in the limbic structures of the brain, which play important roles in emotion and cognition[16,17]. For example, preclinical studies have demonstrated that activation of the σ_1 receptor has an antidepressant-like effect and alleviates cognitive dysfunction[18,19]. Interestingly, some SSRIs including fluvoxamine, sertraline, and escitalopram have high to moderate affinity for σ_1 receptor, and among these fluvoxamine has the highest potency[20]. Furthermore, positron emission tomography using [^{11}C]SA4503, a selective σ_1 receptor agonist[21,22], has revealed high occupancy of the σ_1 receptor in the brain following the administration of therapeutic doses of fluvoxamine to healthy male volunteers[23]. These reports suggest that the σ_1 receptor may be involved, at least in part, in the pharmacological effects of fluvoxamine. Therefore, as part of study to clarify the mechanisms involved in the interaction between fluvoxamine and yokukansan mentioned above[13], we examined whether yokukansan influences the effects of σ_1 receptor agonist on anxiety estimated by the contextual fear conditioning paradigm in mice.

Ⅱ. Material and methods

1. Animals

Male ICR mice (Japan SLC Inc., Shizuoka, Japan) weighing 25−30 g were housed at a room temperature of 23 ±1℃ with a 12 h light-dark cycle (light on 7:00 a.m. to 7:00 p.m.). Food and water were available ad libitum. All experiments were carried out during the light period.

This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by

the Committee on the Care and Use of Laboratory Animals of the International University of Health and Welfare, which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

2. Drugs

Yokukansan is composed of seven dried medicinal herbs: 4.0 g of Atractylodis lanceae Rhizoma (*Atractylodes lancea* De Candolle), 4.0 g of Poria (*Wolfiporia cocos* Ryvarden et Gilbertson (*Poria cocos* Wolf)), 3.0 g of Cnidii Rhizoma (*Cnidium officinale* Makino), 3.0 g of Uncariae Uncis cum Ramulus (*Uncaria rhynchophylla* Miquel), 3.0 g of Angelicae Radix (*Angelica acutiloba* Kitagawa), 2.0 g of Bupleuri Radix (*Bupleurum falcatum* Linné), and l.5 g of Glycyrrhizae Radix (*Glycyrrhiza uralensis* Fischer). These herbs are registered in the Pharmacopeia of Japan ver. 17. The powdered water extract of yokukansan used in the present study was manufactured according to the formulation previously reported[1,24] and supplied by Tsumura & Co. (Tokyo, Japan). SA4503, a selective σ_1 receptor agonist, was purchased from Tocris Bioscience (Bristol, UK). Yokukansan and SA4503 were dissolved in purified water and saline, respectively. The dosage and injection route of drugs were decided based on previous reports[19,25].

3. Apparatus and procedure for the contextual fear conditioning paradigm

For the experiments, we used a plastic box (20 × 18 × 30 cm high) with a stainless steel grid floor. Intermittent inescapable electric foot-shocks were delivered through the grid floor by an isolated shock generator (Muromachi Kikai, Co., Ltd., Japan).

The contextual conditioned fear stress procedure was performed over 2 days in accordance with our previous reports[26,27] with a minor modification; i.e., a day for the conditioning session and a day for the test session. In the conditioning session, mice were placed in the box and subjected to 36 inescapable foot-shocks (intensity 1 mA, duration 1 s) at 1−10 s intervals. After the last foot-shock, mice were immediately returned to their home cage. Twenty-four hours or a week later, mice were used in the test session. In the test session, the mice were again placed in the same box without being exposed to foot-shocks, and the duration of freezing behavior was recorded for 360 sec. The duration of freezing behavior was recorded by an activity-monitoring system (SUPER-MEX, Muromachi Kikai). SA4503 (1 mg/kg) or saline (10 ml/kg) was administered intraperitoneally (i.p.) 30 min prior to the start of the test session (Figs. 1A and 2A). Yokukansan (1 g/kg) or distilled water (10 ml/kg) was administered orally (p.o.) once a day for 6 days from the day after the conditioning session (Fig. 2A).

4. Statistical analysis

All data are expressed as the mean ± S.E.M. Data presented in Figs. 1B and 2B were analyzed by two-way ANOVA, with factors of treatment and time, followed by post-hoc Bonferroni multiple comparison tests. Data presented in Figs. 1C and 2C were analyzed by Student's *t*-test and one-way analysis of variance (ANOVA) followed by post-hoc Student-Newman-Keuls multiple comparison tests, respectively. Probability values of less than 0.05 were considered to indicate statistical significance.

Ⅲ. Results

1. Effects of SA4503, a selective σ_1 receptor agonist, on freezing behavior induced by contextual fear conditioning in mice

Effects of SA4503 on the freezing behavior induced by contextual fear conditioning were examined in mice. As shown in Fig. 1A, mice were exposed to foot-shock (conditioning session), and 24 hour later, the duration of freezing behavior was recorded (test session). SA4503 or saline was administered 30 min prior to the start of the test session.

Fig. 1B shows the time-course of changes in freezing behavior induced by contextual fear conditioning in mice.

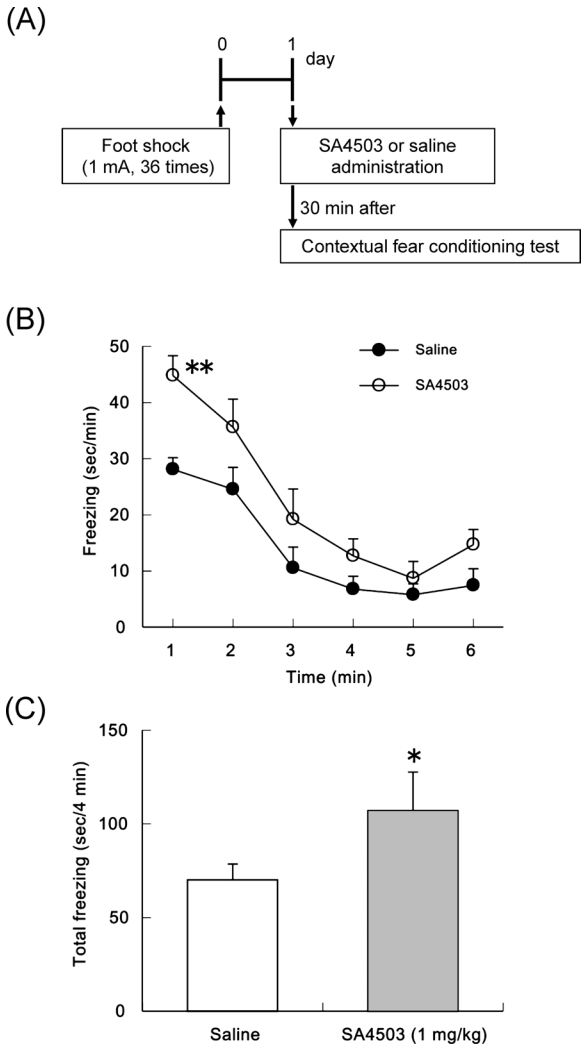

Fig. 1　A: Experimental schedule. B and C: Effects of SA4503 on the time-course of changes (B) in and total duration (C) of freezing behavior induced by contextual fear conditioning in mice. Values are the mean ± SEM of 9−10 mice. **p<0.01 vs. saline group.

Two-way ANOVA indicated a significant effect of drug treatment ($F_{1,102}$=18.47, p<0.0001); a single administration of SA4503 (1 mg/kg, i.p.) before the test session enhanced freezing behavior in mice. Furthermore, a post-hoc test revealed a significant increase in the duration of freezing behavior in the SA4503 (1 mg/kg, i.p.)-treated group during the first minute after mice were placed in the test box.

Fig. 1C shows the total duration of freezing behavior during the first 4 minutes after mice were placed in the test box. In this analysis, freezing behavior was significantly enhanced in mice that were treated with SA4503 (1 mg/kg, i.p.).

2. Influence of repeated pretreatment with yokukansan on the effect of SA4503 on freezing behavior induced by contextual fear conditioning in mice

Influence of repeated pretreatment with yokukansan on the effect of SA4503 on freezing behavior induced by contextual fear conditioning were examined in mice. As shown in Fig. 2A, mice were exposed to foot-shock (conditioning session), and from the next day, yokukansan or distilled water was administered orally once a day for 6 days. Twenty-four hours later, the duration of freezing behavior was recorded (test session). SA4503 or saline was administered 30 min prior to the start of the test session.

Fig. 2B shows the time-course of changes in freezing behavior induced by contextual fear conditioning in mice. Two-way ANOVA indicated a significant effect of drug treatment ($F_{2,168}$=8.67, p=0.0003); a single administration of SA4503 (1 mg/kg, i.p.) before the test session enhanced freezing behavior in mice. In contrast, this enhancing effect of SA4503 on freezing behavior was suppressed in mice that had been repeatedly pretreated with yokukansan (1 g/kg, p.o.) once a day for 6 days after the conditioning session. A post-hoc test also revealed that yokukansan (1 g/kg, p.o.) significantly inhibited the enhancement of freezing behavior induced by SA4503 (1 mg/kg, i.p.) during the first minute after mice were placed in the test box.

Fig. 2C shows the total duration of freezing behavior for the first 4 minutes after mice were placed in the test box. Freezing behavior was significantly enhanced in mice administered SA4503 (1 mg/kg, i.p.), but this enhancement was not seen in mice that had been repeatedly pretreated with yokukansan (1 g/kg, p.o.).

Ⅳ. Discussion

Rodents show a response characterized by a period of crouching and complete immobility when re-exposed to the same environment where they had been previously exposed

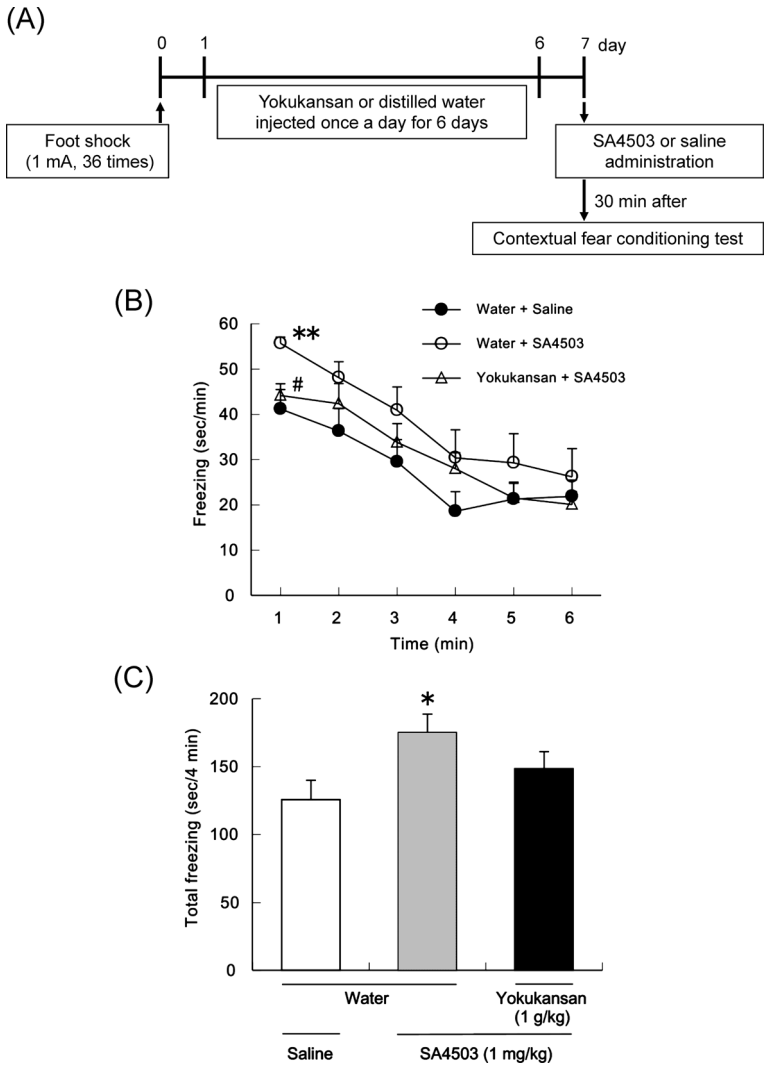

Fig. 2　A: Experimental schedule. B and C: Influence of repeated pretreatment with yokukansan on the effect of SA4503 on freezing behavior induced by contextual fear conditioning in mice.
YKS: yokukansan. Values are the mean ± SEM of 10−11 mice. *p<0.05, **p<0.01 vs. water plus saline group. $^{\#}p$<0.05 vs. water plus SA4503 group.

to aversive stimuli, including inescapable foot-shock. This behavior is called conditioned fear stress-induced freezing behavior, and can be used as a model of anxiety[28]. The freezing behavior induced by contextual fear conditioning has been reported to be attenuated by both benzodiazepine anxiolytics and non-benzodiazepine anxiolytics including SSRIs[26,27,29], indicating that this animal model may be useful for evaluating the efficacy of drugs for the treatment of anxiety disorders. In this study, we examined whether σ_1 receptor is involved in the expression of the conditioned fear response in mice. Treatment with the selective σ_1 receptor agonist SA4503 before the test session in the contextual fear conditioning paradigm enhanced freezing behavior in mice. This finding suggests that σ_1 receptor may positively regulate the expression of the conditioned fear response. It has been reported that σ_1 receptor plays an important role in cognitive function. For example, fluvoxamine was shown to facilitate NMDA receptor-dependent cognition in mice, and this effect was antagonized by the σ_1 receptor antagonist NE-100[18,30]. More recently, (4R,5S)-2-(5-methyl-2-oxo-4-phenyl-pyrrolidin-1-yl)-acetamide (E1R), a positive allosteric modulator of σ_1 receptor,

reversed muscarinic receptor antagonist-induced cognitive impairment[31]. The freezing behavior observed in the test session of the contextual fear conditioning paradigm is an emotional response that reflects the recall of fear memories during conditioning. Taken together, the present findings suggest that σ_1 receptor may be involved in not only cognition but also in the recall of fear memories, which may be related to the expression of anxiety.

Next, in a combination study, the enhancing effect of SA4503 on freezing behavior was significantly inhibited in mice that had been repeatedly pretreated with yokukansan once a day for 6 days after the conditioning session. This finding suggests that repeated treatment with yokukansan may reduce σ_1 receptor-mediated signaling, which positively modulates the conditioned fear response. Again, fluvoxamine has affinity for and acts as an agonist for σ_1 receptor[16,17,20]. Therefore, the downregulation of σ_1 receptor may be involved, at least in part, in the synergistic effect of yokukansan and fluvoxamine on anxiety via a reduction in the recall of fear memories. To our knowledge, the present finding may be the first evidence that yokukansan can affect the function of σ_1 receptor. Although there are several possibilities for the mechanism of yokukansan, noteworthy is the partial agonist activity for 5-HT_{1A} receptors[1]. Previous behavioral and/or neurochemical studies have reported the possibility that 5-HT_{1A} and σ_1 receptors synergistically interact to influence brain function[32,33]. Thus, the sustained activation of 5-HT_{1A} receptor by repeated treatment with yokukansan might result in the downregulation of σ_1 receptor in terms of maintaining homeostasis. However, further studies will be needed to explore this hypothesis.

Ⅴ. Conclusion

We recently found that the anxiolytic effect of fluvoxamine is enhanced by repeated pretreatment with yokukansan. However, how yokukansan enhances the anxiolytic effect of fluvoxamine is not known. Because fluvoxamine also functions as σ_1 receptor agonist, in the present study, we examined the effect of σ_1 receptor activation on anxiety-like behavior of mice. The results demonstrated that pharmacological activation of σ_1 receptor enhanced freezing behavior in the contextual fear conditioning paradigm in mice. This finding provides the new insight that σ_1 receptor may positively regulate anxiety related to the recall of fear memory. Furthermore, the present study provides behavioral evidence that repeated pretreatment with yokukansan downregulates σ_1 receptor, i.e. repeated pretreatment with yokukansan suppressed the anxiogenic-like effect of SA4503. The present findings suggest that yokukansan enhances the anxiolytic-like effect of fluvoxamine by reducing anxiogenic role of σ_1 receptors.

Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

Author contributions

Designing research (RO, HM, MT), performing research (RO, HM, AS, KM, KK), analyzing data (RO, HM), writing paper (RO, HM, MT), proofreading (HT).

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