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Role of 5-HT_7 receptors in context- and tone-dependent fear conditioning in mice

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Abstract

The present study examined whether serotonin (5-HT)$_7$ receptors are involved in the mechanism of fear conditioning using the selective 5-HT_7 receptor antagonist 2*a*-[4-(4-phenyl-1,2,3,6-tetrahydropyridyl)butyl]-2*a*,3,4,-tetrahydrobenzo(*c*,*d*)indol-2-(1*H*)-one (DR4004). Conditioning was performed in a third trial in which a tone was followed by an electrical foot-shock. Context- and tone-dependent fear was examined in tests conducted 24 and 48 hr after conditioning, respectively. DR4004 (5 and/or 10 mg/kg), when administered intraperitoneally (i.p.) either 30 min before or immediately after conditioning, caused a significant decrease in context- and tone-dependent fear. In contrast, neither of the doses of DR4004 (5 and 10 mg/kg, i.p.) when administered 30 min before the tests had significant effects. Additionally, DR4004 (5 and 10 mg/kg, i.p.) did not modify the sensitivity toward painful electrical stimuli. These results suggest that 5-HT_7 receptors may play an important role in the acquisition and retention of fear conditioning.

Introduction

Fear conditioning is an associative learning paradigm for studying the neurobiological mechanisms of aversive learning memory and also for understanding the root of fear-related disorders[1-3]. In conditioning in this paradigm, a neutral conditioned stimulus (CS) such as a novel environment or sound is learned in association with an aversive unconditioned stimulus (US) such as an electrical foot-shock. After conditioning, CS elicits a defensive reaction in the absence of US. For example, rodents that are re-exposed to the same conditioned environment (smell, grid of the floor, shape) show freezing behavior that is characterized by a period of crouching and complete immobility. This is called context-dependent fear conditioning, since the conditioned environment act as a CS. Furthermore, rodents also exhibit freezing behavior when they are exposed to a sound even through they are not placed in a different novel environment. This phenomenon is called tone-dependent fear conditioning. An advantage of this model is that context- and tone-dependent fear can be acquired in a single learning trial and can be assessed independently from each other. Additionally, the single-trial procedure enables the exact timing of drug treatment in relation to the acquisition, retention and expression of fear conditioning.

The brain serotonin (5-hydroxytryptamine : 5-HT) nervous system has been implicated in various brain functions as well as in the pathophysiology and treat-

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ment of a wide variety of neuropsychiatric disorders[4)5)]. Recent studies have reported that 5-HT-related agents, such as 5-HT_{1A} receptor agonists, 5-HT2 receptor antagonists and selective 5-HT reuptake inhibitors, are effective in the treatment of anxiety disorders and depression[5)]. Molecular cloning studies have revealed the existence of 14 different genes, each encoding a distinct 5-HT receptor subtype[6)]. The 5-HT_7 receptor is the most recently identified member of the family of G-protein-coupled 5-HT receptor subtypes[7)8)]. The development of selective ligands for 5-HT_7 receptors will be of utmost importance in determining the physiological role of this receptor subtype. 2*a*-[4-(4-Phenyl-1,2,3,6-tetrahydropyridyl)butyl]-2*a*,3,4,5-tetrahydrobenzo(*c*,*d*)indol-2-(1*H*)-one (DR4004) is a selective 5-HT_7 receptor antagonist that was developed by Kikuchi et al[9)], which displaces the binding of [3H] 5-carbox-amidotryptamine with high affinity and selectivity, and also inhibits the 5-HT-induced stimulation of cyclic AMP accumulation in a mammalian cell line (COS-7 cells) expressing 5-HT_7 receptors. This compound has recently been used as a tool for determining the actual functions of 5-HT_7 receptors *in vivo*[10)11)].

Studies using autoradiography, in situ hybridization, radioligand binding and immunohistochemistry techniques have shown that 5-HT_7 messenger RNA (mRNA) and receptor protein have a similar abundant distribution in various brain regions, i.e., cerebral cortex, hippocampus, thalamus, amygdala and hypothalamus[7)12)13)]. The expression and distribution of mRNA and proteins for 5-HT_7 receptors in the limbic structures suggest that they may play a role in the regulation of emotional as well as cognitive functions. Indeed, recent behavioral studies have suggested that 5-HT_7 receptor antagonists exert anxiolytic[14)15)], antidepressive[14-17)] and precognitive effects[18)]. Furthermore, we recently obtained evidence that 5-HT_7 receptors may play a role in the regulation of emotion by modulating amygdaloid 5-HT and dopamine neuronal transmission[11)]. However, the effects of 5-HT_7 receptor antagonists on fear conditioning have not yet been investigated. Therefore, in the present study, we examined the effects of DR4004 on context- and tone-dependent fear conditioning in mice.

Material and methods

The present studies were 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 Tokyo Medical University and the Japanese Pharmacological Society.

1. Animals

Male ddY mice (Tokyo Experimental Animals, Tokyo, Japan) weighing 30-40 g were housed at a room temperature of 23±1°C with a 12-h light/dark cycle (light on at 0600 to 1800). Food and water were available ad libitum.

2. Apparatus and procedure for the fear conditioning paradigm

For the fear conditioning paradigm, we used a wooden box divided into three compartments by walls (10×30×25 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, Japan).

The conditioned fear stress procedure was performed over 3 days in accordance with the report by Stiedl et al[19)] with a minor modification, i.e. day 1 for the conditioning session, and days 2 and 3 for the context- and tone-dependent fear test sessions, respectively (Fig. 1). In the conditioning session, mice were exposed to each compartment of the fear conditioning box (180 sec), and this was followed by tone presentation (CS, 60 sec, 80 dB). After termination of the tone, a foot-shock (US, 1.0 mA, 2 sec) was delivered through the stainless grid floor. This procedure was repeated three times, and then mice were removed from the box 60 sec after termination of the final exposure to a foot-shock. Non-

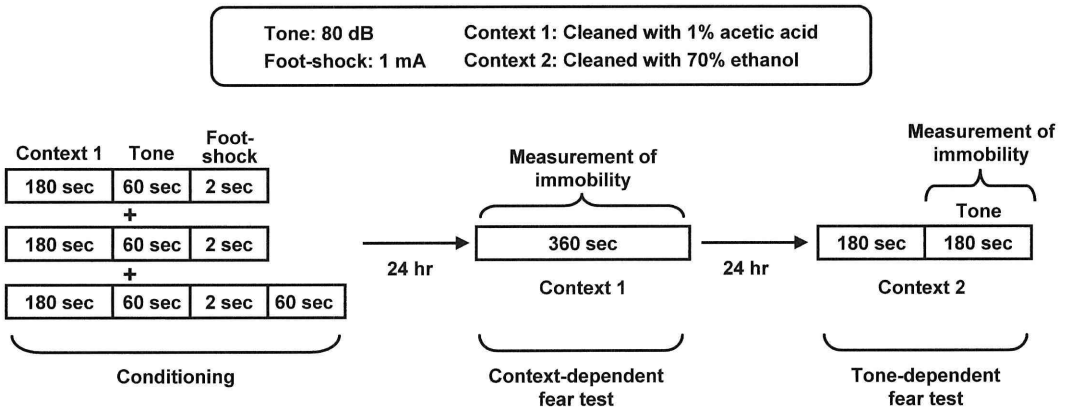

Fig. 1 Experimental paradigms for context- and tone-dependent fear conditioning. For details, see Materials and methods.

conditioned mice were placed in the box and presented a tone, but not subjected to foot-shocks. The box was thoroughly cleaned with 1% acetic acid at the start of each conditioning session. Twenty-four hours later (day 2), context-dependent fear was estimated. The mouse was exposed to the same conditioning chamber that had been cleaned with 1% acetic acid, and the duration of freezing behavior was recorded for 360 sec. Forty-eight hours later (day 3), tone-dependent fear was estimated in a novel context. A transparent plastic cylinder (19 cm in diameter and 25 cm high) that had been cleaned with 70% ethanol was used as a novel context. In the tone-dependent fear test, a 180-sec pause without stimulation preceded a 180-sec period of auditory stimulation. The duration of freezing behavior was recorded for 180 sec during exposure to auditory stimulation. The duration of freezing behavior of mice was recorded by an activity monitoring system (SUPER-MEX, Muromachi Kikai, Japan). DR4004 (5 mg/kg and 10 mg/kg), a selective 5-HT_7 receptor antagonist, or vehicle was injected intraperitoneally (i.p.) 30 min prior to the start of conditioning, immediately after conditioning was finished or 30 min prior to the start of the test session. The timing of drug treatments aimed to examine whether 5-HT_7 receptors are involved in the acquisition, retention and expression of fear conditioning, respectively.

3. Procedure for the measurement of pain sensitivity

The pain sensitivity of mice was estimated as described previously[20]. An electric shock chamber (12×14×15 cm high, Muromachi Kikai) was used to determine the pain threshold to electrical stimuli. Mice were allowed 15 min to habituate to the environment of the chamber before a series of inescapable shocks was delivered. Each series consisted of 10 shocks at the following intensities (mA): 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.6 and 0.8. The shock duration was 2 sec and the shocks were delivered at 30-sec intervals. Thresholds for flinching (forepaws off the grid floor) and jumping (all four paws off the grid floor) were measured. Mice were pretreated with DR4004 (5 and 10 mg/kg, i.p.) 30 min prior to exposure to the electrical stimuli.

4. Drug

DR4004 was provided by Meiji Seika Kaisha (Kanagawa, Japan). It was dissolved in Tween 20 until a clear solution was obtained, and then diluted with saline to reach the proper concentrations. The final concentration of Tween 20 in the solution was 1%. The doses and timing of treatment with DR4004 were determined based on previous reports[11)21)22)].

5. Statistical analysis

The data are presented as the mean±S.E.M. Two-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls multiple comparisons test was used for the statistical evaluation ($P<0.05$ and 0.01).

Results

1. Effects of DR4004 on the context-dependent fear conditioning

The effects of DR4004 on context-dependent fear conditioning are shown in Fig. 2. Vehicle-treated mice that had been subjected to a conditioning session showed stable freezing behavior compared with non-conditioned mice (Figs. 2A-C). This freezing behavior was significantly suppressed by pre- or post-conditioning treatment with DR4004 (5 mg/kg, i.p.), whereas a higher dose (10 mg/kg, i.p.) was ineffective (Figs. 2A and B). In contrast, nether of the doses of DR4004 (5 and 10 mg/kg, i.p.) when administered before the test session affected freezing behavior. (Fig. 2C).

2. Effects of DR4004 on the tone-dependent fear conditioning

The effects of DR4004 on tone-dependent fear conditioning are shown in Fig. 3. Upon exposure to auditory stimulation, vehicle-treated mice that had been subjected to a conditioning session showed a sudden freezing behavior compared with non-conditioned mice, and this change in behavior then gradually disappeared. (Figs. 3A-C). This freezing behavior was significantly suppressed by pre- or post-conditioning treatment with DR4004 (5 and/or 10 mg/kg, i.p.), and a lower dose (5 mg/kg, i.p.) seemed to be more effective (Figs. 3A and B). However, similar to the effect on context-dependent fear conditioning, pre-test treatment with DR4004 (5 and 10 mg/kg, i.p.) did not modify the freezing behavior (Fig. 3C).

3. Effects of DR4004 on the pain sensitivity

The effects of DR4004 on pain sensitivity are shown in Fig. 4. Mice treated with DR4004 (5 and 10 mg/kg, i.p.) displayed no significant differences in the thresholds for flinching and jumping compared with those in vehicle-treated mice.

Discussion

The present study clearly demonstrated that pre- as well as post-conditioning treatment with the selective 5-HT_7 receptor antagonist DR4004 at a low, but not at a high dose, caused a marked decrease in context-dependent fear, tested 24 hours after conditioning. These findings are consistent with a previous report which showed that 5-HT_7 receptor knockout mice exhibited impaired context-dependent fear conditioning[23]. Furthermore, the present study showed that, similar to context-dependent fear conditioning, tone-dependent fear conditioning is also decreased by pre- and post-conditioning treatment with DR4004. To the best of our knowledge, this is the first evidence to indicate that

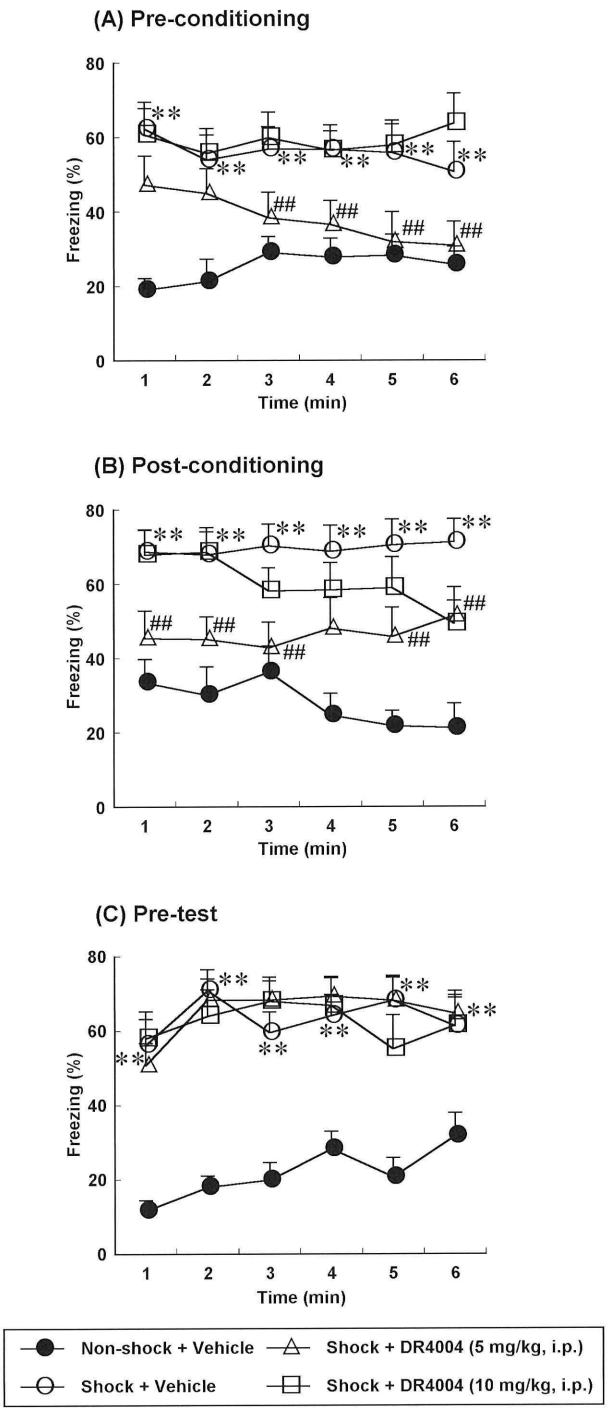

Fig. 2 Effects of DR4004 on context-dependent fear conditioning in mice. DR4004 or vehicle was injected intraperitoneally (i.p.) 30 min prior to the start of conditioning, immediately after conditioning was finished or 30 min prior to the start of the test session. Each point represents the mean with S.E.M. of 15 mice. $^{**}P<0.01$ vs. non-shock plus vehicle group. ## $P<0.01$ vs. shock plus vehicle group.

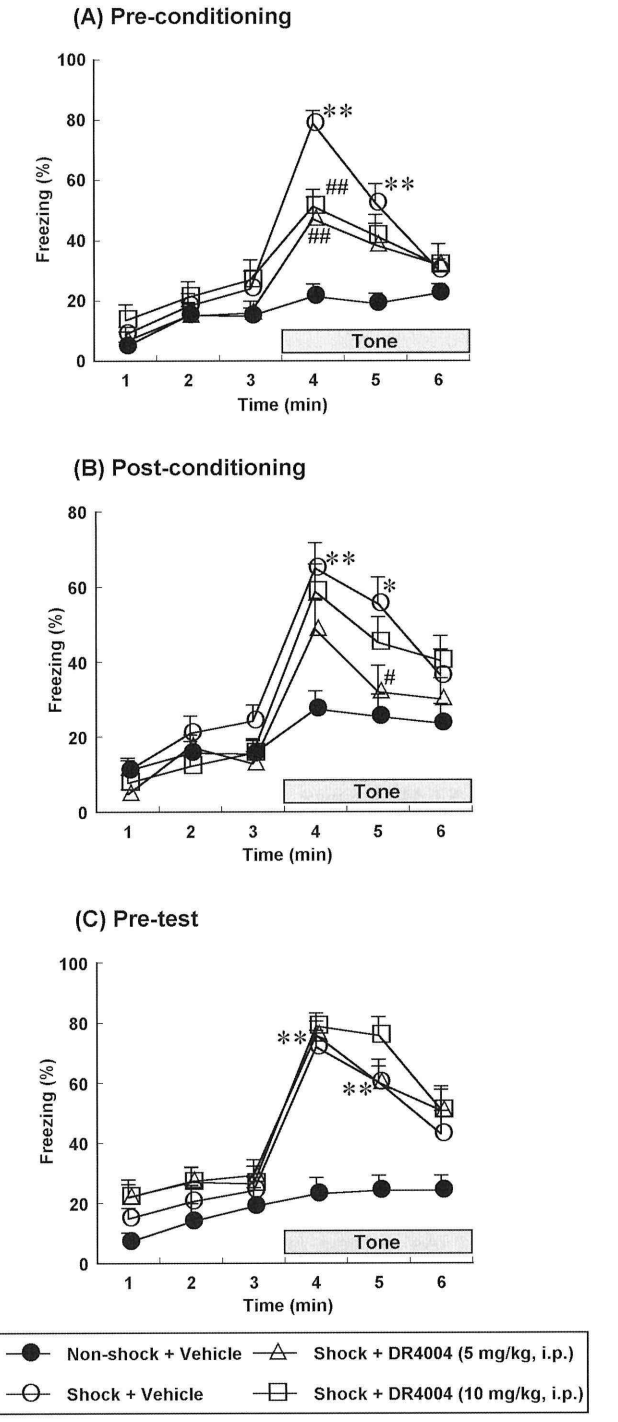

Fig. 3 Effects of DR4004 on tone-dependent fear conditioning in mice. DR4004 or vehicle was injected intraperitoneally (i.p.) 30 min prior to the start of conditioning, immediately after conditioning was finished or 30 min prior to the start of the test session. Each point represents the mean with S.E.M. of 15 mice. $^{*}P<0.05$, $^{**}P<0.01$ vs. non-shock plus vehicle group. # $P<0.05$, ## $P<0.01$ vs. shock plus vehicle group.

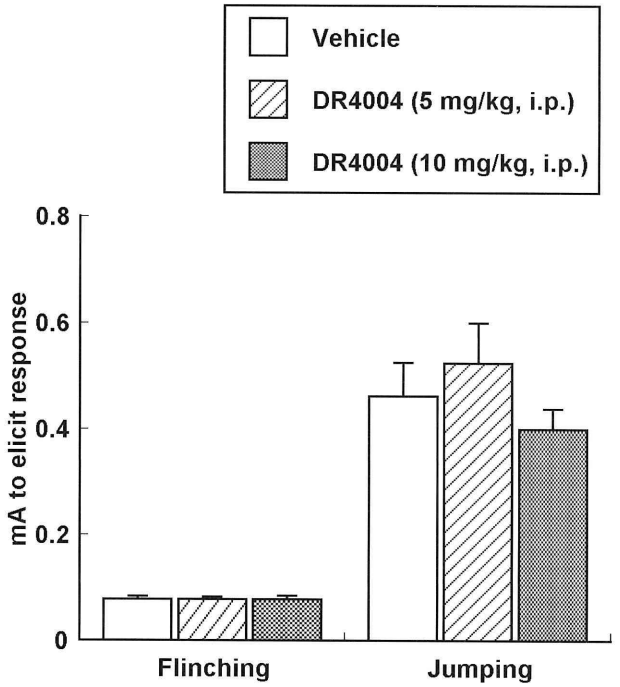

Fig. 4 Effects of DR4004 on pain sensitivity in mice. DR4004 or vehicle was injected 30 min prior to the delivery of electrical foot-shocks. Each point represents the mean with S.E.M. of 8 mice.

both context- and tone-dependent fear conditioning are modulated by 5-HT_7 receptors. However, when administered before the tests, DR4004 showed no effects on both context- and tone-dependent fear conditioning. Taken together, the present findings show that the pharmacological blockade of 5-HT_7 receptor functions disrupts both the acquisition and retention of fear conditioning.

A U-shaped dose-response curve has been reported for 5-HT_7 receptor antagonist in several behavioral studies[14)15)]. As in these previous reports, DR4004 did not show dose-dependent effects in the present study. Although the cause of this result is not yet clear, under the present circumstances, one possibility is that there might be a critical range of 5-HT_7 receptor activity for modulating fear conditioning. Thus, there is an optimal range of 5-HT_7 receptor activity for affecting fear conditioning, whereas too little or too much activity is ineffective. Furthermore, nonserotonergic mechanisms might contribute to the lack of an effect of DR4004 at a high dose. For instance, we previously found that the same dose of DR4004 decreases dopamine turnover in the amygdala[11)], a brain region which plays a critical role in the modulation of fear conditioning[1)2)].

In the fear conditioning paradigm, it is possible that altered pain sensitivity for an electrical stimulus in conditioning may affect behavioral performance in the test. Therefore, to assess whether the observed effects of DR4004 in the present study are because of nonspecific actions on pain sensitivity, we performed a nociception assay using electric current as the nociceptive stimulus. It is unlikely that changes in pain sensitivity are involved in the effects of DR4004 on fear conditioning, since neither of the doses of DR4004 used in the present study modified the threshold for flinching and jumping elicited by the electrical stimuli.

The detailed mechanisms involved in the modulation of fear conditioning by 5-HT_7 receptors are not yet clear. However, previous behavioral studies in rodents in which 5-HT has been depleted both pharmacologically[24)] and genetically[25)] have provided evidence for a possible role of 5-HT in fear conditioning. The brain regions that underlie context- and tone-dependent fear conditioning have recently been investigated in detail, and both types of fear conditioning depend on the integrity of the amygdala, which is considered to be the sensorimotor interface for fear conditioning[1)2)]. Furthermore, the hippocampus is also an important limbic structure that controls fear conditioning in cooperation with the amygdala, whereas its role in tone-dependent fear conditioning is still unclear[1)2)]. Both the amygdala and hippocampus are densely innervated by 5-HT neurons of the raphe nuclei[26)27)], and this area is rich in 5-HT_7 receptors[7)12)13)]. These reports, along with our present findings, suggest that 5-HT_7 receptors play an important role in fear conditioning and that a possible neuronal correlate for such a role is present within the amygdala and/or hippocampus.

The most interesting functional molecule that is expected to be closely associated with the present findings is extracellular signal-regulated kinase 1 and 2 (Erk-1/2). Erk-1/2 belong to a family of mitogen-activated protein (MAP) kinases that integrates signals received by membrane growth factor and G-protein coupled receptors and transfers them to the nucleus[28)]. A growing body of evidence suggests that the activation of an Erk-1/2-mediated signaling pathway of the hippocampus and amygdala is essential for the acquisition and retention of context- and/or tone-dependent fear conditioning[29–31)]. Importantly, it has recently been found that 5-HT_7 receptors, expressed by cultured rat hippocampal neurons as well as human embryonic kidney (HEK) 293 cells, were coupled to stimulation of Erk-1/2[32–34)]. Thus, it has been speculated that blockade of 5-HT_7 receptors reduces Erk-1/2 activity, and as a result fear conditioning is impaired. Further detailed studies on 5-HT_7 receptors, which may functionally interact with Erk-1/2, may be useful for understanding the mechanisms that underlie the regulation of fear conditioning, and also the pathophysiology of fear-related disorders.

In conclusion, the present study demonstrated that pharmacological blockade of 5-HT_7 receptor using a selective antagonist before or after conditioning decreased both context- and tone-dependent fear. These results provide evidence that 5-HT_7 receptors may play a critical role in the acquisition and retention of fear conditioning.

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マウス文脈依存的および音刺激依存的恐怖条件付けにおける 5-HT_7 受容体の役割

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本研究では、選択 5-HT_7 受容体拮抗薬である 2*a*-[4-(4-phenyl-1,2,3,6-tetrahydropyridyl)butyl]-2*a*,3,4,-tetrahydrobenzo(*c*,*d*)indol-2-(1*H*)-one(DR4004) を用いて、マウス恐怖条件付けにおける 5-HT_7 受容体の役割について検討した。音刺激ならびに電撃刺激を用いて条件付けを行い、24 時間では文脈依存的恐怖条件付け反応を、さらに 48 時間後では音刺激依存的恐怖条件付け反応を評価した。その結果、文脈依存的および音刺激依存的恐怖条件付け反応は、ともに DR4004 (5 または 10 mg/kg, i.p.) を条件付け開始の 30 分前あるいは終了直後に投与することにより有意に抑制された。一方、DR4004 (5 または 10 mg/kg, i.p.) を恐怖条件付け反応を評価する 30 分前に投与した場合では、何ら効果は認められなかった。尚、マウスの電撃刺激に対する疼痛反応は、DR4004 (5 または 10 mg/kg, i.p.) の投与では全く影響を受けなかった。以上の結果より、文脈依存的および音刺激依存的恐怖条件付けの獲得・保持過程おいて、5-HT_7 受容体が重要な役割を担っていることが示唆された。

〈キーワード〉 文脈依存的恐怖条件付け、音刺激的恐怖条件付け、5-HT_7 受容体、マウス