Original Article

Involvement of Brain 5-HT$_7$ Receptors in the Formation of Stress Adaptation in Mice

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Abstract

Background/Objectives: Impairment of the ability to adapt to stress in animals may contribute to some stress-related psychiatric disorders, such as anxiety and depression. A growing body of evidence has suggested that the brain's serotonin (5-HT) nervous system may play an important role in the etiology, expression and treatment of anxiety and depression. The aim of the present study was to examine whether brain 5-HT$_7$ receptors are involved in the formation of stress adaptation.

Methods: Male ICR mice were either exposed to repeated restraint stress for 60 or 240 min/day (stressed group) or left in their home cage (non-stressed group) for 1 or 14 days. The emotionality of mice was estimated by the hole-board test. The levels of 5-HT$_7$ receptor expression and extracellular signal-regulated kinase 1/2 (ERK) phosphorylation were assessed by western blot analysis.

Results: A single exposure to restraint stress for 60 min induced a decrease in head-dipping behavior in the hole-board test. This emotional stress response was not observed in mice that had been exposed to repeated restraint stress for 60 min/day for 14 days, which confirmed the development of stress adaptation. In contrast, mice that were exposed to restraint stress for 240 min/day for 14 days did not develop this stress adaptation, and still showed a decrease in head-dipping behavior. Increases in 5-HT$_7$ receptor protein and ERK phosphorylation were observed in the frontal cortex and hippocampus of stress-adaptive, but not stress-maladaptive, mice. The decreased emotionality observed in stress-maladaptive mice was significantly recovered by chronic treatment with LP-12, a selective 5-HT$_7$ receptor agonist, immediately after daily exposure to stress.

Conclusion: The present findings suggest that the brain 5-HT$_7$ receptor-ERK system may play an important role in the formation of stress adaptation. Furthermore, stimulation of 5-HT$_7$ receptors may have a beneficial effect on stress adaptation and alleviate the emotional abnormality observed under conditions of excessive stress.

Keywords: Stress adaptation; 5-HT$_7$ receptor; ERK phosphorylation; Hole-board test; Mouse.

Abbreviations: 5-HT, serotonin; ERK, extracellular signal-regulated kinase 1/2; LP 12, 4-(2-Diphenyl)-*N*-(1,2,3,4-tetrahydronaphthalen-1-yl)-1-piperazinehexanamide; mRNA, messenger RNA; i.c.v., intracerebroventricularly; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MAP, mitogen-activated protein; TrkB, tropomyosin-related kinase B; BDNF, brain-derived neurotrophic factor; mTOR, mammalian target of rapamycin; Cdc42, cell division cycle 42; Cdk5, cyclin-dependent kinase 5; CREB, cAMP response element-binding protein.

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Introduction

The ability to adapt to stress is an important defensive function of living things and impairment of this ability in animals may contribute to some stress-related disorders. Thus, identification of the brain mechanisms that contribute to stress adaptation could help to pave the way for new therapeutic strategies for stress-related mood disorders, such as anxiety and depression. Evidence obtained in our previous studies suggests that the brain's serotonin (5-HT) nervous systems, especially that involving 5-HT$_{1A}$ receptors, may be involved, at least in part, in the development of adaptation to stress.[1–4]

There are now believed to be seven 5-HT receptor families—collectively known as 5-HT$_{1-7}$—that comprise a total of 14 structurally and pharmacologically distinct 5-HT receptor subtypes.[5] The 5-HT$_7$ receptor is the most recently identified member of the

family of G protein-coupled 5-HT receptor subtypes.[6,7] Studies using autoradiography, in situ hybridization, radioligand binding and immunohistochemistry techniques have shown that 5-HT$_7$ messenger (m)RNA and receptor protein have a similar abundant distribution in various brain regions (*i.e.* the cerebral cortex, hippocampus, thalamus, amygdala and hypothalamus).[6,8,9] 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, previous behavioral pharmacological studies have suggested that 5-HT$_7$ receptor antagonists exert anxiolytic and antidepressive effects.[10–13] Interestingly, it has also been recently reported that 5-HT$_7$ receptors are highly co-expressed with 5-HT$_{1A}$ in brain regions implicated in the regulation of emotionality, and these receptors have been shown to form heterodimers both *in vitro* and *in vivo*.[14] Considering our previous findings and these more recent reports led us to speculate that brain 5-HT$_7$ receptor may play a significant role in the development of stress adaptation.

A series of behavioral experiments have demonstrated that repeated exposure to the same type of stress stimuli diminishes acute stress responses. For example, Kennett *et al*.[15–17] reported that rats exposed to a single restraint stress exhibited a reduction in locomotion in an open field and that this behavioral change disappeared after repeated exposure to restraint stress. Other researchers have described similar behavioral adaptive responses to stress stimuli,[18–20] suggesting that this animal model may be useful for investigating the mechanisms of stress adaptation. In addition, to further characterize models of stress adaptation, we recently examined behavioral responses in mice that were produced by either single or repeated exposure to restraint stress for 60 or 240 min.[21]

A single exposure to restraint stress reduces head-dipping behavior of mice in the hole-board test, which is a good index for evaluating emotionality and this stress response is not seen in mice that are exposed to repeated restraint stress for 60 min/day for 14 days, which confirms the development of stress adaptation.[22–26] However, mice that were exposed to restraint stress for 240 min/day for 14 days did not show this adaptive response to stress stimuli, but did show a decrease and increase in the weights of the thymus and adrenal gland respectively. Such maladaptation to stress stimuli and morphological abnormalities in organs suggest that the mice were unable to adapt to stressful conditions. Thus, we can create stress-adaptive and -maladaptive models in mice by repeatedly exposing rats to different degrees of restraint stress.[21]

In the present study, we carried out behavioral and biochemical experiments to obtain evidence that the brain's 5-HT$_7$ receptor plays a role in the formation of stress adaptation using stress-adaptive and -maladaptive modeled mice.

Materials and methods

Animals

Male ICR mice (Japan SLC, Inc., Shizuoka, Japan), weighing 25–30 g, were housed at a room temperature of 23 ± 1 °C 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 in the light phase of the cycle. 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 the International University of Health and Welfare, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Drugs

4-(2-Diphenyl)-*N*-(1,2,3,4-tetrahydronaphthalen-1-yl)-1-piperazinehexanamide hydrochloride (LP-12; Tocris Bioscience, Minneapolis, MN, USA) was used in the present study as a selective 5-HT$_7$ receptor agonist. For experimentation, the LP-12 was dissolved in saline and administered intracerebroventricularly (i.c.v.) in a volume of 4 µL/mouse.

Exposure to restraint stress

Mice were either exposed to repeated restraint stress for 60 or 240 min/day by being inserted into a syringe (50 mL) (stressed group) or left in their home cage (non-stressed group) for 1 or 14 days. After the final exposure to restraint stress, emotionality of the mice was estimated using an automatic hole-board apparatus.[1–4,21,23] In particular, each mouse was placed in the center of the hole-board and allowed to freely explore the apparatus for 5 min. The exploratory behaviors of mice on the hole-board (*i.e.* distance moved, number and duration of rearing, number and duration of head-dips, and latency to head-dips) were automatically recorded. In the experiment for examining the effect of LP-12, the drug (3 or 10 µg/mouse, i.c.v.) or saline was injected immediately after the daily exposure to restraint stress for 240 min/day.

Automatic hole-board apparatus

The automatic hole-board apparatus (model ST-1; Muromachi Kikai Co., Ltd., Tokyo, Japan) consisted of a gray box (50 × 50 × 50 cm) with four equidistant holes, each 3 cm in diameter, in the floor. An infrared beam sensor was installed on the wall to detect the number and duration of rearing and head-dipping behaviors. The distance that mice moved on the hole-board was recorded by an overhead digital video camera; the heads of the mice were painted yellow and the digital video camera followed their center of gravity.

Data from the digital video camera were collected through a custom-designed interface (DVTrack, Muromachi Kikai) as a reflection signal. Head-dipping behaviors were double-checked via an infrared beam sensor and the overhead digital video camera. Thus, head-dipping behavior was counted only when both the head intercepted the infrared beam and the head was detected at the hole by the digital video camera. All of the data were stored in a personal computer and analyzed using analytical software (Comp ACT HBS, Muromachi Kikai).

Western blotting

After the behavioral experiments, brain regions were quickly removed and homogenized in 6 volumes of ice-cold buffer containing 20 mM Tris-HCI (pH 7.4; Wako Pure Chemical Industries, Ltd., Osaka, Japan), 2 mM ethylenediaminetetraacetic acid (EDTA; Wako Pure Chemical), 10 mM ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′,-tetraacetic acid (EGTA; Wako Pure Chemical), 250 mM sucrose (Wako Pure Chemical), 1% Triton (Calbiochem-Novabiochem, San Diego, CA, USA) and a protease inhibitor cocktail (Complete®; Roche Molecular Biochemicals, Mannheim, Germany), and by using a homogenizer (Pellet Pestles® Cordless Motor; Techno Chemical Co., Ltd., Tokyo, Japan). The homogenates were centrifuged at 1,000 × g (3,500 rpm) for 10 min at 4 °C, and the supernatants were collected and stored

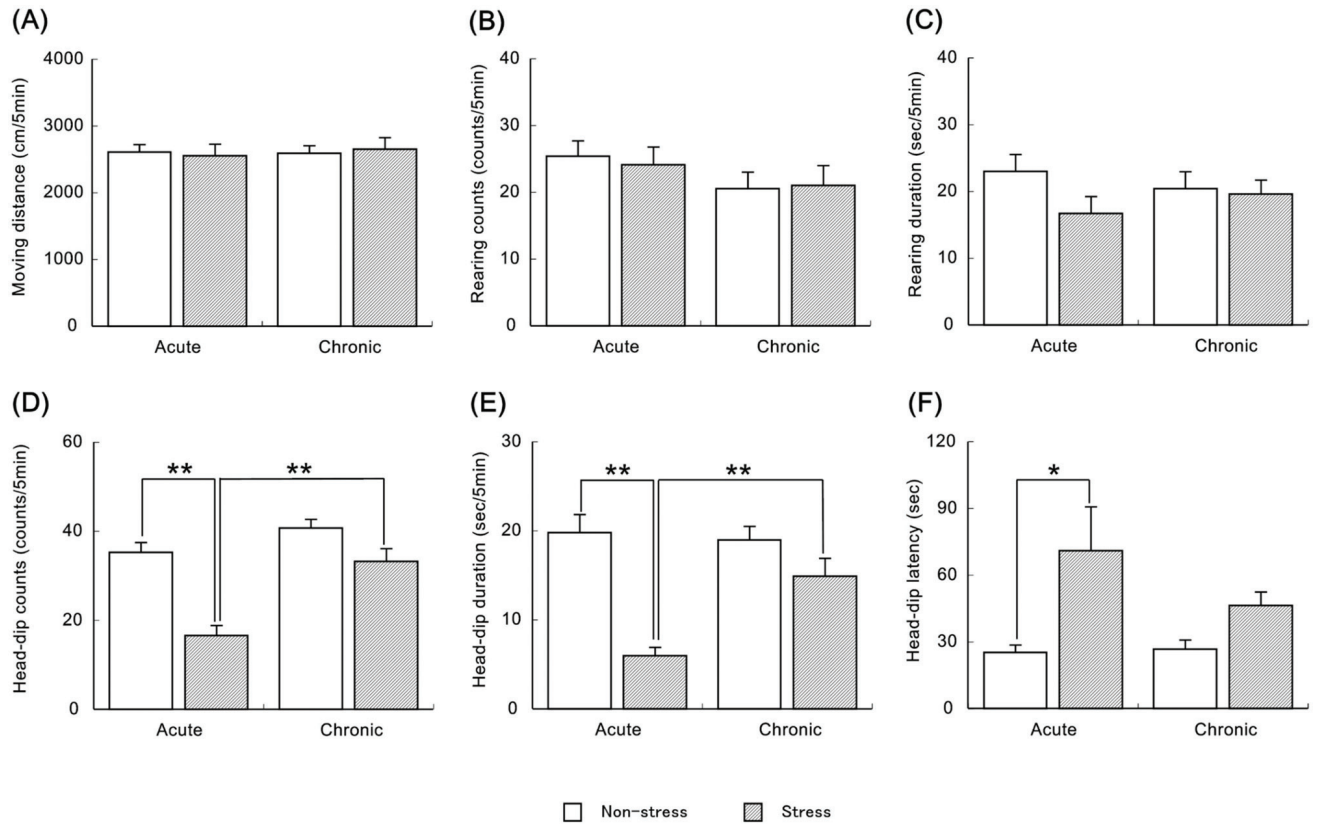

Fig. 1. Effects of exposure to adaptable repeated restraint stress on the exploratory behaviors of mice in the hole-board test. A: Moving distance; B: Number of rearing; C: Duration of rearing; D: Number of head-dips; E: Duration of head-dips; F: Latency to head-dips. Each column represents the mean with SEM of 12–16 mice. *p < 0.05, **p < 0.01.

as test samples at −70 °C for future analysis.

An aliquot of test sample was diluted with an equal volume of electrophoresis sample buffer (Bio-Rad Laboratories, Co., Ltd., Hercules, CA, USA). Proteins were separated by size on 5–20% SDS-polyacrylamide gradient gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories) soaked in 20% methanol (Wako Pure Chemical) with Tris-glycine buffer (Bio-Rad Laboratories) and by using a semi-dry electrophoretic transfer cell (Bio-Rad Laboratories). In addition, molecular markers (Precision Plus Protein™ Dual Color Standards; Bio-Rad Laboratories) were loaded in lanes adjacent to sample lanes before the commencement of each run. For the immunoblot detection of 5-HT$_7$ receptor, extracellular signal-regulated kinase 1/2 (ERK) or phosphorylated ERK, membranes were blocked in 0.05% Tween 20-Tris-buffered saline (TTBS) containing 3% bovine serum albumin (BSA; Sigma-Aldrich, Co., Ltd., St. Louis, MO, USA) for 1 hr at room temperature with agitation. The membrane was incubated with primary antibody for 5-HT$_7$ receptor (1:300 dilution; Imgenex, Co., Ltd., San Diego, CA, USA), ERK (1:1,000; Cell Signaling Technology, Co., Ltd., Danvers, MA, USA) or phosphorylated ERK (1:1,000; Cell Signaling Technology), which were diluted in TTBS containing 3% BSA overnight at 4 °C. The membranes were washed in TTBS and then incubated for 60 min at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2,000; Jackson Immunoresearch Laboratories, Co., Ltd., West Grove, PA, USA), which was diluted in TTBS containing 3% BSA. After this incubation, the membranes were washed in TTBS. The antigen-antibody-peroxidase complex was then finally detected by enhanced chemiluminescence (Santa Cruz Biotechnology, Co., Ltd., Dallas, TX, USA), and scanned, optimized and analyzed by ChemiDoc XRS (Bio-Rad Laboratories). The relative protein levels were compared with the protein level of the appropriate standard (glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for 5-HT$_7$ receptor blots, total ERK for phosphorylated ERK blots) probed on the same membrane, after stripping of the antibody previously used.

Immunohistochemistry

In the immunohistochemical analysis, naive (non-stressed) mice were deeply anesthetized with sodium pentobarbital (70 mg/kg, intraperitoneal (i.p.)) and perfusion-fixed with 4% paraformaldehyde (Wako Pure Chemical) in PBS. The brains were quickly removed after perfusion, and thick coronal sections, including the hippocampus, were initially dissected using brain blocker. The brain coronal sections were postfixed in 4% paraformaldehyde for 2 hr. After the brains were permeated with 20% sucrose for 1 day and 30% sucrose for 2 days, they were frozen in embedding compound (Sakura Finetechnical, Tokyo, Japan) on dry ice and stored at −30 °C until use. Frozen 10 µm-thick coronal sections were cut with a cryostat (Sakura Finetechnical) and thaw-mounted on amino silane-coated glass slides (Matsunami Glass Ind., Ltd., Osaka, Japan).

The mounted brain sections were incubated with 10% normal goat serum in ice-cold PBS for 60 min to block the binding of

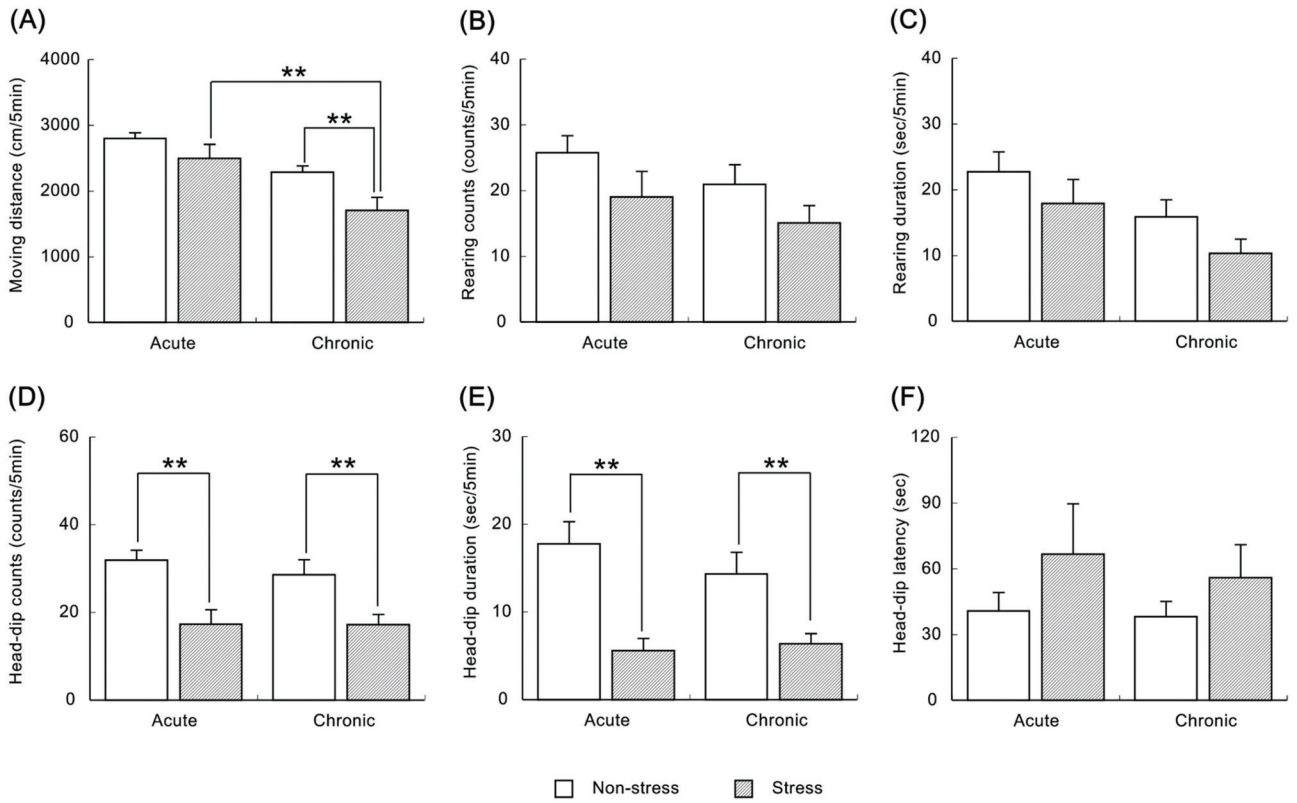

Fig. 2. Effects of exposure to unadaptable repeated restraint stress on the exploratory behaviors of mice in the hole-board test. A: Moving distance; B: Number of rearing; C: Duration of rearing; D: Number of head-dips; E: Duration of head-dips; F: Latency to head-dips. Each column represents the mean with SEM of 8–15 mice. **p < 0.01.

nonspecific antibody. Each primary antibody was diluted in PBS containing normal goat serum (1:100 5-HT$_7$ receptor (Imgenex), 1:100 p-ERK (Cell Signaling Technology)) and incubated for 2 days at 4 °C. The samples were then rinsed with PBS and incubated with the appropriate secondary antibody conjugated with Alexa 488 and Alexa 546 (1:500) for 2 h at room temperature. The slides were then cover-slipped with PermaFluor aqueous mounting medium (Immunon, Pittsburgh, PA, USA). Fluorescence immunolabeling was detected using a confocal laser-scanning microscope (FV1000; Olympus Optical, Tokyo, Japan).

Statistical analysis

Data are presented as mean ± S.E.M. Treatment effects were compared using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls multiple comparisons post-hoc test. Probability values of less than 0.05 were accepted as significant.

Results

Effects of exposure to repeated restraint stress on emotionality of mice as estimated by the hole-board test

The effects of exposure to repeated restraint stress on emotionality of mice as estimated by the hole-board test are shown in Figures 1 and 2. A single exposure to restraint stress for 60 min induced significant decreases in the number and duration of head-dipping behaviors (Fig. 1D and 1E; $p < 0.01$) as well as an increase in latency to head-dips (Fig. 1F; $p < 0.05$) in the hole-board test. These emotional stress responses were not observed in mice that were exposed to the same duration of restraint stress repeatedly once a day for 14 days (Fig. 1D, 1E and 1F). In contrast, mice that were exposed to restraint stress daily for 240 min/day for 14 days continued to show a significant decrease in the number and duration of head-dipping behaviors (Fig. 2D and 2E; $p < 0.01$). The distance mice moved was also significantly decreased (Fig. 2A; $p < 0.01$).

Changes in the expression of 5-HT$_7$ receptor in brain regions of mice induced by exposure to adaptable or unadaptable stress

The changes in expression of 5-HT$_7$ receptor in brain regions of mice induced by exposure to adaptable or unadaptable stress are shown in Figure 3. Western blot analysis revealed two bands with apparent molecular masses of approximately 45 and 50 kDa in extracts of hippocampal tissue (Fig. 3A). Therefore, expression levels of 5-HT$_7$ receptor were assessed in terms of the combination of two molecules. These signals were standardized by normalization with the signal for GAPDH. The expression of 5-HT$_7$ receptor was significantly increased in both the frontal cortex (Fig. 3B; $p < 0.05$) and hippocampus (Fig. 3C; $p < 0.05$) of mice that had been chronically exposed to adaptable stress (60 min/day for 14 days), although such changes in the expression levels were not observed in the other brain regions examined (% of control: amygdala,

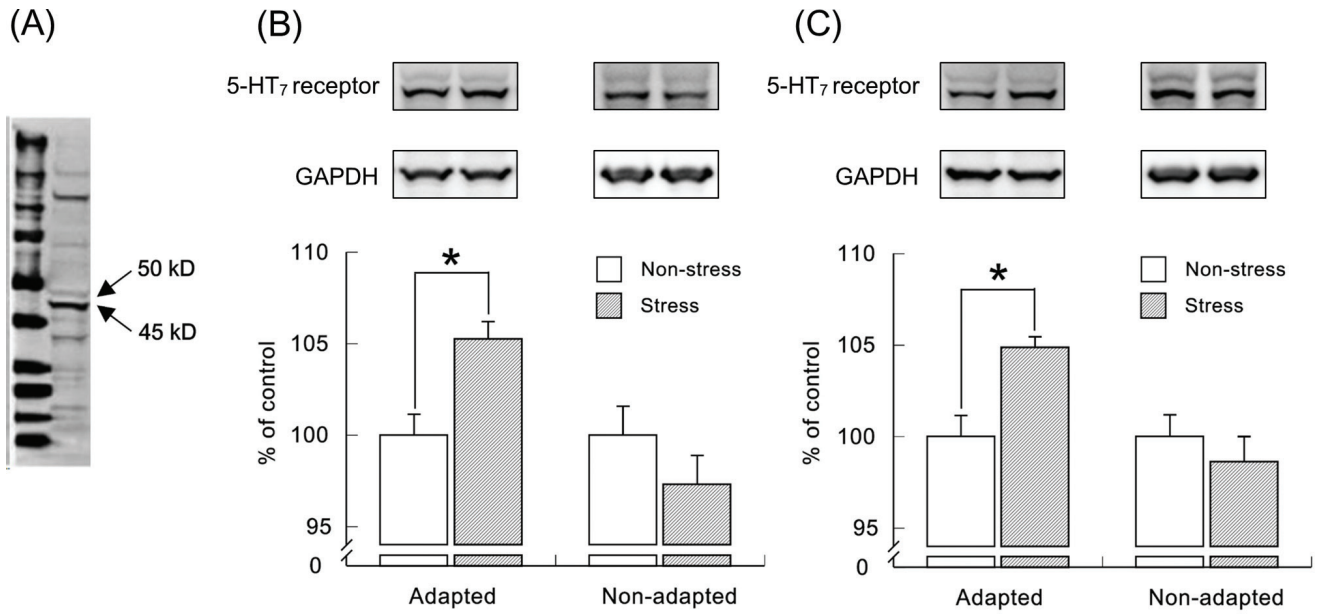

Fig. 3. Changes in 5-HT$_7$ receptor expression in the frontal cortex and hippocampus of mice exposed to adaptable or unadaptable stress. A: Immunoblots of hippocampal tissues showing 5-HT$_7$ receptor. B, C: The expression level of 5-HT$_7$ receptor was assessed in terms of the combination of two molecules in the same tissue: frontal cortex (B) and hippocampus (C). The expression is expressed as the percentage of values measured in the corresponding non-stressed group. Each column represents the mean with SEM of 8–10 mice. *p < 0.05.

non-stressed: 100 ± 1.5, stressed: 99.3 ± 1.3; hypothalamus, non-stressed: 100 ± 0.7, stressed: 97.9 ± 2.2; midbrain, non-stressed: 100 ± 1.6, stressed: 102.4 ± 4.3). In contrast, the expression of 5-HT$_7$ receptor in both the frontal cortex (Fig. 3B) and hippocampus (Fig. 3C) was unchanged in mice that had been chronically exposed to unadaptable stress (240 min/day for 14 days).

Localization of 5-HT$_7$ receptor and phosphorylated ERK in the anterior cingulate cortex and hippocampal CA2/CA3 subfields in mice

The localization of 5-HT$_7$ receptor and phosphorylated ERK in the anterior cingulate cortex and hippocampal CA2/CA3 subfields in mice is shown in Figure 4. Immunohistochemistry was used to localize 5-HT$_7$ receptor and phosphorylated ERK. Abundant immunoreactivity for 5-HT$_7$ receptor and phosphorylated ERK was observed in the anterior cingulate cortex (Fig. 4A) and hippocampal CA2/CA3 subfields (Fig. 4B). Importantly, the expression of 5-HT$_7$ receptor and phosphorylated ERK show almost complete overlap in both brain regions.

Changes in ERK phosphorylation in the frontal cortex and hippocampus of mice induced by exposure to adaptable or unadaptable stress

The changes in ERK phosphorylation in the frontal cortex and hippocampus of mice induced by exposure to adaptable or unadaptable stress are shown in Figure 5. To evaluate ERK activation, the phosphorylated ERK levels were normalized with respect to the total ERK levels in the same membranes. ERK phosphorylation closely paralleled changes in 5-HT receptor expression in stress-adaptive and -maladaptive mice; ERK phosphorylation was significantly increased in the frontal cortex (Fig. 5A; $p < 0.01$) and hippocampus (Fig. 5B; $p < 0.01$) of mice that had been chronically exposed to adaptable stress (60 min/day for 14 days). In contrast, such changes in the phosphorylation levels of ERK were not observed in mice that had been chronically exposed to unadaptable stress (240 min/day for 14 days) (Fig. 5A and 5B).

Effects of LP-12 on the emotional abnormality in mice induced by exposure to unadaptable stress

The effects of LP-12 on the emotional abnormality in mice induced by exposure to unadaptable stress are shown in Figure 6. Repeated exposure to restraint stress for 240 min/day for 14 days induced significant decreases in the number and duration of head-dipping behaviors in the hole-board test (Fig. 6D and 6E; $p < 0.01$). The decrease in head-dipping behavior was dose-dependently and significantly inhibited by chronic treatment with LP-12 (3 and 10 μg/mouse, i.c.v.) immediately after the daily exposure to restraint stress (Fig. 6D and 6E; $p < 0.05$).

Discussion

The hole-board test offers a simple method for measuring the response of an animal to an unfamiliar environment. In the hole-board test, a pronounced inhibition of head-dipping behavior is observed in rats or mice following exposure to stress stimuli.[22,25] We also previously reported that either treatment with benzodiazepine anxiogenics or exposure to acute restraint stress produced a decrease in head-dipping behavior in mice.[1–4,21,23] These findings indicate that head-dipping behavior in the hole-board test is a good index for evaluating emotionality of mice. In the present study, a single exposure to restraint stress for 60 min produced a decrease in the number and duration of head-dipping behaviors of mice in the hole-board test, and these acute emotional responses were re-

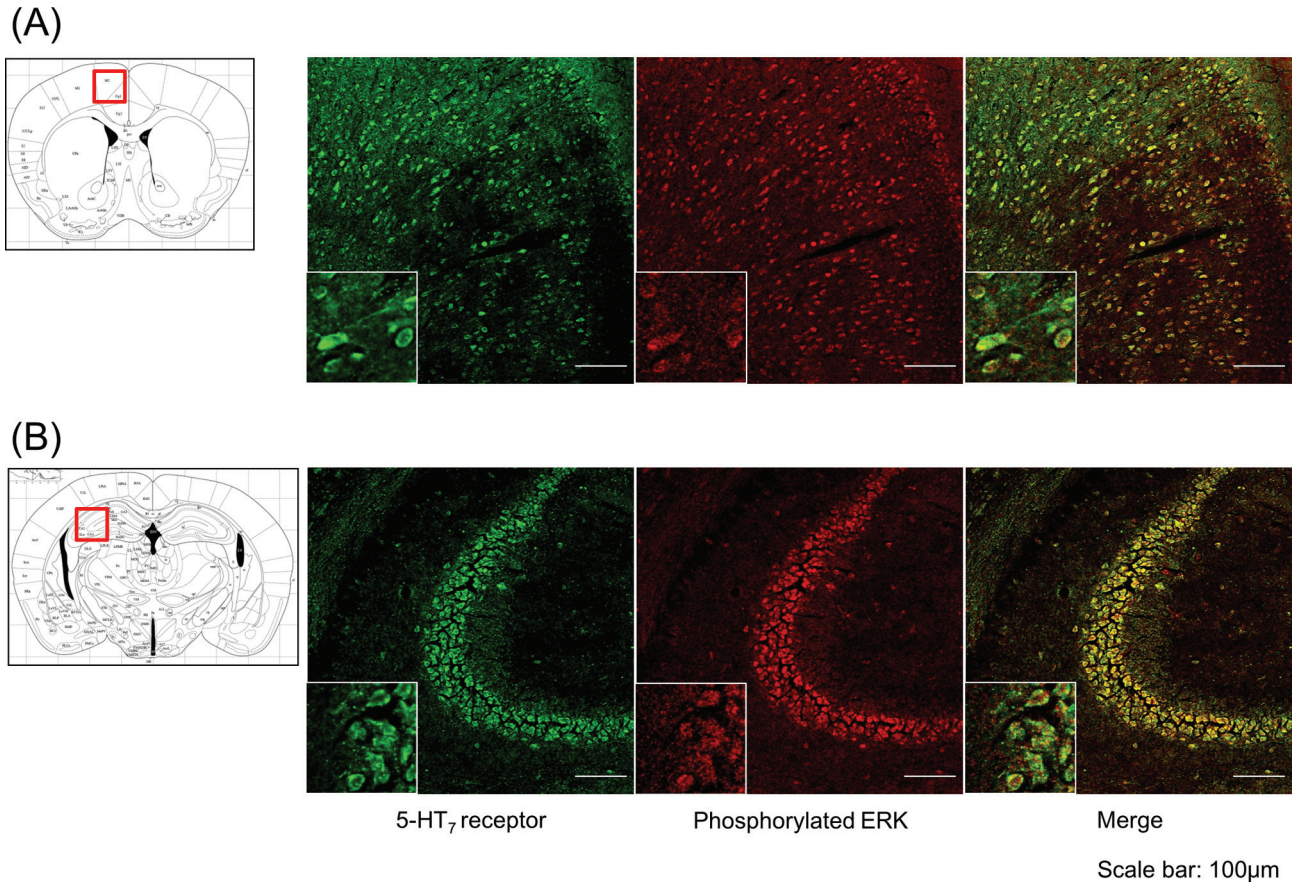

Fig. 4. Localization of 5-HT$_7$ receptor and phosphorylated ERK in the anterior cingulate cortex (A) and hippocampal CA2/CA3 subfields (B) in naive (non-stressed) mice. The 5-HT$_7$ receptor (green) and phosphorylated ERK (red) are almost co-localized. High-magnification images suggest that the 5-HT$_7$ receptor is localized in the membrane of phosphorylated ERK-positive cells. Scale bars: 100 μm.

covered by exposure to repeated restraint stress for 60 min/day for 14 days. In contrast, this development of stress adaptation was not observed in mice that had been exposed to repeated restraint stress for 240 min/day for 14 days, *i.e.* they continued to show a decrease in head-dipping behavior in the hole-board test. These findings are in good agreement with our previous report,[21] and confirm that stress-adaptive and -maladaptive models can be created in mice by repeatedly exposing them to different degrees of restraint stress. Thus, in the subsequent studies, we used the stress-adaptive and -maladaptive mice to examine whether brain 5-HT$_7$ receptors are involved in the development of stress adaptation.

Several splice variants of human (5-HT$_{7(a/b/d)}$), mouse (5-HT$_{7(a/b/c)}$) and rat (5-HT$_{7(a/b/c/e)}$) receptors have been identified. When expressed in cell lines they display similar pharmacological and functional characteristics, and also a similar tissue distribution.[27–29] In western blot analysis using a specific antibody raised against a sequence that is identical for all human receptor splice variants, two bands were detected in various types of cells, in particular the Chinese hamster ovary (commonly known as CHO) cells stably transfected with the human 5-HT$_{7(a)}$ receptor cDNA, the human glioblastoma cell lines and the human microglial MC-3 cell line, with apparent molecular masses of approximately 45 and 50 kDa.[30,31] The 45–50 kDa range corresponds to the anticipated molecular mass of the 5-HT$_7$ receptor, perhaps with different degrees of glycosylation and/or phosphorylation.[32]

Consistent with these previous reports, in the present study, western blot analysis using the same specific antibody confirmed two bands with molecular masses of approximately 45 and 50 kDa in extracts of mouse hippocampal tissue. Thus, the expression levels of 5-HT$_7$ receptor were assessed in terms of the combination of two molecules. The expression of 5-HT$_7$ receptor was significantly increased in the frontal cortex and hippocampus of mice that had been chronically exposed to adaptable stress (60 min/day for 14 days), while such changes were not observed in other brain regions, including the amygdala, hypothalamus and midbrain, which may play a role in the regulation of emotion. In contrast, the expression of 5-HT$_7$ receptor in the frontal cortex and hippocampus was unchanged in mice that had been chronically exposed to unadaptable stress (240 min/day for 14 days). These findings suggest that the up-regulation of cortical and/or hippocampal 5-HT$_7$ receptors may be involved in the development of stress adaptation.

5-HT$_7$ receptor activation activates adenylyl cyclase signaling and consequently the conversion of ATP to cAMP through coupling to Gαs.[6] ERK belongs to a family of mitogen-activated protein (MAP) kinases that integrate signals received by membrane growth factor and G protein-coupled receptors and transfer them to the nucleus.[33] A growing body of evidence suggests that the ERK-mediated signaling pathway in the brain is essential for stress-related mood regulation.[34] It has been found that 5-HT$_7$ receptors, expressed by cultured rat hippocampal neurons as well as human embryonic kidney (commonly known as HEK) 293 cells, are associated with the stimulation of ERK.[35–37]

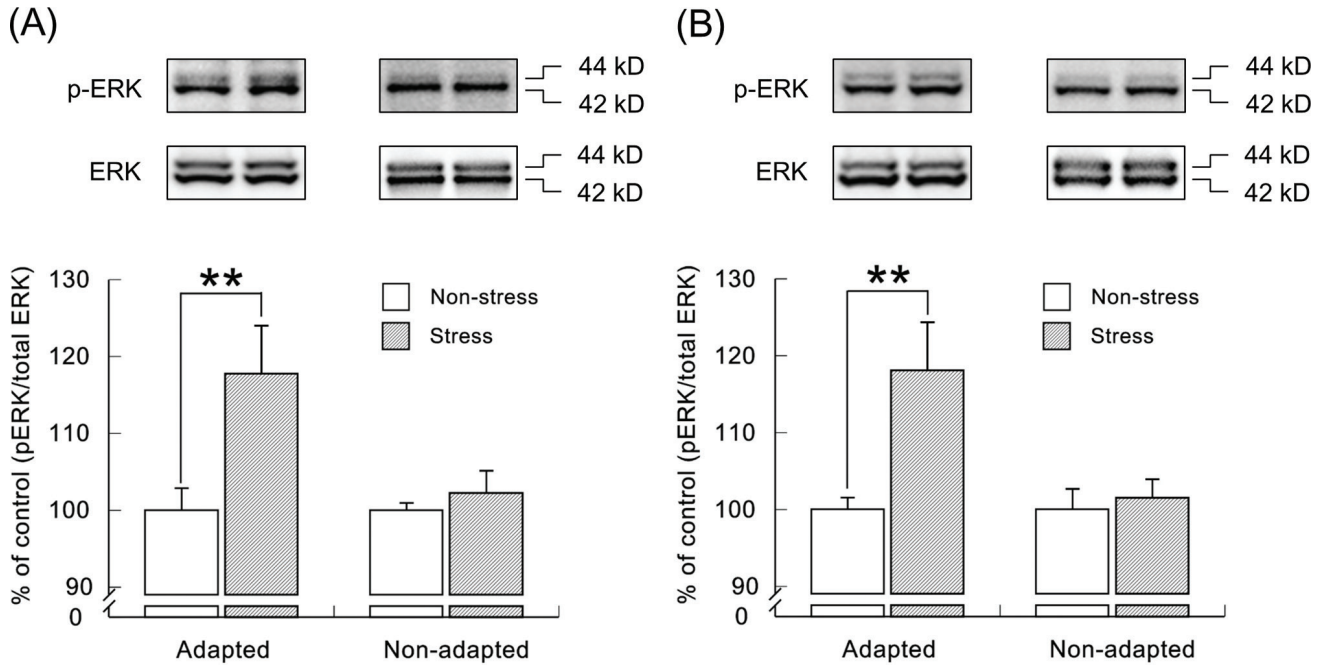

Fig. 5. Changes in ERK phosphorylation in the frontal cortex and hippocampus of mice exposed to adaptable or unadaptable stress. A, B: The level of ERK phosphorylation was measured as the intensity of phosphorylated ERK normalized with respect to that of unphosphorylated ERK in the same tissue: frontal cortex (A) and hippocampus (B). ERK phosphorylation is expressed as the percentage of values measured in the corresponding non-stressed group. Each column represents the mean with SEM of 8–10 mice. **p < 0.01.

In the present study, an immunohistochemical analysis revealed the co-localization of immunoreactivity of 5-HT$_7$ receptor and phosphorylated ERK in the anterior cingulate cortex and hippocampal CA2/CA3 subfields, indicating that ERK plays an important role in intracellular signaling via the 5-HT$_7$ receptor. More importantly, western blot analysis showed that ERK phosphorylation closely paralleled changes in 5-HT receptor expression in stress-adaptive and -maladaptive mice. ERK phosphorylation was significantly increased in the frontal cortex and hippocampus of mice that had been chronically exposed to adaptable stress. In contrast, such changes in the phosphorylation levels of ERK were not observed in mice that had been chronically exposed to unadaptable stress. These findings suggest that a cortical and/or hippocampal 5-HT$_7$ receptor-ERK pathway may play an important role in the development of stress adaptation.

Finally, we examined the effect of LP-12, a selective 5-HT$_7$ receptor agonist, on the abnormality of stress-maladaptive mice. It has been reported that LP-12 has high affinity (Ki = 0.13 nM) as well as high selectivity for the 5-HT$_7$ receptor over the 5-HT$_{1A}$, 5-HT$_{2A}$ and D$_2$ receptors (468-, 11,262- and 1,723-fold, respectively), which represent the receptors that may interfere with the evaluation of actions on emotionality mediated by 5-HT$_7$ receptor.[38] In the hole-board test, the significant decreases in both the number and duration of head-dipping behaviors of mice induced by exposure to unadaptable stress were dose-dependently and significantly inhibited by chronic treatment with LP-12 immediately after daily exposure to stress. This result supports the findings in the present biochemical study suggesting that activation of the 5-HT$_7$ receptor may be critical for the development of stress adaptation.

Recently, several reports have suggested that a 5-HT$_7$ receptor agonist might be a novel therapeutic strategy for neuropsychiatric disorders. Activation of 5-HT$_7$ receptors by agonists was shown to reverse metabotropic glutamate receptor-mediated long-term depression and to correct a synaptic malfunction in Fmr1 knock-out mice, which is a mouse model of Fragile X Syndrome, the most common form of inherited intellectual disability associated with mood disorders.[39,40] In Rett Syndrome, a disorder in which severe symptoms affect cognitive, sensory, emotional, motor and autonomic functions, 5-HT$_7$ receptor agonist was shown to ameliorate the deficits in motor coordination, spatial reference memory, and hippocampal synaptic plasticity in a mice model.[41] Thus, the activation of 5-HT$_7$ receptor appears to restore synaptic plasticity, suggesting that agonists of this receptor might be used as novel pharmacological tools in both diseases. The present findings also raise the possibility that a 5-HT$_7$ receptor agonist might be effective for the clinical treatment of mental illness that results from maladaptive coping with stressful situations, such as adjustment disorder.

Although it is well-recognized that modulation of the 5-HT$_7$ receptor affects mood regulation, the present findings somewhat contradict previous reports that the blockade of 5-HT$_7$ receptor exerts anxiolytic and antidepressive effects.[10–13] While the reason for this discrepancy is not fully understood, differences in the type and duration of stress exposure may be involved. The present study used an animal model of exposure to chronic restraint stress, whereas all of the above-mentioned previous findings were obtained from behavioral paradigms involving acute stress, including forced swimming, tail suspension and the elevated plus-maze test. Therefore, the present findings imply that brain 5-HT$_7$ receptors might play multiple roles in the pathophysiology or treatment of stress-related mood disorders. In support of this hypothesis, it has been reported that chronic treatment with imipramine (a tricyclic antidepressant) or citalopram (a selective serotonin reuptake blocker) decreased the reactivity of hippocampal 5-HT$_7$ receptors, while electroconvulsive shocks, which are known to be effective for treatment-resistant major depressive disorder, increased the

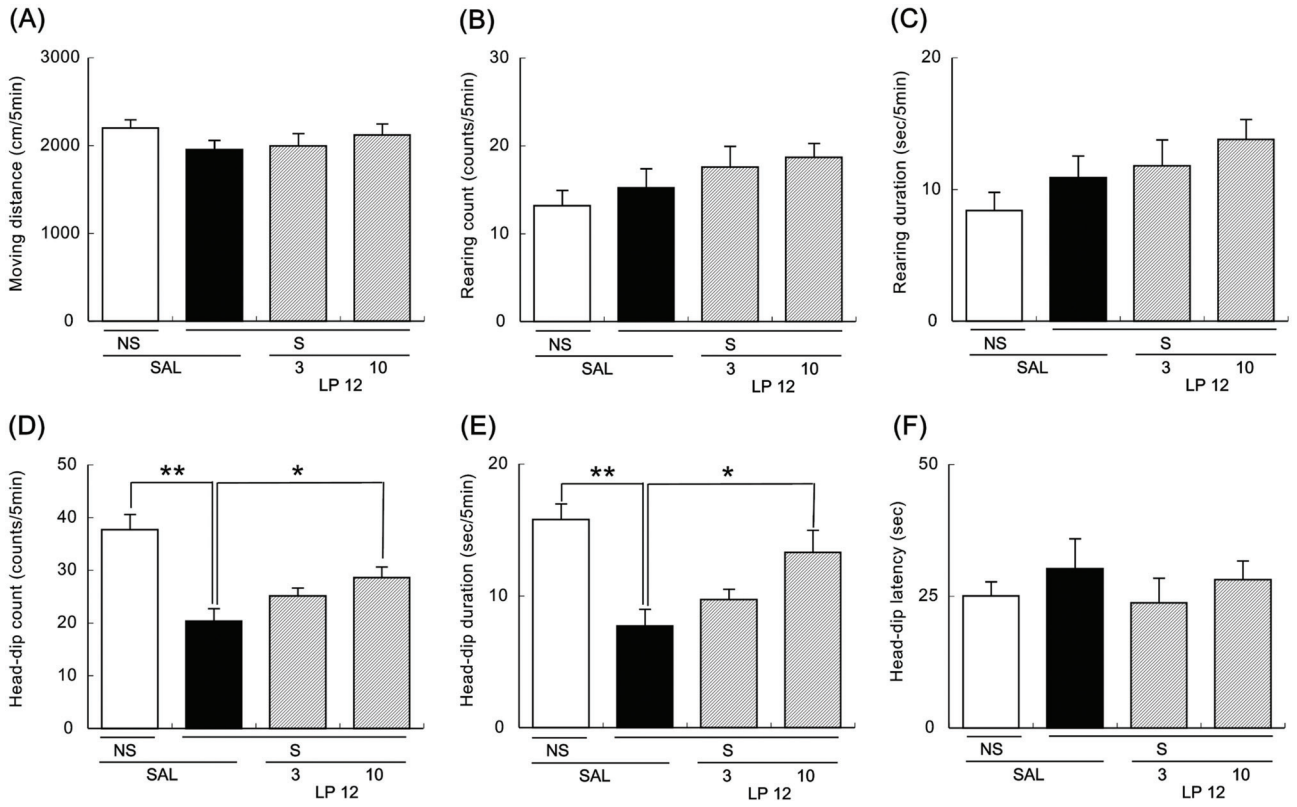

Fig. 6. Effects of LP-12 on the changes in exploratory behaviors of mice in the hole-board test. A: Moving distance; B: Number of rearing; C: Duration of rearing; D: Number of head-dips; E: Duration of head-dips; F: Latency to head-dips. Mice were either exposed to repeated restraint stress for 240 min/day (stressed group: S) or left in their home cage (non-stressed group: NS) for 14 days. LP-12 (3 or 10 μg/mouse, i.c.v.) or saline (SAL) was injected immediately after the daily exposure to restraint stress. Each column represents the mean with SEM of 16–19 mice. *p < 0.05, **p < 0.01.

5-HT$_7$ receptor-dependent response in the hippocampus.[42,43]

Although the distinct mechanisms underlying the role of brain 5-HT$_7$ receptor in stress adaptation are still unclear, one possible explanation is that the modulation of neuronal plasticity and morphology might be involved. For example, it has recently been reported that treatment with 5-HT$_7$ receptor agonist can increase the expression and phosphorylation of tropomyosin-related kinase B (TrkB) receptor.[44] TrkB is a receptor of brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family of growth factors that are involved in both neuronal plasticity and remodeling of neuronal morphology. Thus, these findings suggest that the BDNF signaling may be enhanced by 5-HT$_7$ receptor activation. Another study showed direct evidence that 5-HT$_7$ receptor modulated the neuronal morphology, in that neurite outgrowth was enhanced by the stimulation of 5-HT$_7$ receptor via several signal transduction pathways, such as mammalian target of rapamycin (mTOR), cell division cycle 42 (Cdc42), cyclin-dependent kinase 5 (Cdk5) and ERK, and all of these molecules converge to modulate cytoskeletal reorganization.[45] More recently, it has been reported that 5-HT$_7$ receptor activation increased cAMP and relative phosphorylated cAMP response element-binding protein (CREB) levels and also increased phosphorylation of the GluA1 AMPA receptor subunit in hippocampal neurons; moreover, these biochemical findings were supported by electrophysiological findings in the hippocampus that showed AMPA receptor-mediated neurotransmission was enhanced by stimulation of the 5-HT$_7$ receptor.[46] Importantly, these 5-HT$_7$ receptor-mediated molecular and cellular mechanisms that have been shown to be involved in neuronal plasticity and morphology are consistent with the new insights into neurobiology of stress and mood disorders.[47] Taken together, these findings suggest that further detailed studies focused on neuronal plasticity and morphology, which may be modulated by the 5-HT$_7$ receptor, may be useful for understanding the mechanisms of stress adaptation.

In the context of the present findings, we need further investigation in future studies. First, in order to confirm the significant role of 5-HT$_7$ receptor in the development of stress adaptation, the influence of a 5-HT$_7$ receptor antagonist in stress-adaptive mice need to be investigated. Indeed, we recently carried out a part of such a study, and obtained a preliminary finding that i.c.v. treatment with the 5-HT$_7$ receptor antagonist SB269970 immediately after daily exposure to adaptable stress disturbed the development of stress adaptation and induced abnormality of the anxiety sensitivity in the elevated plus-maze test (*i.e.* excessive increase in time spent in open-arm together with decrease in stretched attend posture; unpublished observation). Second, because LP-12 inhibited the decrease in the emotionality of stress-maladaptive mice, it would be more informative to examine the change in ERK activity induced by LP-12. We speculate that LP-12 may show an effect to increase ERK phosphorylation in the frontal cortex and hippocampus of stress-maladaptive mice. These future studies may be helpful for enhancing the value of the present findings.

In conclusion, the present study demonstrated that 5-HT$_7$ receptor expression as well as ERK phosphorylation were increased in stress-adapted, but not -maladapted, mice. Furthermore, decreases in emotional behaviors of stress-maladapted mice induced by exposure to unadaptable stress were alleviated by the pharmacologi-

cal activation of the 5-HT$_7$ receptor. The present findings suggest that the brain's 5-HT$_7$ receptor-ERK system may play an important role in the formation of stress adaptation. Furthermore, stimulation of 5-HT$_7$ receptors may have a beneficial effect on stress adaptation and alleviate emotional abnormality under conditions of excessive stress. The 5-HT$_7$ receptor may represent a promising target for innovative therapeutical strategies in stress-related neuropsychiatric disorders.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Designing research (KT, MT, HT), performing research (KT, MT, KM), analyzing data (KT, MT, KM), writing paper (KT, MT), proofreading (HT).

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