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Research Article

RESEARCH ARTICLE

Serotonin 5-HT$_7$ Receptor in the Ventral Hippocampus Modulates the Retrieval of Fear Memory and Stress-Induced Defecation

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Abstract

Background: Patients with posttraumatic stress disorder or panic disorder are often troubled by inappropriate retrieval of fear memory. Moreover, these disorders are often comorbid with irritable bowel syndrome. The main aim of the present study is to elucidate the involvement of hippocampal serotonergic systems in fear memory retrieval and stress-induced defecation.
Methods and Results: Microinjection of serotonin$_7$ receptor antagonist, but not other serotonin receptor antagonists (serotonin $_{1A}$, $_{2A}$, $_{2C}$, $_3$, $_4$, and $_6$), into the rat ventral hippocampus significantly suppressed the expression of freezing behavior, an index of fear memory retrieval, and decreased the amount of feces, an index of stress-induced defecation, in the contextual fear conditioning test. Electrophysiological data indicated that the serotonin$_7$ receptor agonist increased the frequency of action potentials in the ventral hippocampal CA3 pyramidal neuron via the activation of the hyperpolarization-activated nonselective cation current I$_h$. Moreover, in situ hybridization demonstrated that Htr7 mRNA was abundantly expressed in the CA3 compared with other subregions of the hippocampus and that these Htr7 mRNA-positive cells coexpressed hyperpolarization-activated cyclic nucleotide-gated channel 2 and 4 mRNAs, which are components of the I$_h$ channel.
Conclusions: These results indicated that the released serotonin activates the serotonin$_7$ receptor in the CA3 ventral hippocampus subregion, enhances the sensitivity to inputs via hyperpolarization-activated cyclic nucleotide 2 and 4 channels, and thereby facilitates fear memory retrieval. The serotonin$_7$ receptor might be a target of drug development for the treatment of mental disorders involving fear memory and gastrointestinal problems.

Keywords: hippocampus, conditioned fear, defecation, serotonergic

Introduction

Although the retrieval of contextual fear memory is necessary to avoid a previously encountered threat to life, patients with psychiatric disorders, such as posttraumatic stress disorder and panic disorder, are often troubled by inappropriate retrieval of fear memory. Moreover, these disorders are often comorbid with irritable bowel syndrome (Gros et al., 2009; White et al., 2010). Therefore, the neural mechanisms underlying fear memory and stress-induced defecation need to be

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elucidated to efficiently examine the clinical treatment of these disorders.

The hippocampus is a major brain region that regulates fear memory (Holt and Maren, 1999; Trivedi and Coover, 2004; Sotres-Bayon et al., 2012), it is innervated by serotonergic fibers from the dorsal and median raphe nuclei (MRN) (Azmitia and Segal, 1978). We have previously found that serotonin (5-HT) release in the ventral hippocampus, but not in the dorsal hippocampus, increased during fear memory retrieval and that the blockade of corticotropin-releasing factor (CRF) receptor-2 in MRN suppressed both 5-HT release in the ventral hippocampus and fear memory retrieval (Ohmura et al., 2010); however, this previous study did not directly prove the causal relationship between 5-HT release in the ventral hippocampus and fear expression. Therefore, the main aim of the present study is to test this relationship and determine the subtype of 5-HT receptors in the ventral hippocampus responsible for fear memory retrieval.

It has been demonstrated that hippocampal lesions impaired fear-conditioned defecation in rats (Antoniadis and McDonald, 2000) and that serotonergic projections to the hippocampus controlled stress-induced defecation (Robertson et al., 2005). Therefore, another aim of the present study is to determine the subtype of 5-HT receptors in the ventral hippocampus responsible for stress-induced defecation.

The hippocampus expresses almost all types of 5-HT receptors in rodents (Pompeiano et al., 1992, 1994; Kinsey et al., 2001; Morales and Wang, 2002; Vilaro et al., 2005; Tanaka et al., 2012). Therefore, we injected several antagonists for 5-HT receptors (5-HT$_{1A}$, $_{2A}$, $_{2C}$, $_{3}$, $_{4}$, $_{5A}$, $_{6}$, and $_{7}$) that are sufficiently expressed in the ventral hippocampus and have been associated with anxiety, fear, or learning/memory (Nichols and Nichols, 2008). To assess the levels of anxiety/fear, we used the contextual fear conditioning test and the elevated plus-maze test as memory-dependent and -independent tasks, respectively. In addition, we measured the amount of feces during the above behavioral tests.

After specifying the 5-HT receptor subtype responsible for fear memory retrieval, we examined the effects of the stimulation of the receptor subtype on the electrophysiological properties of pyramidal neurons in the ventral hippocampus using whole-cell patch-clamp recording techniques in slices of brain tissue. To elucidate the detailed mechanisms, we further determined the relationships between the specified 5-HT receptor and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels using whole-cell patch-clamp recording and in situ hybridization.

Methods

Behavioral Experiments

Animals

For behavioral experiments, the subjects were male adult Wistar/ST rats (10–13 weeks old) supplied by Nippon SLC Co., Ltd. (Hamamatsu, Japan). They were housed in groups of 2 or 3 rats under an alternating light-dark cycle (light from 7:00 PM to 7:00 AM) at approximately 21°C. All testing was performed in the dark period. The treatment of animals complied with the NIH Animal Care Guidelines and the guidelines of the Animal Research Committee of the Hokkaido University Graduate School of Medicine for the care and use of laboratory animals.

Drugs

For behavioral experiments, WAY100635 (5-HT$_{1A}$ receptor antagonist), ondansetron (5-HT$_{3}$ receptor antagonist), SB258585 (5-HT$_{6}$ receptor antagonist), and SB269970 (5-HT$_{7}$ receptor antagonist) were dissolved in saline. MDL11939 (5-HT$_{2A}$ receptor antagonist), SB242084 (5-HT$_{2C}$ receptor antagonist), GR113808 (5-HT$_{4}$ receptor antagonist), SB69951 (5-HT$_{5A}$ receptor antagonist), and LP 44 (5-HT$_{7}$ receptor agonist) were dissolved in saline containing 5% dimethyl sulfoxide (DMSO). WAY100635, ondansetron, and GR113808 were purchased from Sigma (St. Louis, MO). The others were purchased from Tocris Bioscience (Bristol, UK). The dose of each drug was as follows: WAY100635, 1 μg; ondansetron, 1 μg; SB258585, 2.5 μg; SB269970, 2 μg; MDL11939, 0.3 μg; SB242084, 0.5 μg; GR113808, 0.5 μg; SB69951, 0.25 μg; and LP 44 μg in 0.5 μL vehicle. These doses were similar to those in previous studies (Higgins et al., 1991; Wesolowska et al., 2006; Monti et al., 2008; Robinson et al., 2008). We used concentrations almost equal to the solubility when there were no previous studies.

Surgical Procedure

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and fixed in a stereotaxic frame (Narishige, Tokyo, Japan). Stainless-steel guide cannulae (24 gauge, 13.5 mm long) were bilaterally implanted 2 mm above the target sites. The stereotaxic coordinates for the ventral hippocampus were as follows: 5.3 mm posterior to the bregma, 5.0 mm lateral to the midline, and 4.2 mm ventral to the dura (Paxinos and Watson, 2005). After surgery, the rats were individually housed and allowed a 1-week recovery period prior to testing.

Microinjection Procedure

Ten minutes before the start of behavioral tests, the 5-HT receptor antagonist or vehicle was injected into the ventral hippocampus using a Hamilton microsyringe with a 30-gauge stainless-steel injector (15.5 mm long) attached to a polyethylene tube. The solution (0.5 μL) was infused over a period of 1 minute at constant flow and implemented by a microinjection pump (CMA100, Carnegie Medicine, Sweden); the injector was left in place for 1 minute after injection to allow diffusion.

Contextual Fear Conditioning Test

Each rat was acclimated in a foot shock box (30.5 × 24.1 × 21.0 cm, Med Associates Inc.) for 5 minutes. This was followed by 2-second foot shocks administered at 30-second intervals. To detect the suppressing effects of 5-HT antagonists on freezing behavior, 10 foot shocks (shock intensity, 0.5 mA) were inflicted. For 5-HT agonist experiments, however, 5 shocks with lower shock intensity (0.3 mA) were used to avoid a ceiling effect, because increased freezing was expected. Thirty seconds after the last foot shock, rats were returned to their home cage. Twenty-four hours later, the drugs were injected. Ten minutes after the injections, each rat was returned to the foot shock box without being shocked. The freezing behavior was defined as the lack of movement except for respiration accompanied by an arched back and retraction of the ears (Fanselow, 1980), and it was used as a measure of fear memory retrieval. In the 30-minute testing period, the presence or absence of freezing was estimated by an automatic system (FreezeFrame, Actimetrics) using a pixel difference method, and the number of feces was counted by hand. The concordance between this automatic system and trained human observers has been measured at >90% (Actimetrics).

Elevated Plus-Maze Test

The apparatus was made of wood and consisted of 2 open arms (50 × 10 cm) and 2 closed arms (50 × 10 cm) that extended from the central platform (10 × 10 cm). Closed arms were surrounded by 40-cm-high side walls. The maze was elevated 50 cm above the floor, and the illumination of the room was set to 200 lux. Rats were placed on the central platform facing an open arm. The behavior of each rat was monitored by a charge-coupled device camera during a 5-minute testing period; and the distance moved in the maze and the time spent in each arm were recorded and automatically analyzed by a software package (LimeLight, Actimetrics). The distance moved in the maze was used as a measure of locomotor activity. The time spent in the open arms was used as a measure of memory-independent fear, because rats innately avoid open spaces (Treit et al., 1993). The time spent in the open arms was quantified as a percentage of the total time spent in the 4 arms. The number of feces was counted by hand.

Verification of Cannula Placements

After the completion of above behavioral experiments, rats were sacrificed under deep anesthesia (urethane, 2 g/kg, i.p.). The brain was rapidly removed and frozen in liquid nitrogen. Coronal sections (50 µm thick) were cut on a cryostat and thaw-mounted onto slides. After drying, the sections were stained with toluidine blue and cannula placements were verified under a microscope according to the atlas (Paxinos and Watson, 2005). Only data from rats with correct injection needle placements were included in the statistical analysis (supplementary Figure 1).

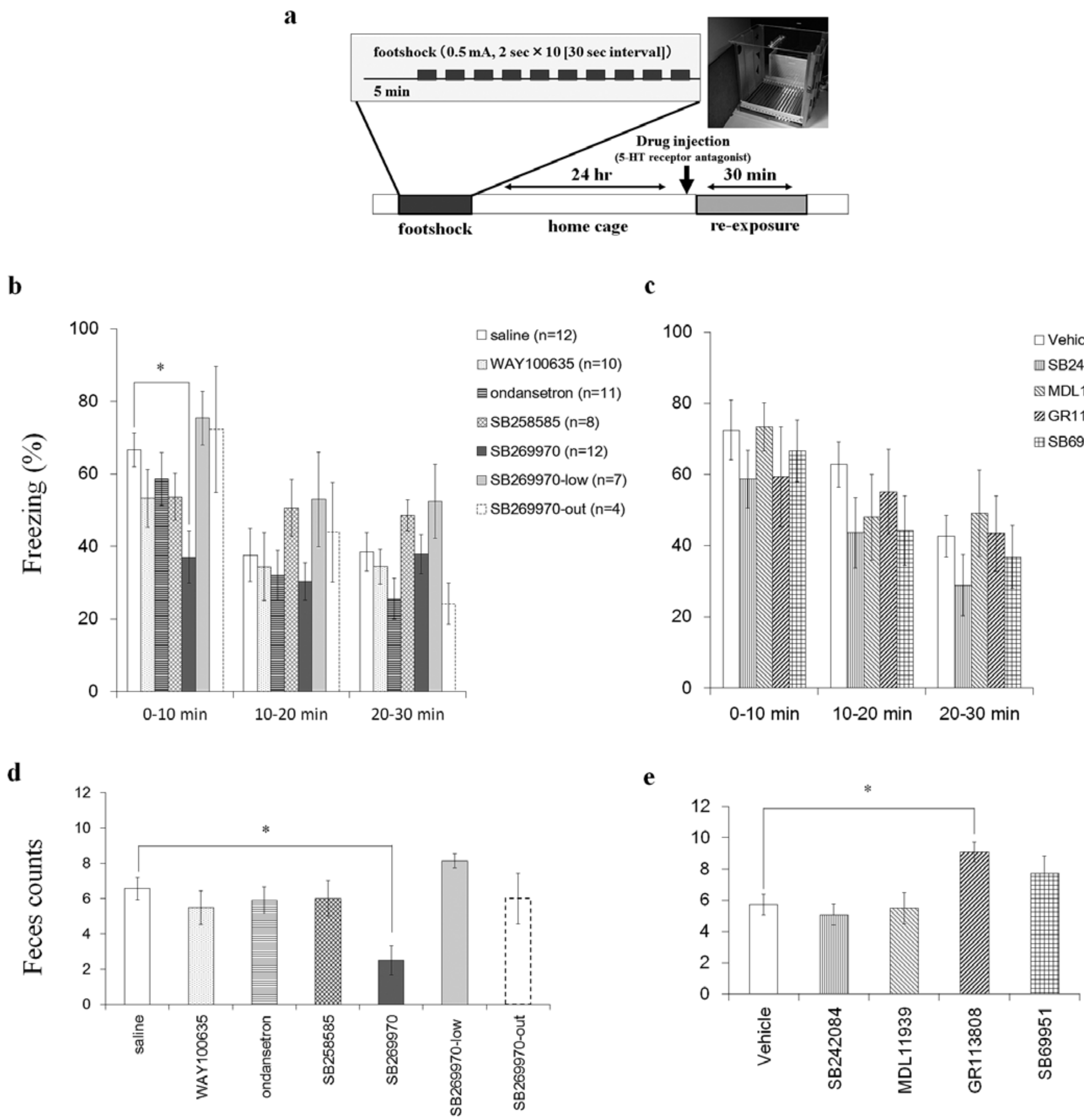

Figure 1. Effect of microinjection of serotonin (5-HT) receptor antagonists into the ventral hippocampus on freezing behavior and stress-induced defecation in a contextual fear conditioning test. (a) The procedure of the contextual fear conditioning test. (b) The effect of microinjection of 5-HT$_{1A}$, $_3$, $_6$, and $_7$ receptor antagonists or saline on freezing behavior. Data from animals that 5-HT$_7$ receptor antagonist was microinjected outside of the ventral hippocampus were indicated by a dotted line. Note that the data were not included in statistical analysis. (c) The effect of microinjection of 5-HT$_{2A}$, $_{2C}$, $_4$, and $_{5A}$ receptor antagonists or vehicle (saline containing 5% dimethyl sulfoxide [DMSO]) on freezing behavior. Ten minutes before the start of behavioral tests, 5-HT receptor antagonists or vehicles were injected. (d) The effect of microinjection of 5-HT$_{1A}$, $_3$, $_6$, and $_7$ receptor antagonists or saline on the amount of feces. Data from animals that 5-HT$_7$ receptor antagonist was microinjected outside of the ventral hippocampus were indicated by a dotted line. Note that the data were not included in statistical analysis. (e) The effect of microinjection of 5-HT$_{2A}$, $_{2C}$, $_4$, and $_{5A}$ receptor antagonists or vehicle (saline containing 5% DMSO) on the amount of feces. Data are given as mean ± SEM. *$P < .05$ with Dunnett's method.

Statistical Analysis

For the contextual fear conditioning test, freezing rate was divided into 3 time phases (0–10, 10–20, 20–30 minutes) and analyzed by a 2-way ANOVA with the time effects as the within-subject factor and the effects of microinjected drugs as the between-subject factor. In the case where time × drug interaction was significant, 1-way ANOVA was conducted for each time period. For antagonist study, multiple comparisons with Dunnett's method (Dunnett, 1964) were also conducted following each ANOVA. For agonist study, multiple comparisons with Holm's correction (Holm, 1979) were also performed after ANOVA.

Two-tailed unpaired *t* tests were performed to examine the effects of drugs on behavior in the elevated plus-maze test.

The alpha level was set at 0.05 for all comparisons. All statistical procedures were conducted using SPSS (version 15.0 J).

Electrophysiological Experiments

Animals

Juvenile male Wistar/ST rats (4–5 weeks old) were used for electrophysiological experiments. They were reared as described in Behavioral Experiments section.

Drugs

For electrophysiological experiments, LP44 (1 μM), SB 269970 (5 μM), and ZD7288 (10 μM, HCN channel blocker) were dissolved in artificial cerebrospinal fluid (aCSF).

Contextual Fear Conditioning

Some groups of rats received 10 foot shocks (shock intensity, 0.5 mA) in a foot shock box as described in Behavioral Experiments section. Approximately 24 hours after the foot shocks, these stressed rats were used for electrophysiological recording without reexposing to the foot shock box. Other groups of rat did not experience foot shocks or exposure to the box and these naïve rats were used for electrophysiological recording.

Electrophysiological Recording

Slice patch-clamp recordings were conducted as previously described (Shikanai et al., 2012). Wistar/ST rats (28–35 days old) were decapitated following CO_2 anesthesia, and brains were rapidly removed and placed in chilled ice-cold low-Na^+ solution containing (in mM) 120 choline-Cl, 3 KCl, 8 $MgCl_2$, 1.25 NaH_2PO_4, 28 $NaHCO_3$, and 22 glucose and bubbled with 95% O_2 and 5% CO_2 (pH 7.4). Transverse ventral hippocampal slices (300 μm thick) were cut with a Leica VT1000S slicer in ice-cold low-Na^+ solution. For tissue recovery, slices were incubated for 60 minutes in a normal bathing solution (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 20 glucose, pH 7.4, which was continuously bubbled with a mixture of 95% O_2 and 5% CO_2 at 35°C. Whole-cell patch-clamp recordings were made from the CA3 pyramidal neurons in acute transverse slices using an upright microscope (BX51WI; Olympus) equipped with an infrared charge-coupled device camera system (IR filter-removed DP72; Olympus) in normal bathing solution at 32°C. The resistance of the patch pipette was 3 to 6 MΩ when filled with standard intracellular solution (in mM): 6 KCl, 130 K_D-gluconate, 10 NaCl, 10 HEPES, 0.5 EGTA, 0.1 $CaCl_2$, 2 $MgCl_2$, 4 Na-ATP, and 0.4 Na-GTP, pH 7.3, adjusted with KOH. After whole-cell recording of the CA3 pyramidal neurons held at a membrane potential of –70 mV and switched to current-clamp recording mode, membrane potentials were recorded with an Axopatch 200B amplifier (Molecular Devices, CA) and achieved by stepwise current injections (from –0.2 to 0.5 nA, duration 500 ms). Capacitance compensation and bridge-balance adjustment were simultaneously made. The pCLAMP 9 software (Molecular Devices) was used for stimulation and data acquisition. Signals were filtered at 3 kHz and digitized at 20 kHz. The liquid junction potential was approximately 11 mV between the pipette solution and normal bath solution, which was subtracted from the recorded data. Drugs were applied to the brain slice in the aCSF by peristaltic pump (1 mL/min) and for at least 15 minutes to establish equilibrium in the tissue. aCSF served as a vehicle control in all experiments.

Statistical Analysis

For electrophysiological data, firing frequency was analyzed by repeated measures 2-way ANOVA with the intensity of electrical stimulation (nA) and the effects of applied drugs as the within-subject factors. In the case where intensity × drug interaction was significant, 1-way ANOVA was conducted for each intensity. Multiple comparisons with Holm's correction (Holm, 1979) were also performed after each ANOVA. As for the data of rats received foot shocks, shock conditions (naïve or stressed) were included as between-subject factors.

Histological Experiments

Animals

Adult male Wistar/ST rats (10–13 weeks old) were used for histological experiments. They were reared as described in the Behavioral Experiments section.

In Situ Hybridization

Rats were sacrificed under deep anesthesia (urethane, 2 g/kg, i.p.). The brain was rapidly removed and frozen in dry ice. Coronal sections (20 μm thick) were cut on a cryostat and thaw-mounted onto slides. Mouse cDNA fragments of hyperpolarization-activated cation channel 1 (HCN1) (414–1047, NM_010408), HCN2 (2045–2640, NM_008226), HCN3 (1749–2460, NM_008227), and HCN4 (2261–2999, NM_001081192) were subcloned into the pBluescript II plasmid vector. Mouse cDNA fragments of Htr7 (30–1478, NM_008315) that were subcloned into the pCR4-TOPO plasmid vector were kindly donated by Dr. Kenji F. Tanaka (Keio University). The preparation of digoxigenin- or fluorescein-labeled cRNA probes and procedures for chromogenic and fluorescent in situ hybridization were previously reported (Yamasaki et al., 2010; Kudo et al., 2012). Chromogenic in situ hybridization images were taken using a light microscope (BZ-9000; Keyence, Japan) and a PlanApo (4 x/0.20) objective lens (Nikon). Fluorescent in situ hybridization images were taken using a confocal laser-scanning microscope (FV1000; Olympus, Tokyo, Japan) equipped with an HeNe/Ar laser and a PlanApo (20x/0.70) objective lens (Olympus). All images contained single optical sections (640 × 640 pixels).

Results

Effects of the Microinjection of 5-HT Receptor Antagonists into the Ventral Hippocampus on Memory-Dependent Fear in the Contextual Fear Conditioning Test

To prove the causal relationship between 5-HT release in the ventral hippocampus and fear expression and determine the subtype of 5-HT receptors in the ventral hippocampus responsible for fear memory retrieval, we injected several antagonists for 5-HT receptors (5-$HT_{1A, 2A, 2C, 3, 4, 5A, 6}$, and $_7$) into the ventral hippocampus 10 minutes before the start of the 30-minute testing period of the contextual fear conditioning test.

As a 2-way ANOVA indicated a significant time × drug interaction [$F_{(10, 108)} = 2.33$, $P < .01$] for drugs dissolved in saline, a 1-way ANOVA was conducted for each time period. The effect of drug on the freezing behavior was significant only in the 0- to 10-minute phase of the 30-min testing phase [$F_{(5, 54)} = 3.56$, $P < .01$) (Figure 1a). Moreover, posthoc comparisons showed that only SB269970, a 5-HT_7 receptor antagonist, significantly suppressed the freezing behavior ($P < .01$) (Figure 1a, left panel), indicating that 5-HT_7 receptors in the ventral hippocampus are involved in the retrieval of fear memory. As for other 5-HT receptor antagonists dissolved in saline containing 5% DMSO, 2-way ANOVA revealed a significant main effect of time [$F_{(2, 86)} = 31.66$, $P < .01$] but not drug [$F_{(4, 42)} = 0.69$, *NS*] (Figure 1b).

Effects of the Microinjection of 5-HT Receptor Antagonists into the Ventral Hippocampus on Stress-induced Defecation in the Contextual Fear Conditioning Test

To determine the subtype of 5-HT receptors in the ventral hippocampus responsible for stress-induced defecation, we counted the number of feces in the 30-minute testing period of the contextual fear conditioning test and examined the effects of 5-HT receptor antagonists on it. One-way ANOVA indicated a significant main effect of drug [$F_{(5, 54)} = 5.51$, $P < .01$ for drugs dissolved in saline; Figure 1c; $F_{(4, 43)} = 3.99$, $P < .01$ for drugs dissolved in saline containing 5% DMSO; Figure 1d]. Moreover, posthoc comparisons showed that the 5-HT_7 receptor antagonist decreased the amount of feces during fear memory retrieval (Figure 1c, $P < .05$), whereas the 5-HT_4 receptor antagonist increased the amount of feces (Figure 1d, $P < .05$).

Effects of the Microinjection of 5-HT_7 Receptor Antagonist into the Ventral Hippocampus on Memory-Independent Fear and Stress-Induced Defecation in the Elevated Plus-Maze Test

To discriminate between memory-dependent and -independent fear, we injected the 5-HT_7 receptor antagonist (SB269970) into the ventral hippocampus 10 minutes before the start of the elevated plus-maze test, which is a memory-independent task.

Unpaired *t* tests revealed that SB269970 did not affect the time spent in open arms or total distance traveled in the elevated plus-maze test [$t_{(21)} < 0.4$, *NS*] (Figure 2b-c), indicating that microinjection of the 5-HT_7 receptor antagonist into the ventral hippocampus did not alter memory-independent fear or locomotor activity. However, SB269970 significantly decreased the amount of feces during the elevated plus-maze test [$t_{(21)} = 2.27$, $P < .05$] (Figure 2d), which was also observed in the contextual fear conditioning test.

Effects of the Microinjection of 5-HT_7 Receptor Agonist into the Ventral Hippocampus on Memory-Dependent Fear and Stress-Induced Defecation in the Contextual Fear Conditioning Test

To further confirm the roles of 5-HT_7 receptor in fear memory and stress-induced defecation, we injected 5-HT_7 receptor agonist into the ventral hippocampus 10 minutes before the start of the 30-minute testing period of the contextual fear conditioning

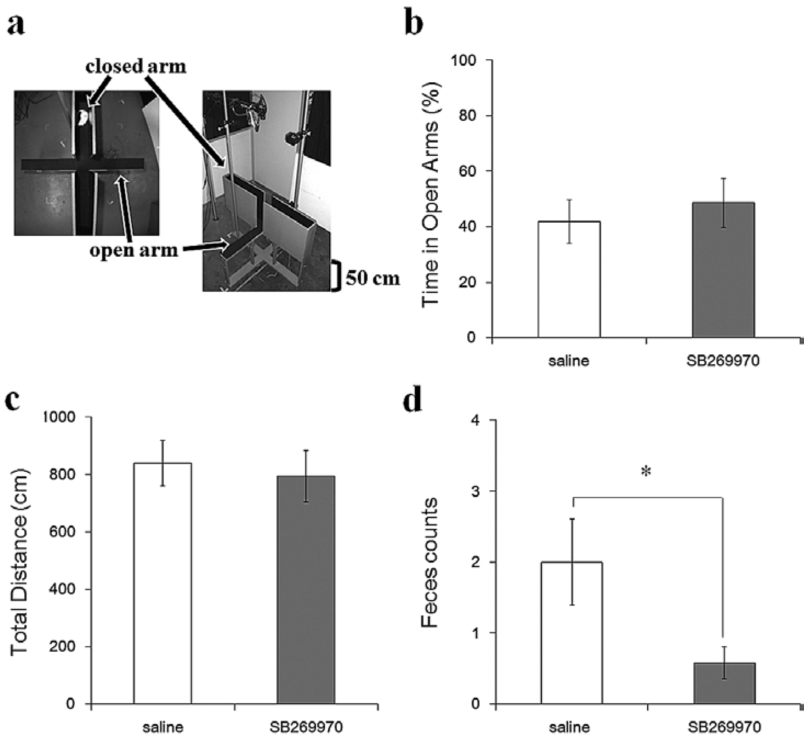

Figure 2. Effect of microinjection of SB269970 (a serotonin [5-HT_7] receptor antagonist) into the ventral hippocampus on parameters in the elevated plus-maze test. Ten minutes before the start of behavioral tests, the 5-HT_7 receptor antagonist or saline was injected. (a) The setup of the elevated plus maze test. (b) Effect of microinjection of the 5-HT_7 receptor antagonist on memory-independent fear expression. (c) Effect of microinjection of the 5-HT_7 receptor antagonist on locomotor activity. (d) Effect of microinjection of the 5-HT_7 receptor antagonist on stress-induced defecation. Data are given as mean ± SEM. *$P < .05$ with unpaired *t* test.

test. A dose response curve for 5-HT_7 agonist was an inverted U-shape (Figure 3b). Two-way ANOVA revealed a significant main effect of time [$F_{(2, 68)}$ = 22.13, P < .01] or drug [$F_{(3, 34)}$ = 6.73, P < .01] on the freezing behavior, respectively. Posthoc comparisons regarding the drug effect showed that 0.1 µg of the LP-44, a 5-HT_7 receptor agonist, increased the freezing behavior (Figure 3b, P < .05), indicating that the stimulation of 5-HT_7 receptors in the ventral hippocampus could facilitate the retrieval of fear memory. However, LP-44 injection did not significantly affect the amount of feces during fear memory retrieval, although there was a trend [$F_{(3, 34)}$ = 2.16, P = .11] (Figure 3c).

Ex Vivo Electrophysiology

To elucidate the detailed mechanisms by which the stimulation of 5-HT_7 receptor facilitates fear memory retrieval, we examined the effects of the stimulation of 5-HT_7 receptor on the electrophysiological properties of pyramidal neurons in the ventral hippocampus.

A previous study showed that 5-HT_7 receptor mRNA expression in the hippocampus was highest in the CA2 and CA3 subregions, with much lower expression in the dentate gyrus and CA1 subregion (Tanaka et al., 2012). To investigate the effects of the activation of 5-HT_7 receptors on neuronal activities, whole-cell patch-clamp recordings were performed from ventral hippocampal CA3 pyramidal neurons in acute coronal slices. Bath application of the 5-HT_7 agonist (LP-44, 1 µM) caused slight, but significant, depolarization (+4.7 mV; paired t test, P < .05) and lowered input resistance (−20.7 MΩ; paired t test, P < .05) without affecting other membrane potential parameters (Table 1).

Moreover, the 5-HT_7 agonist increased the firing rate evoked by current injection (Figure 4b). Because 2-way ANOVA indicated a significant intensity of stimulation (nA) × drug interaction [$F_{(9, 81)}$ = 2.83, P < .01], 1-way ANOVA was conducted for each intensity of stimulation (nA). The effects of drugs on firing rate were significant in 0.4, 0.5, and 0.7 nA [$F_{(1, 9)}$ = 5.34, 5.60, and 6.22, respectively, P < .05] (Figure 4b).

Furthermore, this effect was completely blocked by the 5-HT_7 antagonist (SB269970, 5 µM) (Figure 4c). Two-way ANOVA indicated a significant intensity of stimulation (nA) × drug interaction [$F_{(18, 108)}$ = 5.01, P < .01], but the following 1-way ANOVA for each intensity of stimulation (nA) did not show a significant effect of drugs [$Fs_{(2, 12)}$ < 2.35, NS].

Because it was known that 5-HT_7 receptors primarily depolarize neurons by increasing hyperpolarization-activated nonselective cation current (I_h) mediated by hyperpolarization-activated and cyclic nucleotide-gated (HCN) channel in the thalamus (Chapin and Andrade, 2001), we used a selective HCN channel blocker (ZD7288, 10 µM). Bath application of ZD7288 elicited significant suppression of the firing frequency and 5-HT_7-activated firing enhancement (Figure 4d). Two-way ANOVA indicated a significant intensity of stimulation (nA) × drug interaction [$F_{(18, 198)}$ = 7.74, P < .01], and the following 1-way ANOVA for each intensity of stimulation (nA) indicated a significant effect of drugs

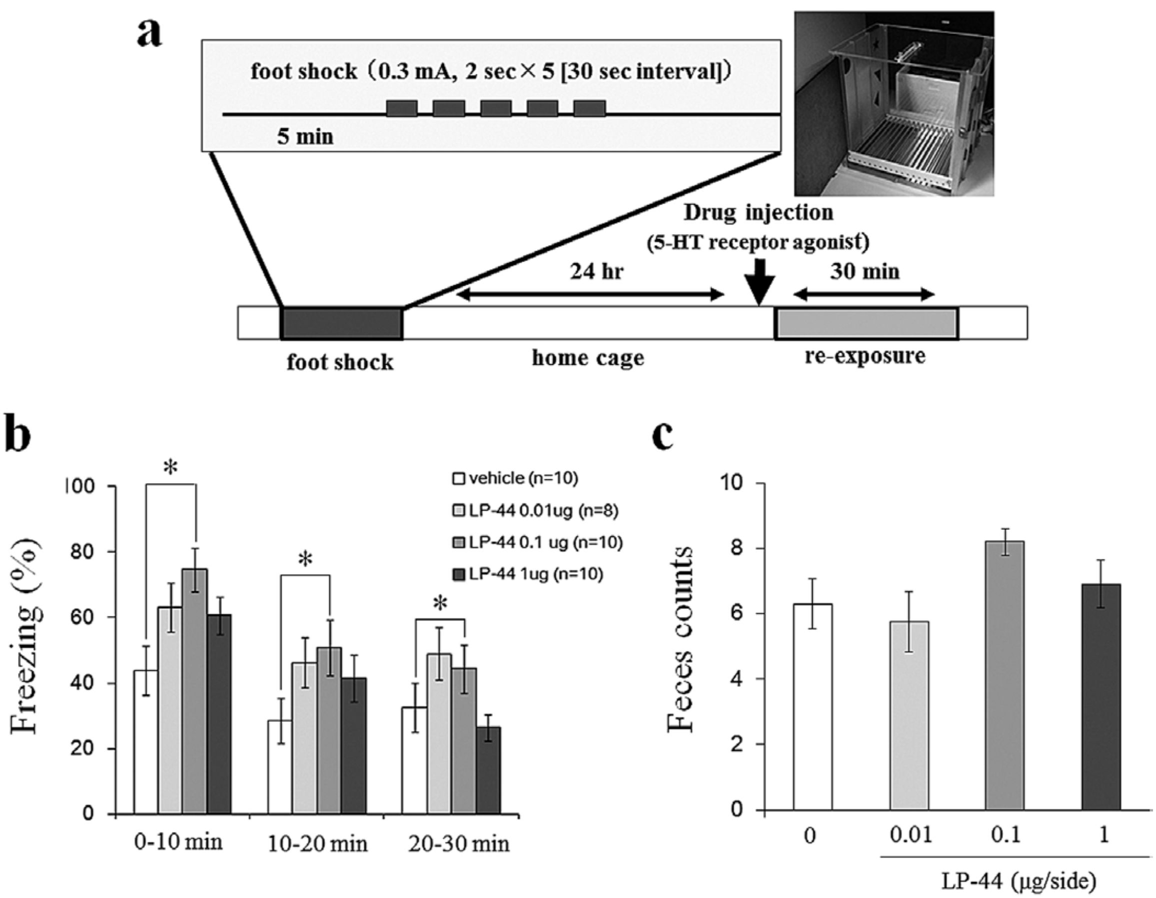

Figure 3. Effect of microinjection of serotonin (5-HT) receptor agonist into the ventral hippocampus on freezing behavior and stress-induced defecation in a contextual fear conditioning test. (a) The procedure of the contextual fear conditioning test. (b) Effect of microinjection of the 5-HT_7 receptor agonist on freezing behavior. Ten minutes before the start of behavioral tests, the 5-HT_7 receptor agonist or vehicle was injected. (c) Effect of microinjection of the 5-HT_7 receptor agonist on the amount of feces. Data are given as mean ± SEM. *P < .05 with Holm's method.

Table 1. Effects of the 5-HT$_7$ Agonist on Membrane Potential Parameters

	Treatment	
	Control (n = 10)	LP 44 (n = 10)
Resting membrane potential (mV)	–74.4 ± 1.6	–69.7 ± 2.1*
Input resistance (MΩ)	93.4 ± 6.5	72.6 ± 6.8*
Action potential threshold (mV)	–40.8 ± 1.1	–39.6 ± 1.6
Action potential amplitude (mV)	87.1 ± 5.8	83.9 ± 6.4
Action potential overshoot (mV)	46.2 ± 5.3	44.3 ± 5.4
Fast afterhyperpolarization amplitude (mV)	14.6 ± 1.4	17.0 ± 1.8
Slow afterhyperpolarization amplitude (mV)	3.6 ± 0.6	4.6 ± 0.4
Half-width of Action Potential (ms)	0.72 ± 0.047	0.71 ± 0.044
Rise time 10–90% (ms)	0.32 ± 0.029	0.34 ± 0.027
Decay time 90-10% (ms)	0.54 ± 0.027	0.50 ± 0.023

The dose of LP 44 was 1 μM. All recordings were conducted in pyramidal neurons in the CA3 subregion of the ventral hippocampus. Data are given as mean ± SEM. *P < .05.

on firing rate in 0.7, 0.8, 0.9, and 1.0 nA [$F_{(2, 22)}$ = 4.98, 7.85, 9.35, and 11.48, respectively, $P < .05$] (Figure 4d). Posthoc comparisons showed that ZD7288 alone decreased the firing frequency in 0.7 and 1.0 nA.

To further confirm this result, the order of drug application was reversed. Two-way ANOVA indicated a significant intensity of stimulation (nA) × drug interaction [$F_{(18, 144)}$ = 2. 34, $P < .01$], and the following 1-way ANOVA for each intensity of stimulation (nA) indicated a significant effect of drugs on firing rate in 0.7, 0.8, 0.9, and 1.0 nA [$F_{(2, 16)}$ = 4.09, 3.85, 4.63, and 4.20, respectively, $P < .05$] (Figure 4e). Posthoc comparisons showed that the 5-HT$_7$ agonist increased the firing frequency in 0.7, 0.8, 0.9, and 1.0 nA, almost consistent with the results in Figure 4b. We did not find any significant difference in firing rate between control and LP44 + ZD7288, indicating that the 5-HT$_7$-activated firing enhancement was attenuated by the blockade of HCN channel.

Furthermore, we examined the effects of foot shock protocol on the electrophysiological properties of CA3 neurons and whether 5-HT$_7$ blockade could normalize their firing activity. Two-way ANOVA indicated a significant intensity of stimulation (nA) × shock conditions interaction [$F_{(9, 450)}$ = 8.33, $P < .01$], and the following 1-way ANOVA for each intensity of stimulation (nA) indicated a significant effect of shock conditions on firing rate from 0.2 to 1.0 nA [$Fs_{(1, 51)}$ = 4.49, 7.26, 7.30, 8.07, 7.97, 7.54, 8.48, 8.12, and 10.27, respectively, $Ps < .05$] (Figure 5b). 5-HT$_7$ blockade normalized these changes: 2-way ANOVA indicated a significant intensity of stimulation (nA) × drug interaction [$F_{(9, 99)}$ = 15.65, $P < .01$], and the following 1-way ANOVA for each intensity of stimulation (nA) indicated a significant effect of drug on firing rate from 0.6 to 1.0 nA [$Fs_{(1, 11)}$ = 14.41, 18.83, 15.33, 21.39, and 28.68, respectively, $Ps < .05$] (Figure 5c).

Taken together, these results indicate that the stimulation of 5-HT$_7$ receptors in the CA3 ventral hippocampus subregion would enhance neuronal activations by increasing I_h.

Htr7 mRNA and HCN Expression in the Ventral Hippocampus

To examine whether 5-HT$_7$ receptor-expressing cells coexpress HCN channels consistent with above electrophysiological results and to determine the subtypes of HCN channels, we examined the regional expression of Htr7 and 4 I_h channel subunit (HCN1-4) mRNAs in the ventral hippocampus using in situ hybridization (Figure 6a-e). Chromogenic in situ hybridization demonstrated that the expression of Htr7 mRNA was intense in the pyramidal cell layer of Ammon's horn, particularly in the CA3 subregion (Figure 6a). Although mRNAs for all 4 subtypes of the HCN were more or less expressed in the ventral hippocampus, HCN2 and 4 mRNAs were particularly strong (Figure 6b-e). Double-labeling fluorescent in situ hybridization showed that almost all Htr7 mRNA-expressing pyramidal cells in the CA3 subregion coexpressed HCN2 or HCN4 mRNA in the CA3 pyramidal neuron (Figure 6f-g).

Discussion

In the present study, the microinjection of SB269970 (the 5-HT$_7$ receptor antagonist) into the ventral hippocampus significantly suppressed memory-dependent fear expression in the contextual fear conditioning test, whereas microinjections of other antagonists (5-HT$_{1A, 2A, 2C, 3, 4, 5A,}$ or $_6$) did not (Figure 1). The microinjection of SB269970 into the outside of ventral hippocampus did not affect memory-dependent fear expression (Figure 1b), indicating that the observed effects of SB269970 were not due to the leakage to other brain regions. Further, the microinjection of SB269970 did not affect memory-independent fear expression or locomotor activity in the elevated plus-maze test (Figure 2), indicating that 5-HT$_7$ receptors in the ventral hippocampus are selectively involved in fear memory retrieval but not memory-independent fear or locomotor activity. The microinjection of LP-44 (5-HT$_7$ receptor agonist) into the ventral hippocampus significantly enhanced memory-dependent fear expression in the contextual fear conditioning test (Figure 3b). Although LP-44 has low but not negligible affinity for 5-HT$_{1A}$ receptors, it would not explain the enhancement of fear expression, because the stimulation of 5-HT$_{1A}$ receptors in the ventral hippocampus increased locomotor activity (File and Gonzalez, 1996), which could reduce the freezing rate. Therefore, it is highly likely that the 5-HT$_7$ receptor in the ventral hippocampus has a pivotal role in fear memory retrieval.

We found from using patch-clamp recordings that foot shock stress significantly enhanced the sensitivity of hippocampal CA3 neurons to inputs, and the enhancement was normalized by 5-HT$_7$ blockade (Figure 5). In addition, our results showed that 5-HT$_7$ receptors primarily depolarize neurons by increasing I_h mediated via HCN channel in CA3 neurons (Figure 4), consistent with a previous study in the thalamus (Chapin and Andrade, 2001). HCN channels are unique among vertebrate voltage-gated ion channels, because they have a reverse voltage-dependence that leads to activation upon hyperpolarization. Voltage-dependent opening of these channels is directly regulated by the binding of cAMP via activation of the Gs-coupled receptor-adenylate cyclase pathway (Chapin and Andrade, 2001). The current flowing through HCN channels, designated I_h, plays a key role in the control of neuronal processes, including determination of resting membrane potential, dendritic integration, and synaptic transmission. Thus, it is speculated that stress enhances the functions of 5-HT$_7$ receptor-mediated HCN pathways in CA3 pyramidal neurons and enhances the sensitivity of these neurons to the next inputs related to stress previously experienced.

Consistent with a previous study (Tanaka et al., 2012), we confirmed that Htr7 mRNA is abundantly expressed in the CA3 subregion compared with that in the other subregions of the hippocampus. We observed that these Htr7 positive cells coexpressed HCN2 and HCN4 mRNA (Figure 6) though HCN channels are encoded by 4 genes (HCN1–4). Therefore, 5-HT$_7$ receptor-mediated pathways in CA3 pyramidal neurons would depend

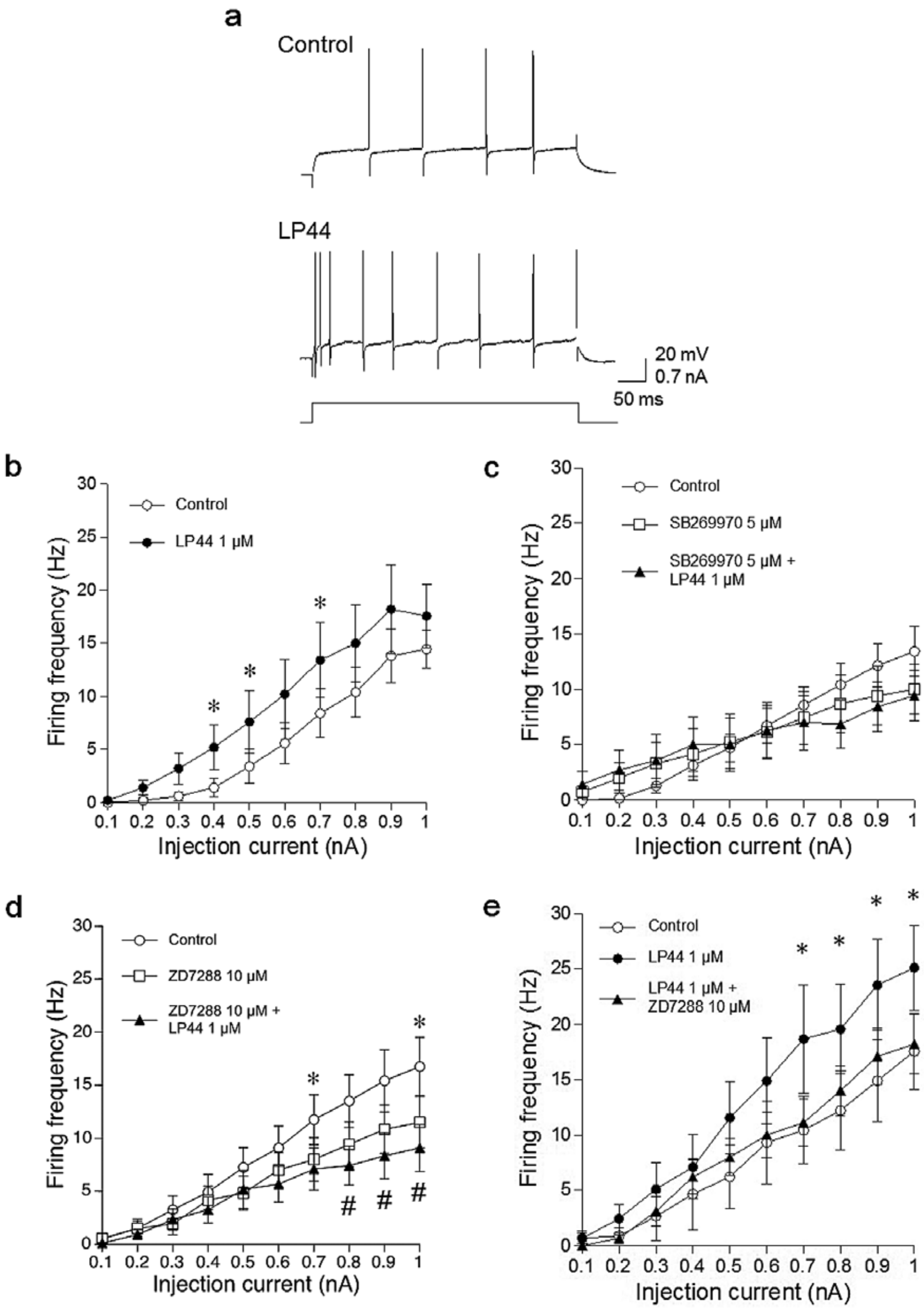

Figure 4. The involvement of serotonin (5-HT$_7$) receptor and hyperpolarization-activated cyclic nucleotide-gated (HCN) channel in electrophysiological parameters of the hippocampal CA3 neurons. Naïve rats were used for whole-cell patch-clamp recording. (a) Representative recordings of membrane potential changes evoked by a positive current injection. (b) Effect of 5-HT$_7$ receptor agonist (LP-44, 1 μM) on firing frequency evoked by current injection. *P < .05. (c) Reversal effect of the 5-HT$_7$ receptor antagonist (SB269970, 5 μM) on 5-HT$_7$ receptor stimulation-induced increased firing frequency evoked by current injection. (d and e) Reversal effect of I_h blocker (ZD7288, 10 μM) on 5-HT$_7$ receptor stimulation-induced increased firing frequency evoked by current injection. Data are given as mean ± SEM. *P < .05 with Holm's correction, control vs ZD7288 alone, or LP44 alone. #P < .05 with Holm's correction, control vs ZD7288+LP44.

mainly on HCN 2 and 4 channels. Although speculative, serotonergic facilitation of I_h via Gs-coupled 5-HT$_7$ receptor stimulation may serve to upregulate spike firing of CA3 pyramidal cells in a positive feedback mechanism (CA3-to-CA3) (Ishizuka et al., 1990), leading to a powerful excitatory influence on the CA1 subregion (CA3-to-CA1) and thereby facilitating fear memory retrieval.

Microinjection of the 5-HT$_4$ receptor antagonist facilitated stress-induced defecation in the contextual fear conditioning test (Figure 1e), but it did not increase the freezing rate (Figure 1c). A possible explanation for this discrepancy between the effects of drugs on freezing and defecation is that the freezing behavior and defecation are controlled by similar but different neural mechanisms though both could be induced by emotional stress (supplementary Figure 2). It is plausible that the neural population modulating the freezing behavior is different from the neural population modulating stress-induced defecation, though both populations exist in the ventral hippocampus. Indeed, a

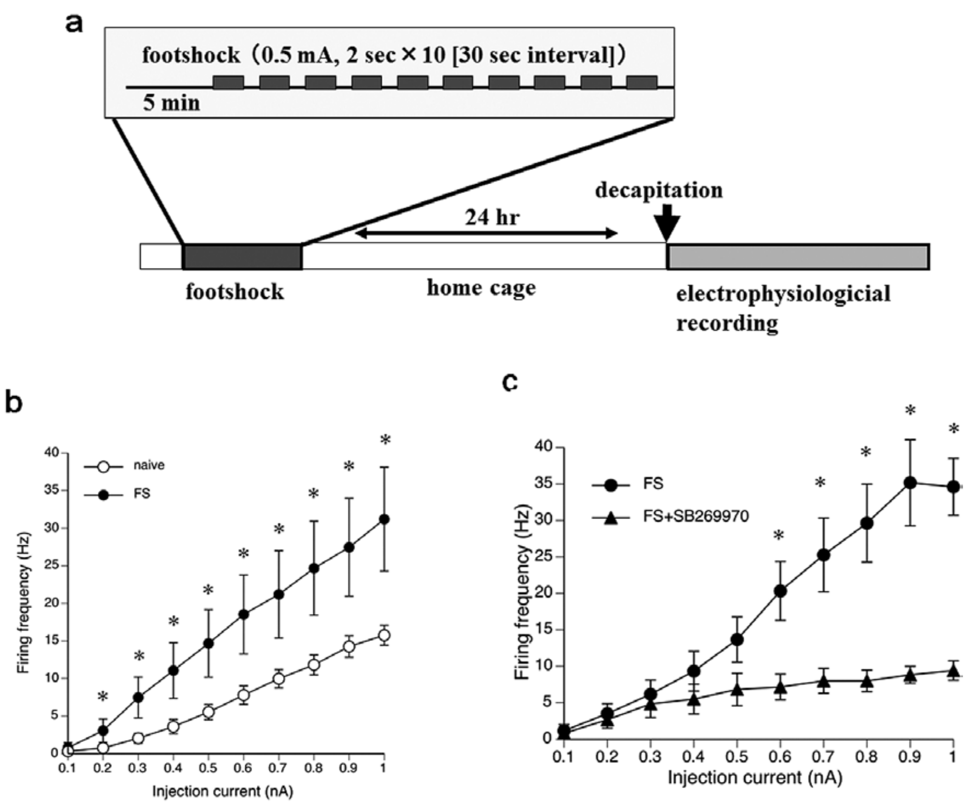

Figure 5. Effect of foot shock stress on serotonin (5-HT$_7$) receptor-mediated electrophysiological parameters of the hippocampal CA3 neurons. Naïve rats or stressed rats (FS) were used for whole-cell patch-clamp recording. (a) The timeline of the foot shock stress and electrophysiological recording. (b) Effect of foot shock stress on firing frequency evoked by current injection. (c) Reversal effect of the 5-HT$_7$ receptor antagonist (SB269970, 5 μM) on stress-induced increased firing frequency evoked by current injection. Data are given as mean ± SEM. *P < .05.

previous study demonstrated that drug injection into the hippocampus altered freezing behavior without affecting defecation (Li et al., 2006).

Microinjection of the 5-HT$_7$ receptor antagonist into the ventral hippocampus significantly suppressed the freezing behavior and attenuated stress-induced defecation in both the contextual fear conditioning test and elevated plus-maze test, respectively (Figures 1b, d and 2c). Microinjection of the 5-HT$_7$ receptor agonist significantly increased the freezing rate and tended to increase the amount of feces though it was not statistically significant (Figure 3). Thus, it is likely that 5-HT$_7$ receptors in the ventral hippocampus are involved in both fear memory retrieval and stress-induced defecation.

Our results filled the gap among previous findings. We previously demonstrated that the blockade of the CRF$_2$ receptor in the MRN reversed 5-HT release in the ventral hippocampus during fear memory retrieval and suppressed memory-dependent fear expression without affecting memory-independent fear expression or locomotor activity (Ohmura et al., 2010). The 5-HT$_7$ receptors in the ventral hippocampus would be stimulated by the fibers from the MRN serotonergic neurons expressing CRF$_2$ receptors. A previous study demonstrated that the ventral hippocampus directly projects to the neurons in the basal amygdala associated with fear expression (Herry et al., 2008). It is also known that the basal amygdala projects to the central amygdala (LeDoux, 2000), which in turn projects to the central gray matter that controls the freezing behavior, and the dorsal motor nucleus of the vagus nucleus ambiguus controlling defecation (Davis, 1992). Taking these results together with previous findings, we can delineate the neural circuits underlying fear memory retrieval and stress-induced defecation (supplementary Figure 2).

It should be noted that the blockade of the 5-HT$_7$ receptors in the ventral hippocampus did not completely suppress the freezing behavior (Figure 1b) as observed with the blockade of the CRF$_2$ receptor in MRN (Ohmura et al., 2010). This indicates that there are also other systems regulating the retrieval of fear memory. For example, the dorsal hippocampus is also involved in fear memory retrieval (Holt and Maren, 1999). However, serotonergic systems in the dorsal hippocampus could not be involved in the retrieval of fear memory under physiological conditions, because 5-HT release in the dorsal hippocampus was not increased during a contextual fear conditioning test (Ohmura et al., 2010). It is likely that other systems such as the glutamatergic and the GABAergic systems in the dorsal hippocampus participated in fear memory retrieval.

Because the microinjection of SB269970 (5-HT$_7$ receptor antagonist) into the ventral hippocampus significantly suppressed both the freezing behavior and defecation in the contextual fear conditioning test (Figure 1b,d), 5-HT$_7$ receptors might be a potential target in drug development for the treatment of mental disorders involving fear memory and gastrointestinal problems. The 5-HT$_7$ receptor is expressed in both the brain and gastrointestinal tract (Sanger, 2008), and a previous study showed that the blockade of the 5-HT$_7$ receptor reversed the 5-HT-induced colonic migrating motor complex (Dickson et al., 2010). The colonic migrating motor complex is necessary for fecal pellet propulsion in the colon. Therefore, the systemic administration of 5-HT$_7$ receptor antagonist might exert its therapeutic effects by acting on both the brain and gastrointestinal tract. Further studies focusing on the 5-HT$_7$

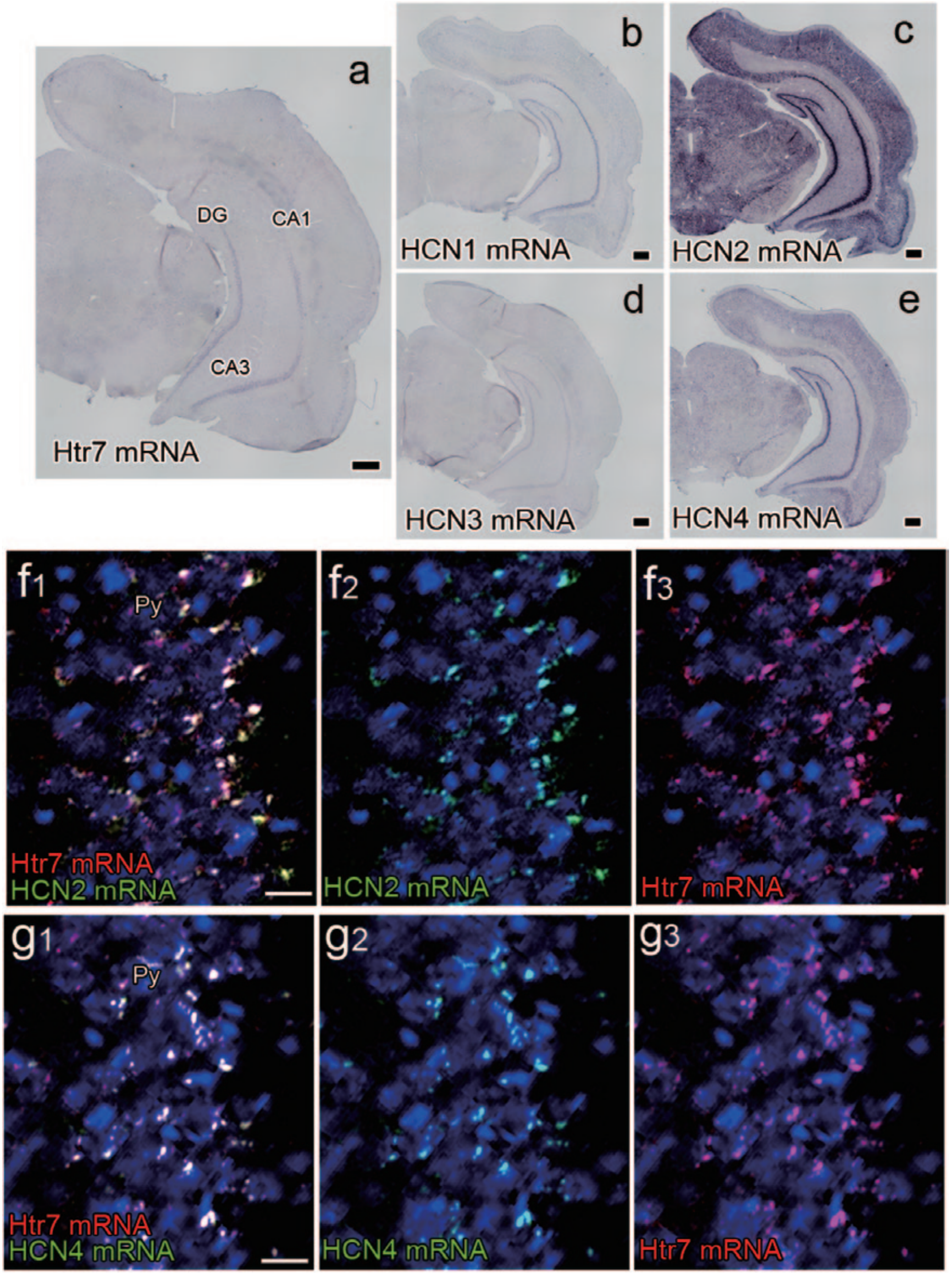

Figure 6. Htr7 and hyperpolarization-activated cyclic nucleotide-gated (HCN) mRNA expression in the ventral hippocampus. Chromogenic in situ hybridization showing regional expression of Htr7 (a), HCN1 (b), HCN2 (c), HCN3 (d), and HCN4 mRNA (e). Double-labeling fluorescence in situ hybridization for Htr7 (red) and HCN2 (f) or HCN4 (g) in the CA3 pyramidal cell layer. TOTO3 was used for nuclear staining (blue). CA1-3, CA1-3 subregions of Ammon's horn; DG, dentate dyrus; Py, pyramidal cell layer. Scale bars, a–e, 500 μm; f-g, 20 μm.

receptors might provide us clues to develop therapeutic drugs for mental disorders involving fear memory and gastrointestinal problems such as posttraumatic stress disorder and panic disorder comorbid with irritable bowel syndrome.

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Statement of Interest

None.

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