Full Paper

Metaplastic Regulation of the Median Raphe Nucleus via Serotonin 5-HT_{1A} Receptor on Hippocampal Synaptic Plasticity Is Associated With Gender-Specific Emotional Expression in Rats

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Abstract. Gender differences in psychiatric disorders are considered to be associated with the serotonergic (5-HTergic) system; however the underlying mechanisms have not been clearly elucidated. In this study, possible involvement of the median raphe nucleus (MRN)-hippocampus 5-HTergic system in gender-specific emotional regulation was investigated, focusing on synaptic plasticity in rats. A behavioral study using a contextual fear conditioning (CFC) paradigm showed that the females exhibited low anxiety-like behavior. Extracellular 5-HT levels in the hippocampus were increased by CFC only in the males. Long-term potentiation (LTP) in the hippocampal CA1 field was suppressed after CFC in the males, which was mimicked by the synaptic response to MRN electrical stimulation. In the MRN, 5-HT immunoreactive cells significantly increased in the females compared with those in the males. Pretreatment with the 5-HT_{1A} receptor agonists tandospirone (10 mg/kg, i.p.) and 8-OH DPAT (3 mg/kg, i.p.) significantly suppressed LTP induction in the males. Synaptic responses to CFC and 5-HT_{1A} receptor interventions were not observed in the females. These results suggest that the metaplastic 5-HTergic mechanism via 5-HT_{1A} receptors in the MRN-hippocampus pathway is a key component for gender-specific emotional regulation and may be a cause of psychiatric disorders associated with vulnerability or resistance to emotional stress.

Keywords: gender difference, serotonin, hippocampus, long-term potentiation, serotonin 5-HT_{1A} receptor

Introduction

Stressful events can lead to psychiatric disorders such as depression and anxiety (1, 2). The serotonin (5-hydroxytryptamine: 5-HT) neural system has been implicated in the etiology of stress-related disorders and is a primary target for psychotherapeutic drugs (3). It is well known that gender difference exists in such psychiatric disorders (4 – 6). For example, major depression is two times more prevalent in women than in men (4, 5).

Clinical studies indicate various factors relevant to the higher incidence of major depression in women. Gender differences exist in the hypothalamic–pituitary–adrenal (HPA) axis responses to psychological stress (7, 8), and gonadal steroid hormone can ameliorate dysregulation of the HPA axis function observed in patients with affective disorders (9). Morphological studies have shown that the total volume of the hippocampus is larger in women than in men (10, 11), whereas a smaller volume of limbic regions was observed in major depressed subjects (12). These findings suggest that endocrinological changes or volumetric differences contribute to a vulnerability for the psychiatric disorders observed in women.

Gender differences are also considered to be associated with the serotonergic (5-HTergic) neural system. For

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example, positron emission tomography (PET) studies have shown that the rate of 5-HT synthesis in the limbic region is higher in men than in women (13). 5-HT_{1A} receptor binding potentials are higher, but 5-HT transporter (5-HTT) bindings are lower in cortical and subcortical regions in women than in men (14). Animal studies also suggest that dimorphisms are implicated in gender differences of cerebral 5-HT levels in the hippocampus and amygdala (15) and the hippocampal 5-HT synthesis (16) is greater in female rats than in the males. Behavioral studies have shown differential sensitivities to various kinds of stresses such as novel environment in the open field test (17, 18), swim stress (19), and eye-blink conditioning (20) between the males and females. These reports consistently indicate that the females exhibit less adaptation to acute stress. Furthermore, gender differences observed from numerous memory tasks depend on neurocircuitry within a specific brain region (21). The 5-HTergic neural system is known to participate in cognition and emotional states by modulating synaptic plasticity. In particular, the midbrain median raphe nucleus (MRN), the primary origin for ascending 5-HTergic projections to the hippocampus, has a critical role in the regulation of stress responsiveness (22). Therefore, it is plausible that the MRN-hippocampus 5-HTergic system contributes to the gender difference in stress response; however, the underlying mechanisms have not been elucidated.

In the present study, the possible involvement of 5-HTergic regulatory mechanisms in the MRN-hippocampus pathway in response to acute fear stress was investigated. A contextual fear conditioning (CFC) paradigm was used to perform behavioral analysis, and a neurochemical approach involving *in vivo* microdialysis was also used. Given the evidence that synaptic plasticity, long-term potentiation (LTP), in the hippocampal CA1 field was influenced by various kinds of stresses (23–26), an electrophysiological study was conducted to investigate 5-HTergic modulation of hippocampal LTP as a response to fear stress. In addition, on the basis of the findings that the hippocampus is enriched with 5-HT_{1A} receptors and that 5-HT_{1A} receptors are located in the MRN as somatodendritic autoreceptors (27), pharmacological approaches were used to examine the underlying 5-HTergic mechanisms, with a focus on 5-HT_{1A} receptors.

Materials and Methods

Animals

Adult male and female Wistar rats (11–14-week-old, body weight of 230–330 g) were used (Sankyo Labo Service, Ltd., Tokyo). The rats were housed in a room

with a 12-h light/dark cycle (lights on 8:00–20:00 h) and a temperature-controlled ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$) environment with access to food and water *ad libitum*. After weaning at 4 weeks of age, litter mate male or female rats were group-housed (2–4 pups per cage) in plastic cages throughout the experiments, with the exception of the rats used for *in vivo* microdialysis and electrophysiological experiments under freely moving conditions, who were single-housed for several days after surgery. All animal procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Animal Research Committee of the Health Sciences University of Hokkaido and were in accordance with National Institutes of Health guidelines.

Contextual fear conditioning

The rats were acclimated to a footshock (FS) box for 5 min and subjected to 5 FS cycles (shock intensity, 1 mA; intershock interval, 30 s; shock duration, 2 s) and then kept in the FS box for 5 min without FS stimuli. After 24-h later, the rats were re-exposed to the conditioning chamber (FS box) without FS stimuli to verify the retrieval of fear memory. The FS box [i.e., conditioning chamber: $50 \times 16 \times 25$ cm (height); grid floor with diameter of rods, 0.5 cm; spacing, 1.0 cm] was composed of opaque acrylic. The box was specially constructed by Nihon Kodens Co., Ltd. (Tokyo) for simultaneous determination of electrophysiological and behavioral parameters. Fear-related freezing behavior was defined as lack of movement with the exception of respiration (28). Freezing expression was evaluated in hand-scoring methods by measuring the presence or absence of freezing behavior every 5 s for 5 min, immediately after FS conditioning (acquisition session) and during re-exposure to the FS box for 15 min (retention session).

Neurochemical analyses

In vivo microdialysis: The dialysis guide cannula was inserted in the dorsal region of the hippocampus, including the CA1 area (5.0-mm anterior, 3.5-mm lateral to the bregma, and 2.0-mm ventral to the skull) under *i.p.* administered pentobarbital anesthesia (60 mg/kg). Seven days later, the rats were subjected to the experimental protocol based on a CFC paradigm. A concentric 2-mm dialysis probe was inserted into the hippocampus of the FS-conditioned rats through the guide cannula and perfused with artificial cerebrospinal fluid (aCSF) at a flow rate of $2 \mu\text{L}/\text{min}$. To obtain a stable baseline, aCSF was perfused for 40–80 min, and successive samples were collected at 20-min intervals. Extracellular 5-HT levels were determined using high-performance liquid chromatography (HPLC) with an electrochemical detector (ECD-300; Eicom, Kyoto). An ion-exchange HPLC

column (4.6- μ m diameter) of silica gel (Eicompack PP-ODS, Eicom) was used, and the working electrode was maintained at 400 mV. The mobile phase consisted of 2.1 mM sodium 1-decanesulfonate, 0.1 mM EDTA-2Na/0.1 M phosphate buffer, pH 6.0, and 1% (v/v) methanol.

Tissue monoamine assay: Brain tissues of the hippocampus, the MRN, and the dorsal raphe nucleus (DRN) were dissected according to the atlas and homogenized by ultrasonification in 0.2 N perchloric acid. The samples were centrifuged (10,000 rpm, -2°C , 10 min), followed by adjustment of the pH to 3.0 with 1 M sodium acetate. Serotonin in supernatants were assayed by the HPLC system, which consisted of an EP-300 chromatopump, a reverse-phase ODS column SC-5, and an ECD-300 electrochemical detector (Eicom) with applied voltage of 400 mV v.s. Ag/AgCl. The mobile phase was 0.1 M sodium acetate/citric acid buffer containing 17% methanol, 190 mg/L sodium octanesulfonic, and EDTA-2Na (5 mg/L).

Electrophysiological experiments

Recordings of evoked potentials in the hippocampal CA1 field: Electrophysiological procedures for the electrophysiological experiment to record evoked field potentials in the hippocampal CA1 field were performed with the rats under anesthesia or consciousness. Under urethane (1 g/kg, i.p.) anesthesia, a recording electrode was stereotactically lowered into the CA1 field (5.0-mm posterior, 3.0-mm lateral to the bregma, and 2.3-mm ventral to the dura), and a bipolar stimulating electrode constructed of stainless steel with a tip separation of 500 μ m (diameter, 100 μ m) was placed in the Schaffer collaterals (3.0-mm posterior, 1.5-mm lateral to the bregma, and 2.8-mm ventral to the dura) through holes drilled into the skull according to the atlas. The recording and stimulating electrodes were implanted in the right ipsilateral side. The intensity of the test stimulation was adjusted for each rat to elicit a population spike amplitude (PSA) of approximately 50% of the maximum amplitude, as assessed by input–output curves, and the test stimulation was administered constantly throughout the experiment. The PSA in the CA1 field (frequency, 0.1 Hz; pulse duration, 250 μ s; stimulus interval, 30 s) was obtained from 7 successive stimuli and recorded every 5 min on a Power Lab Data Acquisition System (ADInstruments, Pty., Ltd., Bella Vista, Australia). LTP was induced by high-frequency stimulation (HFS: tetanus, 10 trains of stimuli, 10 pulses at 100 Hz, 10-s intertrain intervals) with the same stimulation intensity used for baseline recordings. Tetanus was given at the same intensity as that of the test stimulus (29, 30).

The electrophysiological experiments combined with behavioral analysis were performed according to the

previously described methods (31). Briefly, a recording electrode and a stimulating electrode were inserted into the CA1 field and Schaffer collaterals of the hippocampus, respectively, under pentobarbital (60 mg/kg, i.p.) anesthesia. The electrodes were anchored by small, electrically grounded screws and affixed to the skull with quick, self-curing acrylic resin (UNIFAST; GC Corp., Tokyo). After surgery, each rat was singly housed to protect the implanted electrode. Seven days later, electrophysiological experiments were performed under freely moving conditions following the experimental protocol based on a CFC paradigm. The stimulating electrode was connected through a cable to an electrical stimulator (SEN-3401, Nihon Koden) and an isolator (SS-203J, Nihon Koden). Another cable from the recording electrode was connected to an amplifier (gain 1000 $\times$, bandpass 0.08 – 10 KHz, MEG-5200 or S-0746; Nihon Koden). The evoked potential was converted from analog to digital data and stored for subsequent off-line analysis using the PowerLab system running the Scope software (version 3, ADInstruments). The intensity of the test stimulation was adjusted for each rat to elicit a PSA of approximately 50% of the maximum amplitude. High-frequency stimulation (HFS: tetanus, 10 trains of stimuli, 10 pulses at 100 Hz, 10-s intertrain intervals) was applied after exposure to CFC (retention session). After completion of the electrophysiological experiments, a direct current (900 μ A, 30-s duration) was applied under deep anesthesia, and the brain was removed to histologically verify electrode placement.

Electrical stimulation of the MRN: A bipolar stimulating electrode with a tip separation of 500 μ m was placed in the MRN (10.3-mm posterior, 0.1-mm lateral to the bregma, angle of 18° , 8.3-mm ventral to the dura) under urethane (1 g/kg, i.p.) anesthesia. A recording and stimulating electrodes were inserted into the CA1 field and Schaffer collaterals, respectively, as described above. Electrical stimulation of the MRN (stimuli intensity, 100 or 300 μ A; pulse duration, 250 μ s; stimulus interval, 30 s) was initiated before tetanus and continued until the end of the experiment. After the experiments, the location of the stimulating electrode was confirmed histologically.

Electrical lesion of the MRN: Behavioral analysis based on a CFC paradigm was performed in male rats in which the 5-HTergic neurons in the MRN were electrically disrupted. A stimulating electrode was inserted into the MRN (10.3-mm posterior, 0.1-mm lateral to the bregma, angle of 18° , 8.3-mm ventral to the dura) through a hole drilled into the skull under pentobarbital (60 mg/kg, i.p.) anesthesia. Electrolytic lesions were made by application of a direct current of 300 μ A for 10 s. Sham treatment was performed to insert the

electrode without any stimulus. Soon after the lesion, the electrode was removed, the hole in the bone was closed with a modified small nail, and the skin was sutured with acrylic cement. At least 4 days after surgery of the MRN lesion, the rats received FS conditioning, and freezing expression was determined in an acquisition and retention session as described above. After the experiments, the rats were overdosed with urethane and the lesion site was determined histologically.

Neuroanatomical analysis

Procedures for staining 5-HT-positive cells were performed by using the avidin–biotin immunoperoxidase technique (32). For fixation, the rats were transcardially perfused by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB), pH 7.2, under pentobarbital anesthesia. After removal from the skull, the brains were immersed overnight in the same fixative at 4°C, placed in 0.1 M PB containing 10%–30% sucrose at 4°C and sectioned at 30- μ m thickness in a Leica HM 300 cryostat. All immunohistochemical incubations were performed at room temperature. After incubation in 10% normal goat serum in 0.1 M PB (Histofine; Nichirei Co., Ltd., Tokyo) for 1 h, the sections were successively incubated with rabbit anti-5-HT antibody (1:500; Zymed Laboratories, Inc., South San Francisco, CA, USA) for 24 h, followed by biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) for 1 h, and avidin–biotinylated horseradish peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories) for 1 h. Immunoreaction was visualized by transferring the sections to 50 mM Tris-HCl buffer, pH 7.6, containing 0.05% diaminobenzidine, 0.6% nickel ammonium sulfate, and 0.01% H₂O₂.

Subsequently, we measured the number of immunoperoxidase-stained cells according to the previously reported procedures (33). We selected representative sections positioned 7.8-mm posterior to the bregma from each rat. Unit areas of the brain were digitally recorded with a CCD camera (CCD-IRIS; Sony, Tokyo) connected to a photomicroscope (BX50; Olympus, Tokyo) and a densitometric video image analysis system (MCID system; InterFocus Imaging, Cambridgeshire, UK). The number of 5-HT-positive cells was counted in 3 rostrocaudal sections every 20 μ m in the 3 dorsal raphe nucleus (DRN) subregion (DRN dorsal parts, DRD; DRN ventral parts, DRV; DRN lateral wings, DRL) and MRN. The cells were then assessed by automated selection of cells within the unit areas (200 $\times$ 200 μ m) that satisfied the criteria used in previous experiments. Finally, the numbers of 5-HT-positive cells were averaged in respective regions of the DRN or MRN.

Pharmacological experiments

A 5-HT_{1A} receptor partial agonist, tandospirone (10 mg/kg, i.p.), and a selective 5-HT_{1A} receptor agonist, 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH DPAT) (0.3 and 3 mg/kg, i.p.) (Sigma, St. Louis, MO, USA), were injected at 20 min before tetanic stimulation under urethane (1 g/kg, i.p.) anesthesia. The selective 5-HT_{1A} receptor antagonist WAY100135 (3 mg/kg, i.p.) (Tocris, Bristol, UK) was injected at 20 min before tandospirone administration. Tandospirone citrate was kindly donated by Dai-nippon Sumitomo Pharmaceuticals (Tokyo). The injection route and dosage of these drugs were chosen on the basis of the previous (29, 34–38) and preliminary studies. Especially, we used a relatively high dose of tandospirone (10 mg/kg, i.p.) based on our previous study (34).

Data analysis and statistics

All data were expressed as the means $\pm$ standard errors of the means (S.E.M.). Electrophysiological data were expressed as the percentage of baseline values before tetanus. Neurochemical data (in vivo microdialysis) were expressed as the percentage of baseline values before CFC exposure. The area under the curve (AUC; % min/1000) of the time course changes was determined to evaluate the ensemble effects of PSA.

Multiple group comparisons were performed using one-way ANOVA or two-way ANOVA with Bonferroni's or Dunnett's *t* post hoc test, when the difference of variance in these multiple groups was not significant. Especially, in vivo microdialysis data (time-course) was analyzed with two-way ANOVA with repeated measure (CFC $\times$ time). The alpha level was set at 5%. When significant, Mann-Whitney's *U*-test was used with the appropriate alpha level according to the number of comparison (Bonferroni correction). Differences between two groups were compared using Student's *t*-test when the difference in the variance of two groups was not significant. When significant, comparisons were performed using Mann-Whitney's *U*-test. The alpha level was set at 5%.

Results

Freezing behavior during exposure to CFC

Fear-related freezing behavior immediately after FS conditioning (i.e., acquisition session) in the females [91.23% $\pm$ 4.19% (*n* = 8)] was identical to that in the males [92.09% $\pm$ 3.81% (*n* = 9)] [*t* = 0.14, *df* = 15, not significant (n.s.)], indicating that the two groups acquired fear learning in a similar fashion (Fig. 1A). During exposure to CFC (i.e., retention session), significant decreases in the expression of freezing were observed in

the females [$37.50\% \pm 5.85\%$ ($n = 8$)] compared with those in the males [$69.03\% \pm 5.51\%$ ($n = 9$)] ($t = 3.92$, $df = 15$, $**P < 0.01$) (Fig. 1B). In the present study, we used the control group that was naive of any FS as the group-CFC(-). We previously determined freezing behavior during the retention session (10 min) in male rats that underwent the same acquisition procedure but without FS stimuli. Freezing expression was $4.3\% \pm 1.9\%$ (mean $\pm$ S.E.M., $n = 4$) in naive male rats (39), indicating that freezing during the retention session appears to

Fig. 1. Behavioral responses to fear stress. Fear-related behavior was assessed by a contextual fear conditioning (CFC) paradigm in male and female rats ($n = 8-9$ per group). Freezing was determined immediately after footshock conditioning (A, acquisition session) and during exposure to CFC (B, retention session). Each value represents the mean $\pm$ S.E.M. $**P < 0.01$.

reflect retrieval of fear memory link to the FS. In naive females, freezing expression was $3.1\% \pm 1.2\%$ ($n = 4$) during the retention session (10 min), which did not significantly differ from that in the males. These data suggest that the females exhibit low anxiety-like behavior during the CFC retention phase.

Extracellular 5-HT levels in the hippocampus induced by CFC

To elucidate changes in endogenous 5-HT levels in the hippocampus following CFC, extracellular 5-HT levels were measured by use of in vivo microdialysis. There was no significant difference of the basal 5-HT levels between the males (0.50 ± 0.09 pg/20 min) and females (0.45 ± 0.06 pg/20 min), although the values were not corrected by probe recovery.

As shown in Fig. 2, extracellular 5-HT levels were increased at 40 and 60 min after exposure to CFC in the males [CFC (+)]. Two-way ANOVA with repeated measure showed that exposure to CFC enhanced the extracellular 5-HT levels as demonstrated by the significant effect of CFC ($F_{1,8} = 14.23$, $*P < 0.05$, $**P < 0.01$), time ($F_{5,40} = 3.54$, $*P < 0.05$, $**P < 0.01$), and CFC $\times$ time interaction ($F_{5,40} = 2.62$, $*P < 0.05$, $**P < 0.01$). Significant elevation of the 5-HT levels, assessed by the area under the curve (AUC, $\% \cdot 60 \text{ min}/1000$) was observed in the CFC (+) group [7.33 ± 0.55 ($n = 5$)] compared with those in the CFC (-) group [4.94 ± 0.49 ($n = 5$)] ($t = 3.09$, $df = 8$, $*P < 0.05$). In the females, there were no significant changes in the extracellular 5-HT levels

Fig. 2. Changes in extracellular 5-HT levels in the hippocampus during exposure to CFC. Following the CFC paradigm, extracellular 5-HT levels in the dorsal hippocampus were determined by use of in vivo microdialysis in male and female rats under freely moving conditions. Time-course response (left) and the AUC (right) of the change in 5-HT levels in the males (upper) and females (lower) ($n = 5-6$ per group). AUC ($\% \cdot 60 \text{ min}/1000$) values are presented as the percentage of changes in 5-HT levels and integrated for 60 min after exposure to CFC. CFC (+) and CFC (-): conditioned group with and without exposure to CFC, respectively. Each value represents the mean $\pm$ S.E.M. $*P < 0.05$, $**P < 0.01$.

Fig. 3. Changes in hippocampal synaptic plasticity following CFC. Effects of CFC on high-frequency stimulation (tetanus)-induced LTP in the hippocampal CA1 field were examined in male and female rats. **A:** a representative field potential in the CA1 field evoked by Schaffer collaterals stimulation. The population spike amplitude (PSA) in the CA1 field was defined as the absolute amplitude between the two dotted lines separating the positive line (between the P1 and P2 components) and the negative (N) peak. PSA is defined as the absolute voltage with a latency of 8–10 ms. **B:** diagrams depicting electrode placements of the hippocampus for stimulating the Schaffer collaterals (left) and recording in the CA1 field (right). Scale bar: 1 mm. **C:** Time-course (left) and the AUC (right) of the change of PSA in the CA1 following the CFC paradigm in the males (upper) and females (lower) under freely moving conditions ($n = 5–8$ per group). Tetanus was applied immediately after exposure to CFC (retention session). The time-course response is presented as the percentage change in the PSA with time. AUC (%·60 min/1000) are presented as the percentage of changes in PSA and integrated PSA for 60 min after tetanus. CFC (+) and CFC (–): FS conditioned groups with and without exposure to CFC, respectively. Each value represents the mean $\pm$ S.E.M. * $P < 0.05$.

between the CFC (+) group [5.10 ± 0.51 ($n = 6$)] and the CFC (–) group [4.82 ± 0.39 ($n = 5$)] ($t = 0.59$, $df = 9$, n.s.).

Hippocampal synaptic plasticity associated with CFC

On the basis of previous findings that synaptic plasticity in the CA1 field was influenced by various kinds of stresses (24–26), the effects of CFC on LTP induction was examined under freely moving conditions. Significant change in LTP induction was assessed by AUC (%·60 min/1000). LTP was significantly suppressed following CFC in the males compared with that in the CFC (–) group [7.99 ± 0.79 ($n = 8$); CFC (+) group 5.70 ± 0.65 ($n = 7$)] ($t = 2.19$, $df = 13$, * $P < 0.05$). In the females, CFC-induced synaptic inhibition did not occur and LTP was induced [CFC (–) group, 10.13 ± 1.01 ($n = 7$); CFC (+) group, 9.95 ± 1.80 ($n = 5$)] ($t = 0.09$, $df = 10$, n.s.) (Fig. 3). Notably, the basal levels of PSA in the males were significantly reduced by approximately

10.2% during exposure to CFC (* $P < 0.05$), but not in the females ($t = 0.12$, $df = 10$, n.s.). These results suggest that gender differences in fear-related behavior are associated with changes in synaptic plasticity in the hippocampus.

Effects of MRN stimulation on the hippocampal synaptic plasticity

The possible involvement of the MRN, the primary origin for ascending 5-HTergic projections to the hippocampus (27), was elucidated by evaluating the effects of MRN stimulation on LTP in the CA1 field under anesthesia. In the males, stimulation of the MRN suppressed LTP in an intensity-dependent manner [MRN stimulation (–), 10.10 ± 0.55 ($n = 8$); MRN stimulation (+), 100μ A, 7.19 ± 0.58 ($n = 6$); MRN stimulation (+), 300μ A, 6.32 ± 0.25 ($n = 9$)] ($F_{2,20} = 20.39$, ** $P < 0.01$ followed by Bonferroni's *post hoc*). MRN

Fig. 4. Effects of MRN stimulation on hippocampal synaptic plasticity. Tetanus-induced LTP in the hippocampal CA1 field was examined during electrical stimulation of the MRN in male and female rats under anesthesia. Electrical stimulation of the MRN (100 and 300 μ A in the males; 300 μ A in the females) was performed from 20 min before tetanus and continued until the end of the experiment. Time-course response and the AUC of the change of PSA in the CA1 in the males (upper) and females (lower) ($n = 6 - 9$ per group). The time-course response is presented as the percentage change in the PSA. AUC (%·60 min/1000) are presented as the percentage of changes in PSA and integrated PSA for 60 min after tetanus. Stim (+) and stim (-): groups with and without MRN stimulation, respectively. Each value represents the mean $\pm$ S.E.M. $**P < 0.01$.

stimulation (300 μ A) tended to augment the magnitude of LTP in the females, but there was no significant difference of LTP magnitude among the females with or without MRN stimulation [MRN stimulation (-), 8.15 ± 0.18 ($n = 6$); MRN stimulation (+), 300 μ A, 9.63 ± 1.70 ($n = 7$)] ($t = 0.80$, $df = 11$, n.s.) (Fig. 4). The effects of MRN stimulation with 100 μ A were not examined in the females since MRN stimulation inhibited LTP induction in an intensity-dependent manner in the males, suggesting that MRN stimulation with 100 μ A, as well as 300 μ A, does not affect LTP induction in the females.

Effects of MRN lesion on freezing expression in the males

Given the findings that the synaptic changes induced by CFC observed in the males and females were mimicked by those induced by MRN stimulation, the MRN-hippocampus 5-HTergic systems appear to be involved in the underlying mechanisms of gender differences. On the basis of this hypothesis, behavioral analysis using the CFC paradigm was performed in the males with electrically disrupted MRN. As shown in Fig. 5, freezing behavior during the acquisition session was similar between groups [sham group, $90.28\% \pm 2.77\%$ ($n = 6$); MRN lesion group, $82.78\% \pm 4.07\%$ ($n = 6$)] ($t = 1.53$, $df = 10$, n.s.), but the behavior during exposure to CFC (retention session) significantly decreased in the MRN-lesioned group [$39.22\% \pm 12.02\%$ ($n = 5$)] compared with that in the sham-operated controls [$75.56\% \pm 6.77\%$ ($n = 6$)] ($t = 2.76$, $df = 9$, $**P < 0.01$). Reduction of freezing in the MRN-lesioned males

appeared to be similar to the behavioral pattern observed in the females. These data strengthen that the MRN-hippocampus 5-HTergic pathway is involved in the gender differences in behavior and synaptic plasticity in response to fear stress.

5-HT immunoreactive cells of the raphe nuclei

On the basis of the electrophysiological findings, we next immunohistochemically analyzed the expression of the 5-HT positive cells of the raphe nuclei using intact animals (naive of any behavioral experiments). As shown in Fig. 6, the number of MRN 5-HT positive cells ($\times 25$ cells/mm²) in the females [21.56 ± 1.17 ($n = 6$)] were significantly higher than those in the males [14.39 ± 1.10 ($n = 6$)] ($t = 4.44$, $df = 10$, $**P < 0.01$). There were no significant differences of 5-HT positive cells in any region of the DRN between the males and females ($n = 6$, each) [DRD: males, 17.22 ± 2.42 , and females, 14.08 ± 2.16 ($t = 0.97$, $df = 10$, n.s.); DRV: males, 28.53 ± 2.62 , and females, 24.14 ± 2.01 ($t = 1.33$, $df = 10$, n.s.); DRL: males, 21.86 ± 1.88 , and females, 20.89 ± 1.64 ($t = 0.39$, $df = 10$, n.s.)].

5-HT contents of the raphe nuclei and hippocampus

The tissue contents of 5-HT (ng/g wet weight) in the MRN showed that higher levels in the females [70.53 ± 7.34 ng/g ($n = 5$)] were observed compared with those in the males [47.90 ± 3.97 ng/g ($n = 5$)] ($t = 2.71$, $df = 8$, $P < 0.05$). There were no significant differences in 5-HT levels in the DRN between the males [95.48 ± 16.37 ng/g ($n = 4$)] and females [87.89 ± 1.81

Fig. 5. Effects of MRN lesion on behavioral responses to CFC. Inactivation of the MRN area was accomplished by electrolytic lesion of the MRN in male rats under anesthesia; and 4 days later, the rats were subjected to the CFC paradigm. The left figure is a schematic representation of the locations of the tips of the electrical electrodes for the MRN lesion (solid square). Scale bar: 1 mm. Fear-related freezing behaviors were determined immediately after FS (acquisition session) and during exposure to CFC (retention session) ($n = 5 - 6$ per group). Lesion and sham: male rats with or without electrolytic MRN lesion, respectively. Each value represents the mean $\pm$ S.E.M. ** $P < 0.01$.

Fig. 6. Serotonin-positive cells in the raphe nuclei. The numbers of 5-HT-positive cells in the raphe nuclei were determined by immunohistochemical staining in male and female rats. **A:** A low-power photograph of 5-HT immunolabeling in the raphe nuclei. Scale bar, 1 mm. **B:** High-power photographs of 5-HT immunolabeling in the MRN of the males (left) and females (right), respectively. Scale bar, 50 μ m. **C:** The column graphs show the numbers of 5-HT-positive cells in the MRN and in each area of the DRN ($n = 6$ per group). Each value represents the mean $\pm$ S.E.M. ** $P < 0.01$.

ng/g ($n = 4$) (n.s.). In the hippocampus, 5-HT contents levels in the females [14.18 ± 2.36 ng/g ($n = 5$)] were significantly higher than in the males [5.70 ± 2.02 ng/g ($n = 5$)] ($t = 2.73$, $df = 8$, $P < 0.05$).

Effects of 5-HTergic intervention of 5-HT_{1A} receptors on the hippocampal synaptic changes

To further elucidate the underlying 5-HTergic mechanism associated with gender difference, pharmacological

experiments were performed with focusing on 5-HT_{1A} receptors. Pretreatment with tandospirone (10 mg/kg, i.p.) led to suppression of basal synaptic transmission and tetanus-induced LTP induction in the males [control group, 9.43 ± 0.52 ($n = 8$); tandospirone group, 6.59 ± 0.65 ($n = 8$)]. Tandospirone-induced LTP suppression was not found in the presence of the selective 5-HT_{1A} receptor antagonist WAY100135 (3 mg/kg, i.p.) [tandospirone + WAY100135 group, 9.05 ± 1.54 ($n = 7$)].

Fig. 7. Effects of tandospirone on hippocampal synaptic plasticity. Possible involvement of 5-HT_{1A} receptors in synaptic plasticity, tetanus-induced LTP in the hippocampal CA1 field, was examined by application of a 5-HT_{1A} receptor partial agonist, tandospirone, under anesthesia. The time course and the AUC of the PSA in the CA1 following tandospirone (10 mg/kg, i.p.) administration in the presence or absence of a selective 5-HT_{1A} receptor agonist, WAY100135 (3 mg/kg, i.p.), in the males (upper) and females (lower) ($n = 6 - 8$ per group). Tandospirone was administered at 20 min before tetanic stimulation, and WAY100135 was injected at 20 min before tandospirone treatment. Each value represents the mean $\pm$ S.E.M. * $P < 0.05$. Cont: untreated control, Tand: tandospirone-treated group, Tand + WAY: tandospirone plus WAY100135-treated group, WAY: WAY100135-treated group.

WAY100135 alone did not affect the LTP [9.35 ± 1.61 , ($n = 5$)] (* $P < 0.017$). These results indicate that tandospirone-induced synaptic inhibition was mediated via 5-HT_{1A} receptors. In the females, however, tetanus-induced LTP was not affected by pretreatment with tandospirone [control group, 8.15 ± 0.18 ($n = 6$); tandospirone group, 9.27 ± 0.66 ($n = 6$)] (n.s.) (Fig. 7). Thus, it appeared likely that the 5-HT_{1A} receptor function mediated via 5-HT_{1A} receptors contributed to the gender differences observed in the synaptic plasticity and fear-related behavior in response to CFC. To confirm this suspicion, further experiments were performed by application of a selective 5-HT_{1A} receptor agonist 8-OH DPAT. In the males, 8-OH DPAT (0.3 and 3 mg/kg, i.p.) suppressed the LTP in the CA1 field [control group, 10.10 ± 0.57 ($n = 8$); 8-OH DPAT, 0.3 mg/kg group, 7.60 ± 0.58 ($n = 5$); 8-OH DPAT, 3 mg/kg group, 6.92 ± 1.58 ($n = 5$)] ($F_{2,15} = 3.77$, * $P < 0.05$ followed by Dunnett's *t post hoc*), whereas a high dose (3 mg/kg, i.p.) of 8-OH DPAT did not affect LTP induction in the females [control group, 8.15 ± 0.18 ($n = 6$); 8-OH DPAT, 3 mg/kg group, 9.33 ± 1.60 ($n = 6$)] (n.s.) (Fig. 8).

Discussion

In the present study, female rats showed a decrease in freezing behavior during exposure to CFC compared with that in the males, which was consistent with the results reported by Mitsushima et al. (40). The in vivo microdialysis study showed that extracellular 5-HT levels in the hippocampus were increased by CFC in the males, but not in the females. Hippocampal LTP was suppressed by CFC in the males, which was mimicked by the MRN stimulation. Thus, the metaplastic 5-HT_{1A} receptor mechanism in the MRN-hippocampus pathway is critical for gender-specific behavioral responses under stressful conditions.

Low anxiety-like behavior observed in the females may be responsible for alteration of neural circuitry underlying learning and memory processes, since behavioral distinction between anxiety and memory impairment is not always clear. Indeed, Shors and Miesegaes (41) reported that exposure to acute stress could enhance learning in the males and impair learning in the females. However, the low levels of freezing observed in the

Fig. 8. Effects of 8-OH DPAT on hippocampal synaptic plasticity. Involvement of 5-HT_{1A} receptors in synaptic plasticity, tetanus-induced LTP in the hippocampal CA1 field, was further assessed by application of a full agonist 8-OH DPAT under anesthesia. The time course and the AUC of the PSA in the CA1 following 8-OH DPAT (0.3 or 3 mg/kg, i.p.) administration in the males (upper) and females (lower) ($n = 5 - 8$ per group). The time-course response is presented as the percentage change in the PSA with time. AUC (%·60 min/1000) are presented as the percentage of changes in PSA and integrated PSA for 60 min after tetanus. Each value represents the mean $\pm$ S.E.M. * $P < 0.05$.

females is unlikely to reflect the acquisition deficits of fear conditioning because freezing immediately after FS conditioning was similar to that in the males. Although we did not analyze the behavior during the initial free 5-min acclimatization period, we examined the innate behavior using the elevated-plus maze (EPM) test. There was no significant difference in total arm entries between the males and females, indicating that there were no significant differences in locomotor and explorative behavior between the males and females. The notion that the females exhibited low anxiety-like behavior is further supported by the previous studies using the EPM test (42) and open field test (43).

Synaptic plasticity, LTP, in the hippocampus is known to be suppressed by various kinds of stresses (23 – 26). In the present study, there was no significant difference in the magnitude of LTP between the males and females, consistent with the *in vitro* study; basal excitatory postsynaptic potential (EPSP) amplitude and LTP magnitude in the CA1 region are similar between the males and females (44). On the other hands, LTP induction in the CA1 field was completely suppressed following CFC, and basal synaptic transmission was also reduced during exposure to CFC in the males. Tada et al. (45) reported that spontaneous activity of CA1 pyramidal neurons was reduced during exposure to CFC. Interestingly, decreases in neural activity recovered to the baseline after freezing behavior disappeared. Thus, CFC-induced synaptic inhibition may alter the threshold

for LTP induction, and consequently lead to LTP impairment. In the females, basal synaptic transmission and LTP induction did not change during and after exposure to CFC, respectively. Moreover, LTP in the CA1 region was not influenced by estrogen modulation (46). Although we have no direct evidence to fully explain the relationship between freezing expression and hippocampal synaptic changes in response to CFC, they are closely related to emotional regulation as key players.

Synaptic changes in the CA1 field following CFC in the males and females were mimicked by the synaptic response induced by MRN electrical stimulation; electrical stimulation of MRN induced LTP impairment in the males, but not females. Considering the similarity of synaptic changes, these results strongly suggest that 5-HTergic function in the MRN-hippocampus pathway is involved in gender differences in response to fear stress. These findings were supported by the results observed in lesion studies in the males; freezing expression during exposure to CFC was reduced by electrical lesion of the MRN, as reported in the males with neurotoxic lesions of the MRN (47). Reduction of freezing in the MRN-lesioned males is similar to the CFC-induced behavioral pattern in the females. These results support the involvement of the MRN-hippocampus 5-HTergic pathway in low anxiety-like behavior in the females. In other words, the 5-HTergic function in this pathway is critical for the gender differences underlying synaptic plasticity and behavioral changes in response to fear stress.

An important finding in the present study is that different patterns of synaptic plasticity mediated via the 5-HT_{1A} receptor were observed in the males and females. In the males, pretreatment with a 5-HT_{1A} receptor partial agonist, tandospirone, suppressed LTP; and this inhibition was reversed by a 5-HT_{1A} receptor selective antagonist, WAY100135. These results indicate that stimulation of 5-HT_{1A} receptors lead to suppression of LTP, which was supported by the data showing that a selective 5-HT_{1A} receptor agonist, 8-OH DPAT, also impaired LTP induction. Thus, the MRN-hippocampus 5-HT pathway via 5-HT_{1A} receptors could certainly influence the synaptic plasticity in the males. On the other hand, neither tandospirone nor 8-OH DPAT affected LTP induction in the females. Although the precise mechanism underlying the different synaptic responses mediated via 5-HT_{1A} receptors is still unclear, one possibility may be due to different sensitivities of the somatodendritic 5-HT_{1A} autoreceptors located in the MRN. The synthesis and release of 5-HT in the hippocampus is known to be under inhibitory control via somatodendritic 5-HT_{1A} receptors (22). Dominguez et al. (48) demonstrated that 5-HTergic activity in the MRN decreased by exposure to the EPM test. Rozeske et al. (49) reported that tail shock stress caused functional desensitization of somatodendritic 5-HT_{1A} receptors in the DRN. Therefore, fear stress appears to alter the sensitivity of 5-HT_{1A} autoreceptors and influences the 5-HTergic function in its terminal regions. Interestingly, we observed that the 5-HT immunoreactive cells in the MRN, in addition to MRN 5-HT contents, were higher in the females compared with those in the males. These results suggest that inhibitory control mediated via 5-HT_{1A} autoreceptors may be exaggerated in response to fear stress in the females.

Another possibility is that the functional changes of postsynaptic 5-HT_{1A} receptors in the hippocampus may contribute to the different synaptic responses observed in the males and females. Schiller et al. (50) reported that the females had lower postsynaptic 5-HT_{1A} receptor binding compared with that in the males. On the basis of these data, they suggested that gender differences in postsynaptic 5-HT_{1A} receptor binding may be relevant to hippocampus-dependent behaviors. Several studies (51, 52) have shown that exposure to stress increased the hippocampal 5-HT_{1A} receptor mRNA in the males, but decreased those in the females. These findings lead us to speculate that fear stress induces downregulation of postsynaptic 5-HT_{1A} receptors in the females and ultimately causing failure of 5-HT_{1A} receptor-mediated synaptic inhibition. The 5-HT_{1A}-N-methyl-D-aspartate (NMDA) receptor interaction is known to provide potential mechanisms underlying the 5-HTergic regula-

tion in emotional processes (53, 54). Therefore, their interaction may contribute to the different patterns of 5-HT_{1A} receptor-mediated synaptic responses. Indeed, 5-HT decreases EPSP by eliciting a 5-HT_{1A} receptor-mediated increase in potassium conductance (55–57). Hyperpolarization of postsynaptic membrane via 5-HT_{1A} impairs expulsion of Mg²⁺ ions from NMDA receptors (58). Thus, activation of postsynaptic 5-HT_{1A} receptors could impair LTP induction at the NMDA-dependent synapse. In other words, different sensitivities of postsynaptic 5-HT_{1A} receptors may be associated with dimorphic synaptic changes mediated via 5-HT_{1A} receptors. Speculatively, increase in the sensitivity of 5-HT_{1A} autoreceptors and/or decrease in the sensitivity of postsynaptic 5-HT_{1A} receptors are attributable to the dysregulation of 5-HTergic synaptic inhibition observed in the females.

It should be noted that the gender differences observed in the present study may be caused by gonadal hormone estrogen or stress hormones glucocorticoids. Indeed, some studies have suggested that enhanced fear conditioning in the males is dependent on the presence of glucocorticoids, whereas impaired conditioning in the females is dependent on the presence of estrogen (19, 59). Ovarian hormones also can affect anxiety-like behavior as shown by the EPM test (60, 61) or open field test (62). Toufexis et al. (63) showed that estrogen receptor agonists can disrupt conditioned fear in the females. These reports suggest that the presence of estrogen in the females may disrupt their ability to acquire the learning and memory associated with stressful events. This hypothesis could be confirmed by ovariectomy surgery for the purpose of eliminating gonadal influences as previously reported (64). However, our preliminary experiment (S. Inoue, unpublished data) showed no differences in fear-related freezing expression during exposure to CFC between sham-operated and ovariectomized females at 8 weeks of age. Although further studies are required, circulating gonadal hormones are unlikely to explain our findings, i.e., gender differences in behavioral and synaptic responses to fear stress.

Mitsushima et al. (40) reported that increases in 5-HT levels in the basolateral amygdala (BLA) in response to restraint stress were greater in the females than males. They also showed that the basal levels of extracellular 5-HT were higher in male rats than in females. The BLA, a crucial regions for conditioned fear stresses (65), is one of the terminal regions of 5-HTergic projection from the midbrain DRN. An electrophysiological study revealed that firing activity of the 5-HT neurons in the DRN, but not MRN, was higher in the females than males (48). Additionally, several studies revealed that

gonadal steroid hormones such as androgen and estrogen can regulate neural activity in the DRN in a gender-specific manner (66, 67). Thus, the region-specific 5-HTergic mechanisms in the neural circuit including the BLA and hippocampus may underlie gender differences in emotional regulation (68, 69).

More recently, Renoir et al. (70) demonstrated that the hippocampal 5-HT levels were lower in female mice compared with male mice. Hypothermia responses to 8-OH DPAT, as a measure of 5-HT_{1A} receptor function, were smaller in the female mice. They also reported that the hippocampal 5-HT level was reduced while 8-OH DPAT-induced hypothermia was enhanced in female model mice with depressive-like endophenotypes. On the basis of their findings, they suggest that female-specific phenotype may be associated with disrupted 5-HT signaling due to alteration of 5-HT_{1A} receptor function. In contrast, our present results demonstrated that the 5-HT concentration was increased both in the hippocampus and the MRN accompanied with increased 5-HT-immunoreactive cells in the MRN in the female rats. Hippocampal synaptic responses to 5-HT_{1A} receptor agonist in the male rats were not observed in females. The discrepancy observed in mice and rats possibly indicate the 5-HT_{1A} receptor-mediated mechanism as a key component for gender-specific emotional regulation and/or psychiatric disorder.

In conclusion, the present study revealed that the MRN-hippocampus 5-HTergic mechanisms via 5-HT_{1A} receptors are critical for the gender differences underlying emotional regulation and are associated with changes in hippocampal synaptic plasticity. In other words, metaplastic dysregulation of the MRN via 5-HT_{1A} receptor on hippocampal synaptic plasticity may be a key factor in vulnerability or resistance to emotional stress, and it may be a cause of the gender differences observed in psychiatric disorders. Further clarification of the 5-HTergic mechanisms underlying gender-specific emotional regulation may provide a better understanding of the pathogenesis of psychiatric disorders, which in turn may be useful in the development of novel therapeutic strategies.

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