the identification of objects including identification of features such as color, shape and faces, whereas the occipitoparietal pathway, or "dorsal stream," is crucial for spatial perception and observation of the movements of objects in space such as motion and information about location (Ungerleider et al 1998). The activation of the dorsal and ventral pathways was detected only among the men in this study as a subtraction between the reactions to both stimulations in this kind of analysis. These results suggest that men tended to pay attention to differences in size and shape between the distorted images of their own bodies and real images of their bodies, while women tended to pay the same attention to the distorted images of their own bodies and real images of their bodies.

In a previous study using positron emission tomography (PET), Bailer et al (2004) reported ED patients showed parietal lobe activity on the resting-state experiments. Wagner et al (2003) also reported parietal lobe activity in anorectic patients who viewed distorted body images, but they used a mosaic image as the control. It is difficult to compare our results with the results of previous studies because of differences in methodology.

Another finding of the present study was that the cerebellum was activated in both women and men upon performing the body image task. Neuroimaging studies have indicated that cerebellar regions are not only important in motor control, but also involved in several different types of higher cognitive operations (Kim et al 1994; Allen et al 1997). However, the distinct role of the cerebellar regions in these diverse and complex cognitive functions remains unclear.

Based on comparison of the fMRI images of the brain activation upon performing the fat- versus real-image task or thin- versus real-image task, our study showed that women tend to perceive their own distorted body images by complex cognitive processing of emotion, whereas men tend to perceive their own distorted body images by object and spatial visual processing. On the behavioral level, the female subjects were more sensitive to their fat body image with respect to unpleasant emotion than the male subjects (Table 4). These results and our finding that the amygdala or PFC was activated upon performing the distorted-image task in the women suggest that certain distorted body images in our paradigm may be fearful stimuli to the subjects. There were no significant differences in age, BMI or the EDI-2 total score between the male and female subjects (Table 1). Therefore, the areas of the brain that were activated in each gender may explain their style of cognition of distorted body shape. Taking into consideration the results of previous studies on EDs (Seeger et al 2002; Wagner et al 2003), our study suggests that the cognitive style of visual stimuli of body image in healthy women is more similar to that in ED patients with weight phobia, than to that in healthy men. These results may be related to the fact that women are approximately 10 times more likely to experience a lifetime episode of ED than men and the fact that women tend to be more sensitive to information about body image in daily life than men.

Our study has some limitations. First, we did not perform a structured interview during the selection of subjects for participation in the study. Nevertheless, they had no psychiatric nor neurological illness at the time of their participation in this study, although we can not predict their occurrence in the future. Second, our paradigm was appropriate for detecting neuronal processing of body image in the brain. However, there were some uncertainties in our paradigm: (1) Were the results about the neuronal processing of the distorted self body image, the distorted body image, or just the distorted image? (2) Was the cue itself the negative reward? (3) Was there a difference in the difficulty of the active and control tasks? These are points that are worth studying in the future. Third, although our data suggest that there is differential activation pattern of the brain of men and women when processing distorted body image, these data are not sufficient to conclude that the PFC and the limbic/paralimbic area, which were activated in the women but not the men upon viewing distorted body images of one's own body, are the focal areas responsible for susceptibility to ED.

In conclusion, our neuroimaging results provide evidence that women process visual stimuli of their own body images differently from men, and that women are more emotionally preoccupied with the appearance of their own body shapes than men. Further studies that compare the brain activation of ED patients and healthy controls while performing our tasks are needed to confirm that these brain areas are involved in the mechanism of the onset of ED.

This study was supported by the Research on Psychiatric and Neurological Diseases and Mental Health, Ministry of Health, Labour and Welfare, Japan.

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Brain Research 1024 (2004) 233–243

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Research report

A selective increase in phosphorylation of cyclic AMP response element-binding protein in hippocampal CA1 region of male, but not female, rats following contextual fear and passive avoidance conditioning

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Accepted 10 August 2004
Available online 12 September 2004

Abstract

Cyclic AMP response element-binding protein (CREB), a transcription factor on which multiple signal transduction pathways converge, has been implicated in long-term memory. We examined whether the sex difference in the performance of contextual fear or passive avoidance conditioning is associated with a change in the activation of CREB in the hippocampus, a neural structure important for long-term memory. The activation of CREB in different subregions within the hippocampus in male and female rats was determined immunohistochemically with an antibody that specifically recognizes the phosphorylated form of CREB (pCREB). With respect to the freezing time in contextual fear conditioning and the step-through latency in passive avoidance conditioning, male rats exhibited better performance than female rats. Phosphorylation of CREB (% pCREB) as revealed by the ratio of the pCREB-immunoreactive (pCREB-ir) cell number to the CREB-immunoreactive cell number was increased in the CA1 region, but not in CA3, CA4, or in the dentate gyrus following training for both types of conditioning in males. In females, such an increase in % pCREB was not found in any hippocampal subregion at any time after conditioning or by increasing the intensity of foot shock. Orchidectomy in males did not alter either the performance of contextual conditioning or conditioning-induced CREB phosphorylation in CA1. The close relationship between behavioral performance and CREB phosphorylation in the CA1 region suggests that hippocampal CREB is involved in the sex difference in some forms of learning and memory.

Theme: Neural basis of behavior
Topic: Learning and memory: physiology

Keywords: Sex difference; Cyclic AMP response element-binding protein; Hippocampus; Learning; Memory

1. Introduction

An increased intracellular level of the second messenger cyclic AMP after stimulation by hormones and neurotransmitters induces the expression of a variety of genes, indicating an important role of cyclic AMP in protein synthesis. The transcription factor cyclic AMP response element-binding protein (CREB) is activated by cyclic AMP-dependent protein kinase (PKA)-mediated phosphorylation on Ser133 [19] and binds to a consensus DNA sequence termed cyclic AMP response element (CRE) in promoter regions [55], leading to cyclic AMP-regulated expression of the genes for a wide range of proteins such as somatostatin, c-*fos*, brain-derived neurotrophic factor

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doi:10.1016/j.brainres.2004.08.007

(BDNF), tissue plasminogen activator, and cyclin proteins [35]. In addition to cyclic AMP/PKA, there are a number of upstream regulators of CREB, including calcium–calmodulin-dependent protein kinase II, protein kinase C, and mitogen-activated protein kinase [46,54], suggesting that CREB is a convergence point of multiple signal transduction pathways.

Learning and memory are integrative brain functions based on neuronal plasticity, which involve a variety of molecules such as neurotransmitters, neurotrophins, and their receptors as well as second messengers and protein kinases. Furthermore, the acquisition and consolidation of long-term, but not short-term, memory depend upon gene expression and protein synthesis [15]. When inhibitors of protein synthesis or mRNA synthesis are administered immediately before or after training, conditioned responses are inhibited at 24 h, but not 1 h, after training in contextual fear conditioning and passive avoidance conditioning, paradigms of fear-motivated learning [1,24,44]. There are several lines of evidence suggesting that CREB plays a key role in long-term memory and synaptic plasticity. First, long-term memory is blocked when CREB production is inhibited by maneuvers of microinjection of the CRE sequence in Aplysia [14], a dominant negative CREB transgene in *Drosophila* [56], and targeted deletion of *CREB* gene in mice [10]. Second, training for contextual fear [25] and passive avoidance conditioning [50] and long-term potentiation (LTP) of synaptic transmission [45], a well-known model for strengthening of synaptic efficacy, are accompanied by spatiotemporal changes in CREB phosphorylation in the hippocampus, a crucial neural structure involved in the acquisition and consolidation of many forms of memory [27,39,48,49].

Sex differences in learning and memory are observed in humans and animals. The presence of sex differences and the predominating sex depend upon learning paradigms. Interestingly, various hippocampal-dependent learning tasks exhibit sex differences: male rats perform better at tasks of contextual fear [2,32] and passive avoidance conditioning [16], whereas female rats perform better at tasks of active avoidance [8,16] and trace eyeblink conditioning [53]. However, the sex difference favoring male rats in the Morris water maze spatial task is not consistent [31]. Furthermore, male rats exhibit greater LTP recorded in the dentate gyrus than do female rats [11,32]. The contribution of gonadal steroid hormones to these sex differences also varies depending upon learning paradigms [52]. However, little is known about the neuroanatomical and biochemical pathways responsible for the sex differences in learning and memory.

In the present study, we addressed the issue of whether the sex differences found in hippocampal-dependent learning paradigms are attributable to differences in the phosphorylation of CREB in the hippocampus. We determined the number of phosphorylated CREB (pCREB)-immunoreactive (ir) cells in hippocampal subregions of male and female rats trained for contextual fear and passive avoidance conditioning. These two learning paradigms were chosen as experimental models because they exhibit prominent sex differences favoring one sex in performance and robust acquisition of memory by a single training session, which enables the detection of a rapid and subtle spatiotemporal change in hippocampal CREB. We demonstrated that the sex differences in these types of hippocampal-dependent conditioning were accompanied by changes in immunoreactive pCREB selectively seen in the CA1 region.

2. Materials and methods

2.1. Animals

Eight-week-old Wistar male rats weighing 240–250 g and female rats weighing 160–170 g were purchased from Japan SLC (Shizuoka, Japan) and were maintained individually in a light/dark cycle-controlled (lights on from 06:00 to 18:00 h) and temperature-controlled animal room with free access to laboratory chow and tap water. Rats were allowed to rest undisturbed in their home cages for at least 5 days prior to conditioning experiments. Because vaginal smears had not been taken, females killed after conditioning were not at a specific stage but at random stages of the estrous cycle. All experimental procedures were conducted in accordance with the guidelines of the Ethical Committee of Animal Experiments at the University of Yamanashi. All efforts were made to minimize the number of animals used and their suffering.

2.2. Contextual fear conditioning

A contextual fear-conditioning task was performed in a conditioning chamber placed in a sound-attenuating box during the light phase of the cycle. The conditioning chamber (28 W×21 H×22 D cm^3) was constructed of clear Plexiglas on the top and four sides. The floor of the chamber was lined with 18 stainless steel bars (4 mm in diameter; 1.5 cm spacing), which formed a foot shock grid to deliver scrambled shocks produced by a stimulator (SS-104J Nihon Koden, Tokyo, Japan). The foot shock was a 2-s direct current of 0.75 mA, and served as the aversive unconditioned stimulus (US). Between training and testing sessions, the floor and interior of the conditioning chamber were cleaned with a 75% ethanol solution. The sound-attenuating box (48 W×48 H×48 D cm^3) was provided with a 20-W houselight and a ventilation fan located at the top of the box, supplying background white noise (74 dB). Because hippocampal lesions have been reported to disrupt freezing to a context when an explicit cue is paired with foot shock in that context (background contextual fear conditioning), but have no effect when a context is paired directly with shock (foreground contextual fear conditioning) [40], a discrete tone-conditioned stimulus (CS) was given on

general contextual stimuli. The tone (800 Hz, 20-s duration, 80 dB) was delivered by two speakers located in the lower corner of the sound-attenuating box. Prior to training, rats received 3-day habituation, in which they were placed in the conditioning chamber for 1 s and then returned to their home cage, once a day. On the day of training, the rats were placed in the conditioning chamber and allowed to explore for 3 min. A foot shock was delivered three times at 1, 9, and 18 s after the onset of the tone CS. The rats were then allowed to recover for 30 s in the conditioning chamber and returned to their home cage. An hour later, the rats were again introduced into the conditioning chamber in which they had been trained and were tested for a 5-min period, during which no tone CS was presented. In time course studies, the rats were tested at varying times of 1–24 h after training. Conditioning was assessed by measuring the time spent freezing for each 30 s during the testing period. Freezing behavior was defined as cessation of all but respiratory movement. Data were quantified and presented as the percentage of total freezing time in the 5-min testing period.

2.3. Passive avoidance conditioning

A passive avoidance-conditioning task was performed using a step-through type of conditioning chamber during the dark phase of the cycle. The conditioning chamber was divided into two sections, one light and one dark, by a partition with a closable trap door (light section, 20 W×40 H×20 D cm^3; dark section, 20 W×40 H×15 D cm^3). The light section was illuminated by a 20-W houselight placed on the top of the light section. Both the sections had a 17-bar (4 mm in diameter, 1 cm spacing) foot shock grid floor. The bars in the dark section were connected to a stimulator to deliver scrambled shocks. On days 1 and 2 of the experiment, rats received acclimatization trials, in which they were placed in the light section and allowed to move freely to the dark section. Immediately after the entire body of the rat was within the dark section (defined as step-through), the trap door was closed. After 2 min, the rats were removed from the chamber and returned to their home cage. This acclimatization trial was performed twice each day with a 3-min interval. After two consecutive acclimatization trials on day 2, the rats were placed in the light section for training, and a foot shock (1-s duration, 0.6 mA) was delivered immediately after step-through. The rats was allowed to recover for 2 min in the dark section and then returned to their home cage. Thirty minutes later, the rats were placed in the light section and tested for step-through. The latency for step-through was measured during a maximum testing period of 5 min.

2.4. Immunohistochemistry

Rats were decapitated immediately after completion of testing. Brains were removed within 90 s, frozen rapidly in dry ice-isopentane, and stored at −70 °C until sections were made. About 10-μm-thick coronal sections were cut using a cryostat with reference to the atlas of Paxinos and Watson [38]. Hippocampal sections located 2.9 mm posterior to the bregma suture were placed on 3-aminopropyltriethoxysilane-coated glass slides (Matsunami Glass, Osaka, Japan). The hippocampal sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.0 for 15 min and washed twice in 0.01 M phosphate-buffered saline, pH 7.4 for 10 min each. The sections were then dehydrated with a graded series of 70%, 95%, and 100% ethanol, and rehydrated with a reversed series of the same ethanol concentrations, which was shown in a preliminary experiment to be effective in decreasing background staining. The sections were stored at −20 °C in a cryoprotectant (25% ethylene glycol, 25% glycerin in 0.05 M phosphate buffer) until immunostaining. The sections were treated with 3% H_2O_2 in phosphate-buffered saline for 10 min to block endogenous peroxidase activity and with 10% normal horse serum in Tris-buffered saline, pH 7.4 (TBS) for 30 min to block nonspecific staining. The sections were thereafter immunostained with either anti-pCREB antibody at 1:500 dilution (Cell Signaling Technology, Beverly, MA), which specifically recognizes CREB phosphorylated at Ser133, or anti-CREB antibody, which detects both the phosphorylated and unphosphorylated forms of CREB, at 1:2000 dilution in TBS containing 10% normal horse serum at 4 °C for 48 h. The immunoreactive specificity was confirmed by the result that no staining in hippocampal tissue was found with omission of the primary antibodies. Tissue sections were incubated with biotinylated anti-rabbit IgG (Vector, Burlingame, CA) at 1:200 dilution in TBS containing 10% normal

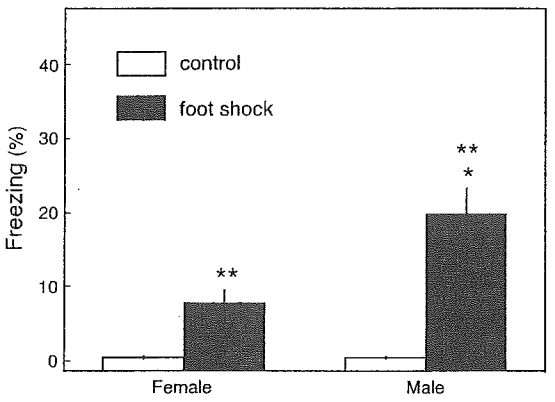

Fig. 1. Freezing after contextual fear conditioning in male and randomly cycling female rats. During training, male and female rats received a foot shock (0.75 mA) while control rats received the same training procedures but without the foot shock. One hour after training, they were tested for freezing behavior. Freezing is expressed as the percentage of the time spent in freezing in a total 5-min testing time. Each bar indicates mean±S.E.M. The numbers of animals are 6 and 5 for control and foot-shocked female groups, and 7 and 7 for control and foot-shocked male groups, respectively. *Significantly different from foot-shocked females; **significantly different from controls at $p<0.05$.

horse serum for 1 h followed by avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit, Vector) in TBS for 1 h. Between incubations, the sections were washed three times for 7 min each in TBS containing 0.3% Tween 20. Peroxidase reaction was performed for 10 min using a DAB Peroxidase Substrate Tablet Set (Sigma, St. Louis, MO) in the presence of 1% nickel ammonium sulfate. The sections were dehydrated with ethanol, cleared with xylene, and were coverslipped with Histomount (Zymed, San Francisco, CA).

Microscopic images of randomized sections were captured into a computer with a high-sensitivity CCD camera (DP-50, Olympus, Tokyo, Japan). Every cell, located within an $800 \times 600\ \mu m^2$ area, in the pyramidal cell layers of CA1, CA3, and CA4 and in the granule cell layer of the inferior blade of the dentate gyrus was subjected to assessment of immunoreactivity by an investigator who did not have information on the sections. Neurons were judged to be immunoreactive and counted when their nuclei were stained as intensely as those seen within several inner lines of the

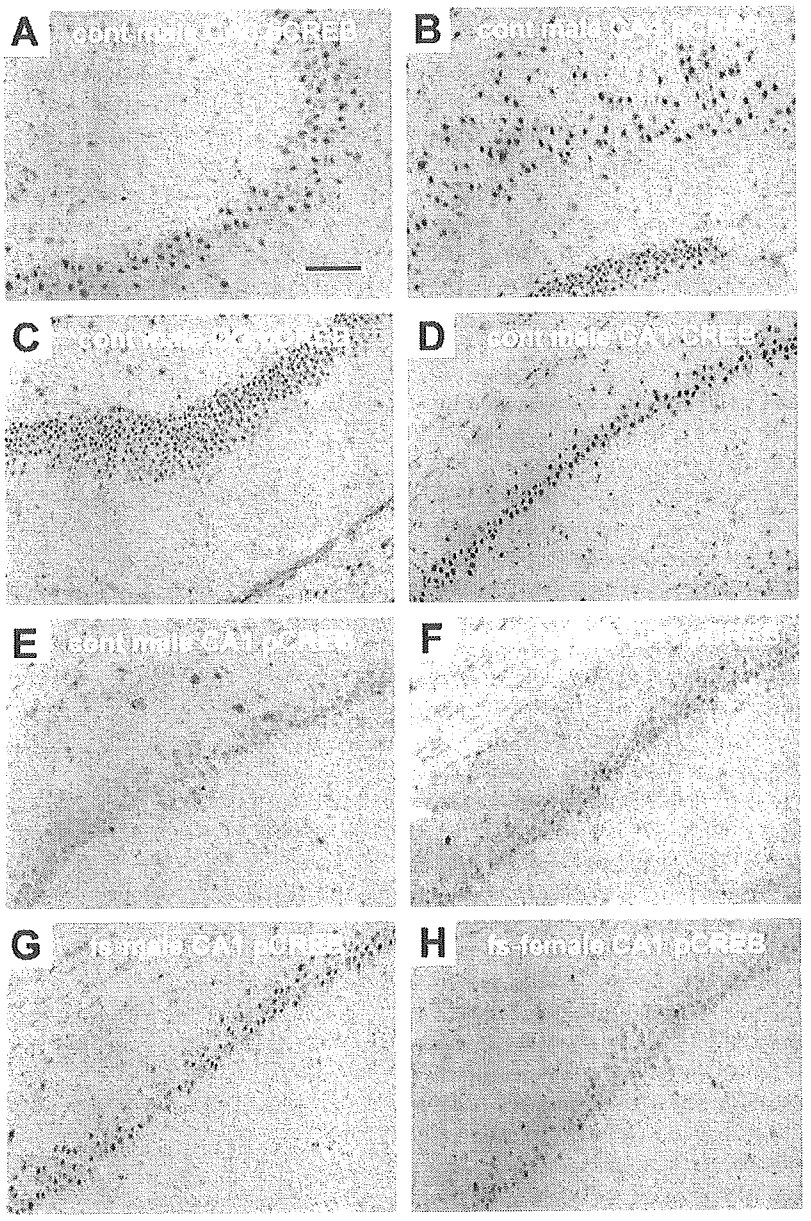

Fig. 2. Photomicrographs showing hippocampal pCREB-ir cells after contextual fear conditioning in male and female rats. During training, male and female rats received a foot shock while control rats received the same training procedures but without the foot shock. One hour after training, they were tested for freezing behavior and then killed for immunohistochemical staining for pCREB and CREB in hippocampal subregions. pCREB-ir cells in CA3 (A), CA4 (B), and dentate gyrus (C) and CREB-ir cells in CA1 (D) in a control male rat. pCREB-ir cells in CA1 in a control male (E), control female (F), foot-shocked (fs) male (G), and fs female rat (H). Scale bar, 100 μm.

granule cell layer in the dentate gyrus. Two independent counts were made from at least two different sections per animal and averaged.

2.5. Statistical analysis

The experimental data were analyzed by nonparametric analysis of Kruskal–Wallis for multiple comparison. Comparisons between pairs of groups were carried out by Mann–Whitney U-test based on the Bonfferoni correction. Differences at $p<0.05$ were considered statistically significant.

3. Results

3.1. Freezing and number of hippocampal pCREB-ir cells after contextual fear conditioning in male and female rats

Control rats that had not received foot shock during training exhibited little or no freezing behavior in response to the context during testing. There was no significant difference in freezing between control male (n=7) and female rats (n=6; Fig. 1). Although both male (n=7) and female rats (n=5) that had received a foot shock with an intensity of 0.75 mA exhibited conditional freezing after 1 h (χ^2=18.9, df=3, $p<0.0001$), freezing was 2.6-fold longer in males than in females (p=0.006).

When the hippocampus from these rats was immunostained using an antibody that specifically recognizes pCREB, numerous intensely immunostained cells were observed in hippocampal subregions including CA3 (Fig. 2A), CA4 (Fig. 2B), and the dentate gyrus in control rats (Fig. 2C). In contrast to these subregions, CA1 contained a marked smaller number of pCREB-ir cells (Fig. 2E and F). No apparent sex difference existed in the distribution of pCREB-ir cells within the hippocampus in control rats. The majority of cells in the granule and pyramidal cell layers of these hippocampal subregions exhibited intense immunoreactivity when stained with a CREB-specific antibody (Fig. 2D). Comparison of adjacent hippocampal sections that were immunostained with pCREB and CREB antibodies revealed that CREB was strongly phosphorylated in many CREB-ir cells in hippocampal subregions except CA1 under control conditions (data not shown). Taking the possibility of variations in the cell density or cell number in hippocampal sections between the sexes into consideration [29,43], we chose the ratio of the pCREB-ir cell number counted in a given area of a section to the CREB-ir cell number in the corresponding area of the adjacent section (% pCREB-ir cell number), rather than the absolute cell number, as a measure of CREB phosphorylation, to minimize the variations. The range of % pCREB-ir cell number was 75–95% in CA3, CA4, and the dentate gyrus in control male and female rats, while it was less than 20% in CA1 (Fig. 3).

In males, the % pCREB-ir cell number was not affected at 1 h after foot shock in any of the CA3, CA4, and dentate gyrus subregions, but was markedly increased in CA1 (χ^2=13.3, df=3, p=0.004; Figs. 2G and 3, lower panel), while the number of CREB-ir cells exhibited no change after foot shock in any hippocampal subregion (Fig. 3, upper panel). In females, there was no significant change in the numbers of pCREB- and CREB-ir cells in any subregion after foot shock (Fig. 2H), leading to a significantly lower % pCREB-ir cell number in CA1 compared to that in males (p=0.001).

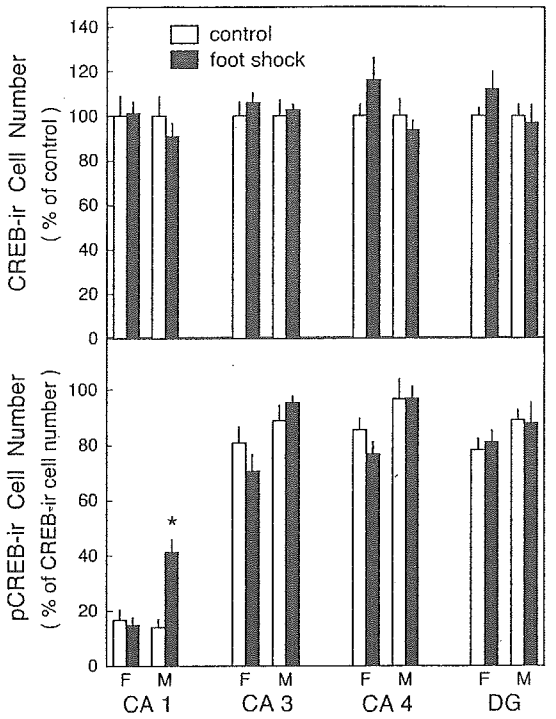

Fig. 3. Numbers of hippocampal CREB- and pCREB-ir cells immediately after contextual fear conditioning in male and randomly cycling female rats. During training, male and female rats received a foot shock (0.75 mA), while control rats received the same training procedures but without the foot shock. One hour after training, they were tested for freezing behavior and then killed for immunohistochemical staining for CREB and pCREB in hippocampal subregions. CREB-ir cell number (upper panel) is defined as the percentage of the CREB-ir cell number in each subregion in control rats. pCREB-ir cell number (lower panel) is defined as the percentage of the pCREB-ir cell number to the CREB-ir cell number counted in adjacent sections in the same subregions. Each bar indicates mean±S.E.M. The numbers of animal are 6 and 5 for control and foot-shocked female groups, and 7 and 7 for control and foot-shocked male groups, respectively. *Significantly different from controls at $p<0.05$. Abbreviations: F, females; M, males; DG, dentate gyrus.

3.2. Time course of freezing and number of hippocampal pCREB-ir cells following contextual fear conditioning in male and female rats

To test whether an increased % pCREB-ir cell number in CA1, as observed 1 h after foot shock in males is maintained thereafter in males and whether it occurs at different time points in females, freezing and the number of hippocampal

pCREB-ir cells were determined at various times after foot shock (each time point group consists of 5–6 animals). Significantly longer freezing was found not only at 1 h but also during a period of 5–24 h after a 0.75-mA foot shock in males compared to that in control males (χ^2=36.8, *df*=7, p<0.0001; Fig. 4, upper left panel). Although % pCREB-ir cell number in CA1 was increased 1 h after foot shock and maintained thereafter at similar levels (χ^2=28.3, *df*=7, p<0.0001), statistically significant differences between the control and trained rats were found only at 1 and 5 h (p=0.002; Fig. 4, lower left panel). In trained females, the slight but significant increase in freezing observed 1 h after foot shock was maintained consistently at all time points of 5–24 h (Fig. 4, upper right panel). Unlike in males, % pCREB-ir cell number in CA1 did not differ between trained and control female rats, at least at the time points examined (Fig. 4, lower right panel). % pCREB-ir cell number in hippocampal subregions other than CA1 did not show any difference between control and trained rats at all time points regardless of the sex (data not shown).

3.3. Effects of increasing foot shock intensity on freezing and number of hippocampal pCREB-ir cells in female rats

Because shorter freezing and the absence of an increase in % pCREB-ir cell number in CA1 following conditioning in females raised the possibility that these sex differences might be due to low sensitivity to foot shock in females, the effects of increased intensity of foot shock on freezing and % pCREB-ir cell number were examined (each intensity group consists of six animals). In females, a foot shock with an intensity of 0.75 mA induced significant freezing 1 h later (p=0.003) as observed in Figs. 1 and 4 (Fig. 5, upper panel). The freezing induced by foot shock tended to increase with an increase in the intensity of foot shock from 0.75 to 1.2 mA, but this rise was not statistically significant (p=0.18). No significant change in % pCREB-ir cell number in CA1 was seen even after increasing the intensity of foot shock (χ^2=1.9, *df*=3, p=0.60; Fig. 5, lower panel).

3.4. Effects of orchidectomy on freezing and number of hippocampal pCREB-ir cells following contextual fear conditioning in male rats

To test whether the male-specific increase in % pCREB-ir cell number in CA1 following conditioning is dependent on circulating testosterone, % pCREB-ir cell number was compared in sham-operated and orchidectomized male rats (each group consists of 6 animals). Orchidectomy affected neither freezing at 1 h after 0.75 mA foot shock (Fig. 6, upper panel) nor % pCREB-ir cell number in all hippocampal subregions including CA1 (Fig. 6, lower panel).

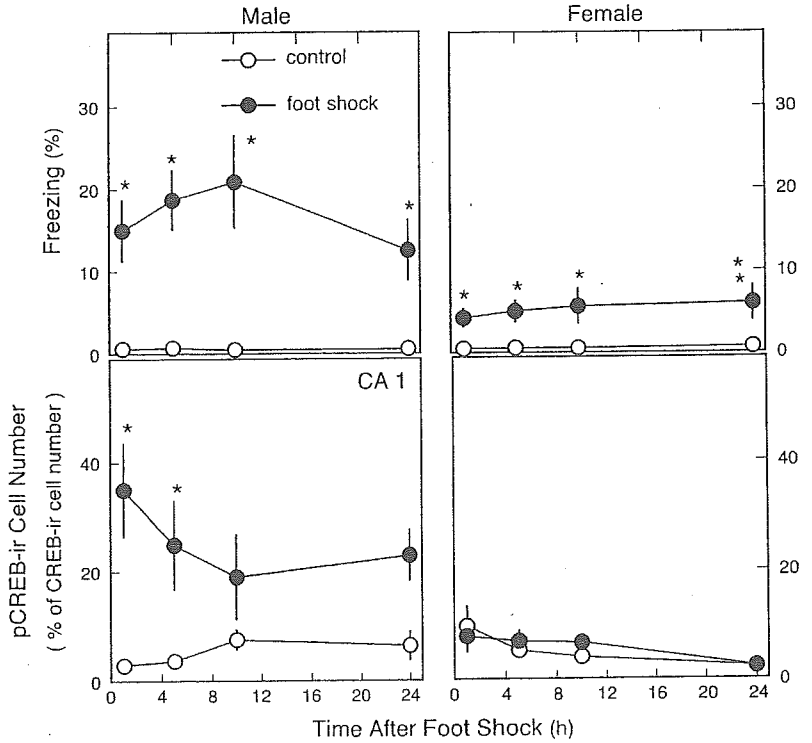

Fig. 4. Time course of freezing and number of CA1 pCREB-ir cells following contextual fear conditioning in male and female rats. Male (left panels) and female rats (right panels) that had received either a foot shock (●; 0.75 mA) or no shock as controls (○) were tested for freezing behavior at various time points after training (upper panels). Immediately after testing, the rats were killed for immunohistochemical staining for pCREB in the CA1 region (lower panels). pCREB-ir cell number is defined in the legend of Fig. 3. Each bar indicates mean±S.E.M. of 5–6 animals. *Significantly different from controls without foot shock at p<0.05.

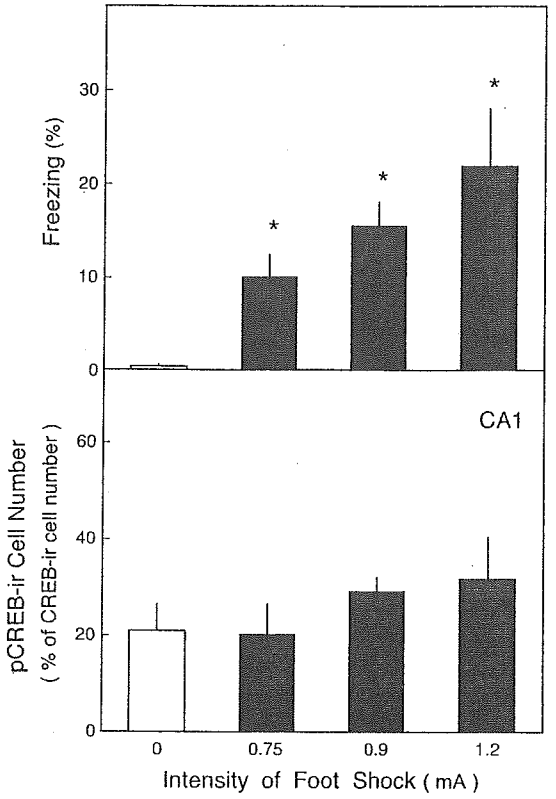

Fig. 5. Effects of increasing foot shock intensity on freezing and number of CA1 pCREB-ir cells in female rats. Female rats that had received a foot shock of various intensity were tested for freezing behavior 1 h after training (upper panel). Immediately after testing, the rats were killed for immunohistochemical staining for pCREB in CA1 (lower panel). pCREB-ir cell number is defined in the legend of Fig. 3. Each bar indicates mean±S.E.M. of six animals. *Significantly different from controls without foot shock at $p<0.05$.

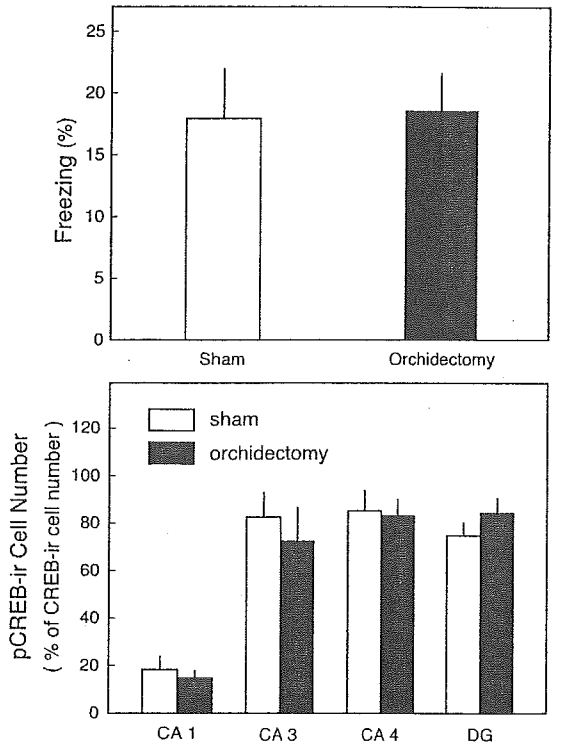

Fig. 6. Effects of orchidectomy on freezing and number of hippocampal pCREB-ir cells following contextual fear conditioning in male rats. Sham-operated and orchidectomized male rats that had received a foot shock (0.75 mA) were tested for freezing behavior 1 h after training (upper panel). Immediately after testing, the rats were killed for immunohistochemical staining for pCREB in hippocampal subregions (lower panel). pCREB-ir cell number is defined in the legend of Fig. 3. Each bar indicates mean±S.E.M. of six animals.

3.5. Step-through latency and number of hippocampal pCREB-ir cells after passive avoidance conditioning in male and female rats

To extend our results of the sex differences obtained in experiments using contextual fear conditioning, behavioral performance and % pCREB-ir cell number in hippocampal subregions were examined in males and females trained for passive avoidance conditioning, another hippocampal-dependent conditioning paradigm that shows a sex difference [16,52]. Control male (n=6) and female rats (n=6) stepped through to the dark section of the conditioning chamber during testing, with similar low step-through latencies (Fig. 7). A foot shock of 0.6 mA increased the step-through latency 12-fold above the control level 30 min later in male rats (n=7, χ^2=12.3, df=3, p=0.006), while there was no significant increase in this measure in female rats (n=7). Male and female control rats exhibited similar % pCREB-ir cell numbers in all hippocampal subregions except CA1, in which % pCREB-ir cell number was significantly higher in males than in females (χ^2=19.1,

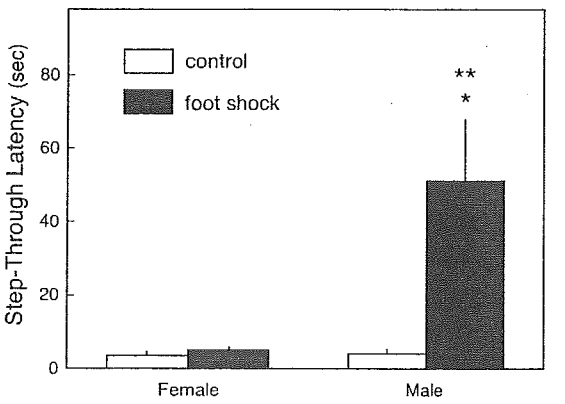

Fig. 7. Step-through latency after passive avoidance conditioning in male and female rats. During training, male and female rats received a foot shock (0.6 mA) in the dark section of a conditioning chamber while control rats received the same training procedures but without the foot shock. Thirty minutes after training, they were tested for stepping through to the dark section. Each bar indicates mean±S.E.M. The numbers of animals are 6 and 7 for control and foot-shocked female groups, and 6 and 7 for control and foot-shocked male groups, respectively. *Significantly different from foot-shocked females; **significantly different from controls at $p<0.05$.

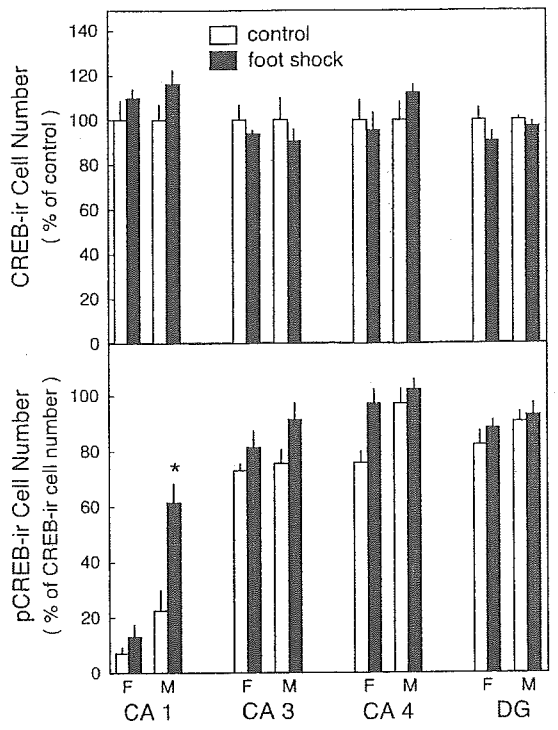

Fig. 8. Numbers of hippocampal CREB- and pCREB-ir cells after passive avoidance conditioning in male and randomly cycling female rats. During training, male and female rats received a foot shock (0.6 mA) in the dark section of a conditioning chamber while control rats received the same training procedures but without the foot shock. Thirty minutes after training, they were tested for stepping through to the dark section and killed for immunohistochemical staining for CREB (upper panel) and pCREB (lower panel) in hippocampal subregions. pCREB-ir cell number is defined in the legend of Fig. 3. Each bar indicates mean±S.E.M. The numbers of animals are 6 and 7 for control and foot-shocked female groups, and 6 and 7 for control and foot-shocked male groups, respectively. *Significantly different from controls at $p<0.05$. Abbreviations: F, females; M, males; DG, dentate gyrus.

df=3, $p<0.0001$; Fig. 8, lower panel). After passive avoidance conditioning, there was a marked sex difference in % pCREB-ir cell number in the hippocampus, which was virtually the same as that observed in contextual fear conditioning (χ^2=19.4, df=3, $p<0.0001$): % pCREB-ir cell number rose 2.7-fold above the control level only in CA1 in trained male rats (p=0.001), while there was no change in % pCREB-ir cell number in any hippocampal subregion in trained female rats. There was no change in number of CREB-ir cells in any of the hippocampal regions following conditioning (Fig. 8, upper panel).

4. Discussion

To determine dynamic spatiotemporal changes in hippocampal CREB activation following conditioning, we assessed the number of pCREB-ir neurons in frozen sections immunohistochemically using a phospho-specific CREB antibody. Our immunohistochemical analysis revealed that in control rats that received no foot shock, most of the CREB-ir neurons were also immunoreactive for pCREB in all hippocampal subregions and many other brain areas examined except CA1. It remains to be elucidated how the notably low pCREB levels in CA1 under basal conditions are related to a selective increase in pCREB following conditioning in this area (discussed below) and the acquisition and consolidation of memory. Following both tasks of contextual fear and passive avoidance conditioning, the number of pCREB-ir neurons was increased selectively in CA1 but not in other hippocampal subregions CA3, CA4, and the dentate gyrus in male rats. There was no change in the number of CREB-ir cells in CA1, suggesting that the increase in pCREB-ir cell number is due to increased phosphorylation of CREB. Our results on the hippocampal distribution of pCREB-ir neurons in males following conditioning are consistent with those by Impey et al. [25], showing that CRE-lacZ gene expression was increased in CA1 and CA3 following contextual fear and passive avoidance conditioning, while an increase in pCREB was found specifically in CA1 after contextual fear conditioning in mice. However, Taubenfeld et al. [50] reported that, in male rats, passive avoidance conditioning induced an increase in pCREB-ir neurons in the dentate gyrus and CA3 in addition to CA1. The discrepancy in the distribution of pCREB-ir neurons between their studies and ours is difficult to explain at present. We chose to use freshly frozen brains for immunostaining because a preliminary experiment has shown a marked reduction of pCREB immunoreactivity in the dentate gyrus and CA regions by transcardiac perfusion with a fixative under anesthesia. Such rapid dephosphorylation of pCREB in the hippocampus might have caused a difference in the detectable amount of pCREB in different histological preparations. Our results do not completely exclude the possibility of a simultaneous increase in pCREB in other areas of the hippocampus after conditioning in male rats. In contrast to CA1, in which undetectable pCREB levels in most cells before conditioning make it easy to detect an increase in pCREB after conditioning, a semiquantitative histochemical analysis based on a parameter of the immunoreactive cell number may mask an additional increase in amounts of pCREB after conditioning in the dentate gyrus and CA3, in which most cells are immunoreactive for pCREB before conditioning. Although the distribution of pCREB-ir neurons in the male hippocampus after conditioning differs somewhat between these two studies, the results in these studies that an increase in pCREB was consistently found in CA1 after both conditioning paradigms emphasize an important role of phosphorylation and activation of CREB in CA1 in emotional learning and memory in males. The specific importance of CA1 in the conditioning paradigms used in the present study has been demonstrated by several studies. Passive avoidance conditioning is impaired in carbon monoxide-induced amnesia [36] and transient ischemia models [4] which selectively damage neurons in

CA1. Furthermore, contextual fear conditioning has recently been shown to be disrupted in CA1-specific *N*-methyl-D-aspartate receptor1-knockout mice [42].

For contextual fear conditioning, males rats exhibited more freezing behavior both at 1 h and later time points 5–24 h after conditioning than did female rats. Because conditioned responses tested at these time points reflect short- and long-term memories, respectively, these results suggest that there is a sex difference in short-term memory obtained by contextual fear conditioning in addition to that in long-term memory as shown by other studies [2,30]. Consistent with this view is our result that step-through latencies, as early as 30 min after passive avoidance conditioning, were higher in males than in females. In the present study, CA1, the sole hippocampal subregion in which the number of pCREB-ir neurons was increased after the two conditioning paradigms in males, also exhibited marked sex differences in CREB phosphorylation; in females, the number of pCREB-ir neurons in this area was not altered at any time after conditioning or by any increase in intensity of foot shock. The positive correlation between pCREB-ir neurons in CA1 and behavioral performance in males and females suggests that a difference in CREB activation is involved in the sex differences in contextual fear and passive avoidance conditioning. Although it is evident that there is a sex difference at the level of CREB phosphorylation itself, it is unknown at present whether a sex difference at some level upstream of CREB is responsible for the difference in CREB phosphorylation. Because calcium–calmodulin-dependent protein kinase II [46] and the mitogen-activated protein kinase cascade [54] regulate the activity of CREB and have been implicated in hippocampal-dependent learning and memory [6,47], the function and regulation of these CREB-upstream regulators in CA1 neurons may differ between the sexes, leading to the sex difference in CREB phosphorylation. Alternatively, a sex difference may exist in neurons that send nerve fibers directly to CA1 neurons such as hippocampal intrinsic neurons in the dentate gyrus and CA3 or in extrinsic neurons, leading to a transsynaptic modification of CREB phosphorylation in CA1. Indeed, consistent with this idea is the finding of Maren et al. [30] that the sex difference in contextual fear conditioning was correlated with that in LTP recorded in the dentate gyrus. Furthermore, neuroanatomical studies have demonstrated that sex differences exist in dendritic density, synaptic connectivity, cell number, and cell layer width in hippocampal subregions other than CA1 [26,29,33,43]. Thus, it remains to be clarified whether there is a sex difference at levels upstream of CREB and what upstream signaling molecules or neurons are involved in the sex differences in behavioral performance.

We postulate that CREB activation and phosphorylation found within 1 h after training contribute to freezing behavior occurring at later times; based on the findings by Bourtchaladze et al. [10] that in mice with targeted disruption of CREB, freezing was decreased at 24 h but not at 30 min after training. This is consistent with the time-dependent effect of protein synthesis blockers; treatment with the blockers 30 min before or immediately after training suppresses freezing at 24 h, but the blockers are not effective when given at 1 h after training, suggesting that only a single short wave of protein synthesis during or immediately after training is required for contextual fear conditioning over 24 h [1]. There are many proteins whose expressions are directed by the CREB–CRE transcriptional pathway and that have been implicated in the formation of multiple types of memory. One of the best studied molecules that likely mediate the CREB action is BDNF, which has been shown to be involved in synaptic plasticity and long-term memory formation as well as the differentiation and survival of neurons [41]. In the CA1 region, LTP is enhanced by BDNF [17], reduced by BDNF antibody [12] or targeted deletion of the *BDNF* gene [28], and associated with increased expression of BDNF mRNA [37]. Recently, rapid and selective induction of BDNF expression has been shown to occur in CA1 after contextual fear conditioning [22]. In this regard, it would be of interest to determine whether males and females exhibit a difference in BDNF expression in CA1 after the conditioning paradigms used in the present study.

Although we demonstrated a close relationship between sex differences in behavioral performance and CA1 pCREB level, the exact neural role of CREB phosphorylation in CA1 in the sexually dimorphic conditioning tasks remains unknown. Although the role of the hippocampus in contextual fear conditioning has been poorly understood and controversial [48], Anagnostaras et al. [3] have proposed that its specific role is the construction and temporary maintenance of a unified representation of a contextual CS, rather than the CS–US association or shock US representation. On the other hand, CREB has been shown to be involved in a variety of learning paradigms in a wide range of species [10,14,56] and in LTP [10,45]. Taking these results together, we prefer to postulate that the sex difference in CREB phosphorylation in CA1 reflects a difference in the learning process itself. This idea is supported by the fact that similar changes in CREB phosphorylation were observed in CA1 following two different learning paradigms of contextual fear and passive avoidance conditioning. However, our results, nonetheless, may be open to an alternative interpretation supported by several lines of evidence. First, at least with regard to passive avoidance conditioning, it has been suggested that the sex difference in performance is attributable not to differences in learning capacity [51], but to differences in locomotor activity between the sexes [23]; females are generally more active than males in open-field tests [7], and the open-field activity is reduced to a greater extent in males than in females after foot shock [23], raising the possibility that the reduced locomotor activity leads to a greater step-through latency in passive avoidance conditioning in males. Second, based on the findings that hippocampal lesions caused increased locomotor activity [9,20], which might interfere with freezing, and disrupted fear-conditioned

freezing but not fear-potentiated startle [34], it has been proposed that the hippocampus is merely involved in behavioral inhibition but is not essential for contextual fear itself. Third, Archer [5] has suggested that male and female rats exhibit different responses to fear stimuli, with males tending to perform an inactive response, e.g., freezing, and females tending to perform an active response, e.g., escaping. Taking these results together, it cannot be completely excluded that the sex difference in pCREB in CA1 observed in the present study reflects a sex difference in the hippocampal dependency of different conditional responses that males and females exhibit. Further studies are needed to determine whether, in females, CREB is activated by conditioning in another brain area such as the amygdala, a major neural structure that is involved in contextual fear conditioning.

Anagnostaras et al. [2] reported that orchidectomy had no effect on the performance of contextual fear conditioning in males. In agreement with their results, we found that orchidectomy affected neither the performance of contextual fear conditioning nor pCREB-ir cell number in CA1 following conditioning in males. These results indicate that the male-specific increase in the number of CA1 pCREB-ir cells following contextual fear conditioning is independent of the activational action of steroid hormones secreted from the testis in adulthood. In females, the performance of contextual fear conditioning is altered during the estrous cycle and by ovariectomy [21,32]. Furthermore, hippocampal LTP, which is also accompanied by changes in CREB phosphorylation [45], is modulated by estrogen [13,18,21]. Despite the prominent effects of estrogen shown in these studies, the present study demonstrates a significant difference in conditioning performance between randomly cycling females and males, suggesting that the estrous cycle-associated changes in performance in females are less marked than its sex difference. The mechanism of the formation of the sex differences in contextual fear conditioning and CREB phosphorylation in CA1 remains to be clarified. Because many of the sex differences in other hippocampal-dependent learning paradigms are determined by perinatal testosterone secreted from the testis [26,43,52], it seems likely that the neural structure underlying the sex differences in contextual fear conditioning and CREB phosphorylation undergoes sexual differentiation under the organizational action of testosterone early in development. This idea is supported by the neuroanatomical finding that treatment of neonatal females with testosterone masculinized the CA1 pyramidal cell field volume and neuronal soma size [26].

Acknowledgements

This work was supported in part by the Ministry of Education, Science, and Culture of Japan (Grant-in-Aid for Scientific Research, 15590206).

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Ginseng enhances contextual fear conditioning and neurogenesis in rats

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Received 18 March 2004; accepted 9 September 2004
Available online 6 November 2004

Abstract

Panax Ginseng is a commonly used galenical known to have an enhancing effect on learning. Neurogenesis in the hippocampus has been shown to be necessary for hippocampus/amygdala-dependent learning tasks. To investigate the role of Ginseng in neurogenesis and learning of rats, we administered both Ginseng and BrdU for five consecutive days. As a result, Ginseng increased the number of BrdU-positive cells in the dentate gyrus in a dose-dependent manner. Further, we administered one dose of BrdU after Ginseng treatment for five consecutive days, and the number of BrdU-positive cells did not increase significantly. However, when one dose of BrdU was given 1 day before the following five consecutive days of Ginseng treatment, the number of BrdU-positive cells markedly increased in the hippocampus. Therefore, it is likely that Ginseng enhances not proliferation but survival of newly generated neurons in the hippocampus. Second, we administered both Ginseng and BrdU to rats for five consecutive days. One day after the last Ginseng and BrdU co-administration, contextual fear conditioning (CFC) was conducted. Ginseng in a dose-dependent manner increased the % freezing time and the number of BrdU-positive cells in the dentate gyrus of rats that received CFC. Thus, an increase in CFC-related neurogenesis may be one mechanism of Ginseng's properties to enhance learning ability.

Keywords: Ginseng; Neurogenesis; Learning; Hippocampus; Dentate gyrus; Contextual fear conditioning

1. Introduction

The Ginseng root (Panax Ginseng) is a common constituent of a large number of traditional oriental medicines. Among its diverse effects on the central nervous system, Ginseng is known to improve learning and memory. Although some of the early studies reported that Ginseng extracts caused learning impairment rather than improvement (Saito et al., 1977, 1979), subsequent studies showed that Ginseng extracts improve performance in active and passive avoidance learning tasks (Lasarova et al., 1987; Petkov et al., 1990, 1992, 1993). This discrepancy may be due to the sedative effect of Ginseng (Koo, 1999), which is observed with acute administration of Ginseng. Those that reported memory impairment by Ginseng examined the acute, but not chronic effects of Ginseng.

Chronic administration of Ginseng extracts or some of its fractions is known to improve learning and memory in several different hippocampus/amygdala-dependent behavioral tasks (Chang et al., 1998; Jaenicke et al., 1991; Jin et al., 1999; Lyubimov et al., 1997; Ni et al., 1993; Nitta et al., 1995a, 1995b; Watanabe et al., 1990; Wen et al., 1996; Yoshimura et al., 1998; Zhao and McDaniel, 1998; Zhong et al., 1998). However, the molecular and cellular mechanisms by which these agents exert behavioral effects remain to be explored.

In recent years, neurogenesis in the subgranular layer of the hippocampus (Gould et al., 1999b) and subsequent

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0168-0102/$ – see front matter © 2004 Published by Elsevier Ireland Ltd and the Japan Neuroscience Society.
doi:10.1016/j.neures.2004.09.004

enhancement of neurotract connections (Nakagawa et al., 2002b) have drawn attention as one of the molecular biological mechanisms of hippocampus/amygdala-dependent learning. The hippocampus is one of few brain regions where production of neurons occurs throughout the lifetime of animals, including humans. Gould et al. (1999a) reported that improvement of a hippocampus-dependent task, such as a spatial learning test, was associated with increase in survival of bromodeoxyuridine (BrdU)-positive cells in rats.

To clarify the mechanism of learning enhancement by Ginseng, we investigated the effects of Ginseng on contextual fear conditioning (CFC), a hippocampus/amygdala-dependent learning task, and on neurogenesis in the hippocampus of rats. Prior to this investigation, we examined the effect of Ginseng on the baseline number of BrdU-positive cells in the hippocampus. For analysis of the phenotype of BrdU-positive cells, double staining with BrdU, a thymidine analog that labels dividing cells in S-phase, and NeuN, a neuronal marker, was used.

2. Materials and methods

2.1. Animals and Ginseng treatment

All experiments were conducted using 60 adult male Wister/ST rats (SLC Japan, 9 weeks old, weighing 270–290 g). They were group-housed (5 rats/group, 12 h light/dark cycle) with ad libitum access to food and water. The animals were treated in accordance with the Guidelines for Animal Experimentation (of the Ethics Review Committee) of the Faculty of Medicine, University of Yamanashi.

For the first experiment, Ginseng powder (supplied by Tsumura Pharmaceutical Co., Tokyo, Japan) was administered orally at doses of 0, 100, and 200 mg/kg/day for five successive days via a gastric tube. Dried Ginseng powder was suspended in tap water just before the use. The group denoted as 0 mg/kg/day received water only. Although in some previous reports Ginseng was administered by intraperitoneal (Mook-Jung et al., 2001) or intracerebroventricular injection (Kim et al., 1998), we considered that oral administration of Ginseng was crucial, because in traditional medicine Ginseng has always been taken orally. In addition, no data are available regarding the disposition of Ginseng in the body, such as absorption and metabolism, and more importantly, biologically active forms are not well identified. Some herbs are known to be metabolized into active forms by intestinal bacteria and then absorbed to exert pharmacological actions (Hasegawa et al., 1996). Thus, non-oral administration of Ginseng may result in non-pharmacological artifacts.

2.2. Contextual fear conditioning

CFC was conducted according to the method of Silva et al. (1998). The CFC task was performed in a conditioning chamber housed in a sound-attenuating box during the light phase of the cycle. The conditioning chamber (28 cm (width) × 21 cm (height) × 22 cm (diameter)) was constructed of clear Plexiglas. The floor of the chamber was lined with 18 stainless steel bars (4 mm in diameter; 1.5 cm spacing), which formed a foot shock grid to deliver scrambled shocks produced by a stimulator. The foot shock was 2 s direct current of 0.75 mA and served as the aversive unconditioned stimulus. The sound-attenuating box (48 cm (width) × 48 cm (height) × 48 cm (diameter)) was provided with a 20 W houselight, and a ventilation fan supplying background white noise (74 dB) was located on the top of the box. A discrete tone conditioned stimulus (CS) was given as a general contextual stimulus. The tone cue (800 Hz, 20 s duration, 80 dB) was delivered by two speakers located in the lower corner of the sound-attenuating box.

Prior to the conditioning, all rats received 3 days of habituation, in which they were placed in the conditioning chamber for 1 min and returned to their home cages once a day. On the day of conditioning, the rats were placed in the conditioning chamber and allowed to explore for 3 min. A foot shock was delivered 18 s after the tone CS. The rats were then allowed to recover for 30 s in the conditioning chamber and returned to their home cages. Two hours later, the rats were again introduced into the conditioning chamber and were tested for a 5 min period, during which no tone CS was delivered. Behavior was evaluated in terms of total freezing time during a 5 min (% freezing time) stay in the conditioning chamber. Freezing behavior was defined as cessation of all but respiratory movement. The rats were sacrificed immediately after % freezing time was measured.

Rats that experienced footshock were allocated to the CFC group and rats that did not experience footshock to the no-CFC group. There were three subgroups in the CFC and no-CFC groups, each of which was treated with 0, 100, or 200 mg/kg/day Ginseng powder. There were five rats in each subgroup.

2.3. Open field locomotion test

To test whether % freezing time was influenced by the sedative effect of Ginseng or not, the open field locomotion test was performed at the end of the treatment period on the sixth day. This test was performed on the rats that were administered 0 or 200 mg/kg/day of Ginseng.

The open field is a 750 mm× 750 mm wooden arena, with 300 mm high walls surrounding the field, painted black on all inner surfaces. Thin white stripes are painted across the floor, dividing it into 25 quadratic blocks. The open field instrument was cleaned after each individual test session to prevent the next rat from being influenced by the odors deposited in the urine and feces of the previous rat.

The rat was placed in the area with its head pointing to a corner. An observer manually quantified the rat's spontaneous ambulatory locomotion in the horizontal plane by

scoring the number of squares entered (crossing into a different adjacent section with all four extremities), during 5 min.

2.4. Immunostaining

On the sixth day, immediately after CFC performance, the rats were deeply anesthetized with sodium pentobarbital (50 mg/kg ip) and perfused transcardially with 350 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. The rat brain was quickly removed, and then post-fixed for 24 h in paraformaldehyde. Then, 40 μm-thick frontal sections were cut on a cryostat and collected in PBS (0.1 M; pH 7.4). In accordance with the rat brain map of Paxinos and Watson (1986), 40 μm-thick free-floating sections were prepared, and 12 sections were collected at 160 μm intervals for staining. DNA denaturation was conducted by incubation for 30 min in 50% formamide/2 × SSC at 65 °C followed by several rinses in 2 × SSC. Sections were then incubated for 30 min in 2N HCl and 10 min in boric acid. After washing in PBS, sections were incubated in 3% H_2O_2 to block endogenous peroxidase for 10 min. After blocking with 10% normal goat serum (NGS), sections were incubated with anti-BrdU (1:1000 Haralan Sera Lab. OBT0030) for 24 h at 4 °C. Sections were then incubated for 1 h with secondary antibody (biotinylated goat, anti-rat IgG; Vector BA9400) followed by amplification with an avidin–biotin complex, before developing the color using DAB.

The same number of free-floating sections from the control and the Ginseng (200 mg/kg/day for 5 days)-treated animals ($n = 6$) surviving 4 weeks after administration of one dose of BrdU before Ginseng treatment was used for analysis of phenotypes. Double immunostaining procedures with fluorescent chromogens were used to evaluate the co-expression of BrdU with neuronal nuclear protein NeuN. In the double-labeling experiment, BrdU was visualized with Streptavidin-Texas Red (Amersham Pharmacia Biotech, 1:100), while the neuronal marker NeuN (Chemicon International Inc., MAB377; 1:500) was visualized with FITC (anti-mouse Ig, fluorescein-linked whole antibody 1:20). Fluorescent signals were viewed using a TCS4D confocal laser-scanning microscope. The emission signals of Texas Red and FITC were assigned to red and green, respectively.

2.5. Quantification of BrdU labeling

Every fourth section throughout the hippocampus was processed for BrdU immunohistochemical study. All BrdU-labeled cells in the dentate gyrus (granular cell layer) and the hilus were counted in each section. To distinguish single cells within clusters, all counts were performed at 400× and 1000× magnification under a light microscope (Olympus BX-60), omitting cells in the outermost focal plane.

A cell was counted as being in the subgranular zone (SGZ) of the dentate gyrus if it was touching or in the SGZ. Cells that were located more than two cells away from the SGZ were classified as hilar cells. The cell number was divided by the area of the dentate gyrus, and then the mean positive cell number per square millimeter was counted.

2.6. Protocol of BrdU administration

To investigate the overall effects of Ginseng on neurogenesis in the dentate gyrus of rats and its association with CFC performance, rats were orally administered both Ginseng (0, 100, 200 mg/kg/day) and BrdU (200 mg/kg/day) simultaneously for five consecutive days. On the sixth day, the animals were subjected to CFC test and then sacrificed for immunohistochemical study as described above (Fig. 1A).

BrdU is taken up into cells that are in the S-phase of DNA synthesis. The S-phase lasts for approximately 2 h (Packard et al., 1973). When one dose of BrdU is given, it is incorporated only into cells in the S-phase. At least one cell cycle is completed in 24 h by cells in the S-phase after the time of BrdU injection (Nowakowski et al., 1989). Therefore, if an animal is given one dose of BrdU and decapitated 2 h later for immunostaining, cells that have newly proliferated are observed. On the contrary, if an animal is given one dose of BrdU and is decapitated a few days later, cells that were produced a few days ago and are still surviving at the time of decapitation are observed. Accordingly, it is possible to differentiate newly proliferated cells from surviving cells by varying the time interval between BrdU administration and decapitation.

In our study, to determine the effects of Ginseng on cell survival, BrdU was given once to drug-naive rats, and Ginseng was given for the following 5 days. Then, the rats were sacrificed for immunohistochemical study (Fig. 1B). On the other hand, to determine the effects of Ginseng on cell proliferation, BrdU was given once on the sixth day, after Ginseng treatment for five consecutive days, and sacrificed 2 h after BrdU labeling (Fig. 1C).

2.7. Statistical analysis

All the results are presented as mean ± S.E.M. One-way analysis of variance (ANOVA) was used to examine the effects of CFC and Ginseng administration on % freezing time because of their interaction, since there was interaction between the two groups (Fig. 6). Two-way analysis of variance (ANOVA) was used to examine the effect of CFC and Ginseng on number of BrdU-positive cells in rats subjected to CFC (Fig. 2). As shown in Fig. 5, Student's *t*-test was used to determine whether the effect of Ginseng treatment on the number of BrdU-positive cells is related to cell proliferation or cell survival. Differences in the values of locomotion measured in the open field test were examined by Student's *t*-test.

A P-value < 0.05 was considered to indicate a statistically significant difference.

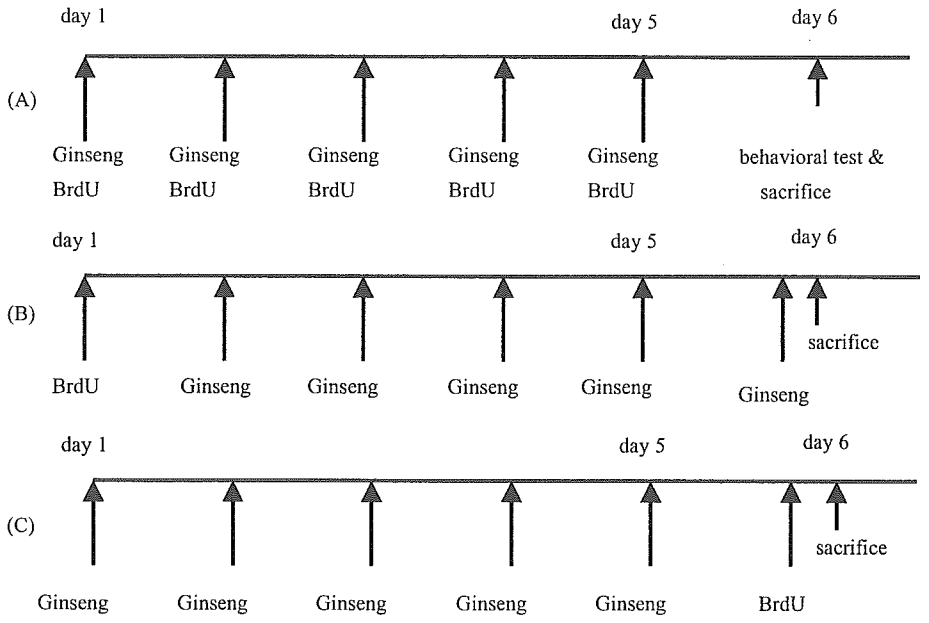

Fig. 1. Experimental procedures to examine effects of Ginseng on CFC-associated neurogenesis, cell survival and cell proliferation in rat hippocampus. Rats were orally administered both Ginseng (0, 100, 200 mg/kg/day) and BrdU (200 mg/kg/day) for five consecutive days. On the sixth day, the animals were subjected to CFC test (A). To determine the effects of Ginseng on cell survival, BrdU was given once to drug-naive rats, and Ginseng was given for the following 5 days. (B) To determine the effects of Ginseng on cell proliferation, BrdU was given once on the sixth day, after Ginseng treatment for five consecutive days, and sacrificed 2 h after BrdU labeling (C).

3. Results

3.1. Effect of Ginseng on baseline number of BrdU-positive cells

To test the effect of Ginseng on neurogenesis in rats, we counted the number of BrdU-positive cells in the dentate gyrus. The number of BrdU-positive cells increased in a dose-dependent manner in the Ginseng treatment group (Figs. 2A and 3).

3.2. Phenotype of BrdU-positive cells

The phenotype of the BrdU-positive cells in the granule cell layer was examined in the Ginseng group 4 weeks after BrdU labeling. We performed immunostaining for BrdU as

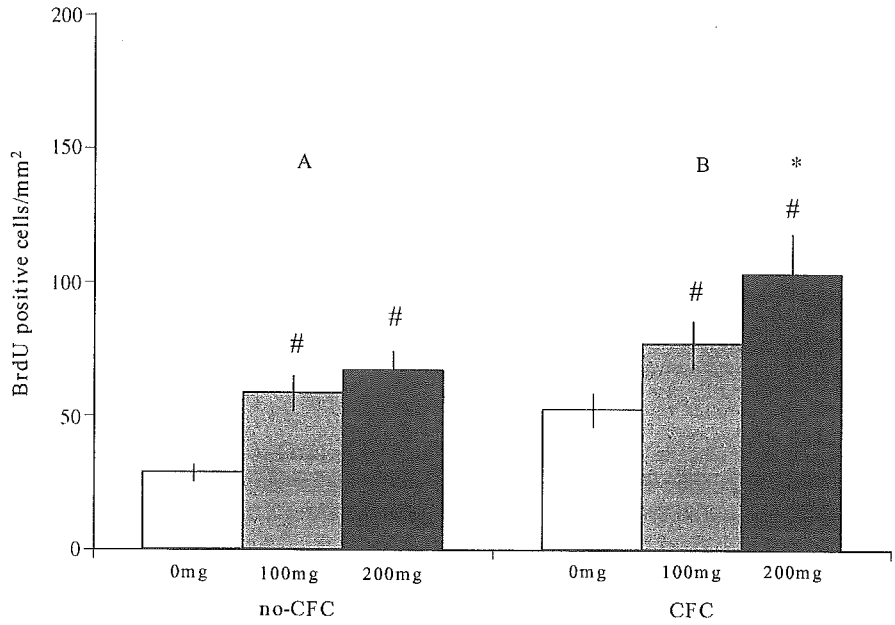

Fig. 2. Change in BrdU-positive cells in rat hippocampus after CFC performance. The number of BrdU-positive cells was higher in the CFC groups than in the no-CFC groups ($P = 0.0113$, $F_{1,24} = 13.718$). The number of BrdU-positive cells was significantly increased in a dose-dependent manner by Ginseng treatment, both in the no-CFC groups (A) and the CFC groups (B) ($P = 0.0001$, $F_{2,24} = 13.538$) (mean ± S.E.M.). (#) $P < 0.05$ vs. 0 mg; (*) $P < 0.05$ for no-CFC vs. CFC.

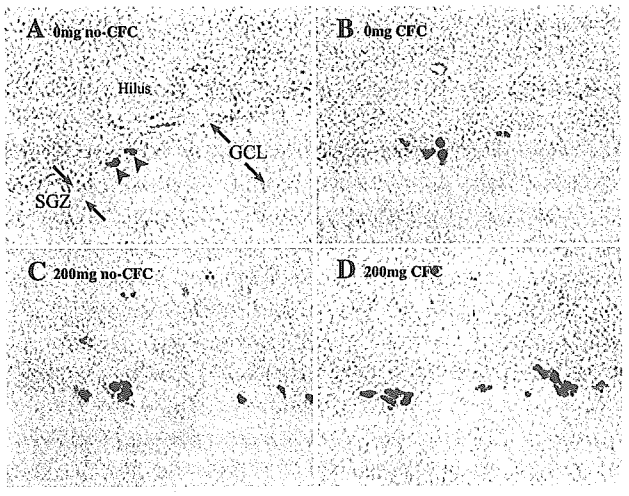

Fig. 3. Optical photomicrographs of changes in number of BrdU-positive cells in the hippocampus after CFC (40× magnification) BrdU-positive cells (arrowhead) were increased in the CFC groups (B and D) compared to the no-CFC groups (A and C). BrdU-positive cells were increased in a dose-dependent manner by Ginseng, in both the CFC groups and no-CFC groups. (A) Ginseng 0 mg and no-CFC; (B) Ginseng 0 mg and CFC; (C) Ginseng 200 mg and no-CFC; (D) Ginseng 200 mg and CFC. The majority of BrdU-positive cells were located in the subgranular zone (SGZ) of the hippocampus—the region between the granular cells layer (GCL) and hilus.

well as for NeuN, a marker for neurons (Fig. 4). It was found that ~80% of BrdU-positive cells expressed NeuN, and no significant difference existed in % NeuN-positive cells regardless of whether Ginseng was administered or not (data not shown).

3.3. Effect of Ginseng on cell survival

The increased neurogenesis may have been due to an increase in cell survival and/or cell proliferation. To investigate the effect of Ginseng specifically on cell survival, one dose of BrdU was given orally to the rats, followed by 5 days' administration of Ginseng (200 mg/kg/day). As a result, Ginseng did significantly increase the number of BrdU-positive cells. This finding showed that Ginseng enhanced the survival rate of newly generated cells in the hippocampus (see Fig. 5A).

3.4. Effect of Ginseng on cell proliferation

On the other hand, in order to investigate the effect of Ginseng on cell proliferation, Ginseng (200 mg/kg/day) was given for 5 days, and then one dose of BrdU was given to the rats 2 h before they were sacrificed for immunostaining. With this administration protocol, the number of BrdU-positive cells did not increase significantly in the dentate gyrus. This result suggested that the Ginseng employed in our experiment did not induce a significant change in cell proliferation in the hippocampus (see Fig. 5B).

3.5. Effect of Ginseng on % freezing time in CFC test and CFC-associated increase in BrdU-positive cells

As shown in Fig. 6A, in the rats that were not given foot shocks in the conditioning chamber (no-CFC group), Ginseng administration did not change % freezing time at any dose. CFC itself increased % freezing time. In the rat groups that received CFC (CFC groups), Ginseng treatment increased % freezing time in a dose-dependent manner (see Fig. 6B). Ginseng at 200 mg/kg significantly increased % freezing time as compared to Ginseng at 0 or 100 mg/kg. This result shows that Ginseng significantly enhances the performance of rats in the CFC, a hippocampus/amygdala-dependent learning task.

When Ginseng and BrdU were co-administered for five consecutive days, the number of BrdU-positive cells increased in a dose-dependent manner in the CFC groups, and the increase was significant at a dose of Ginseng of 200 mg/kg as compared to doses of 0 and 100 mg/kg (Figs. 2B and 3). CFC also increased the number of BrdU-positive cells as compared to the no-CFC groups, even when saline was co-administered with BrdU for 5 days (Figs. 2 and 3).

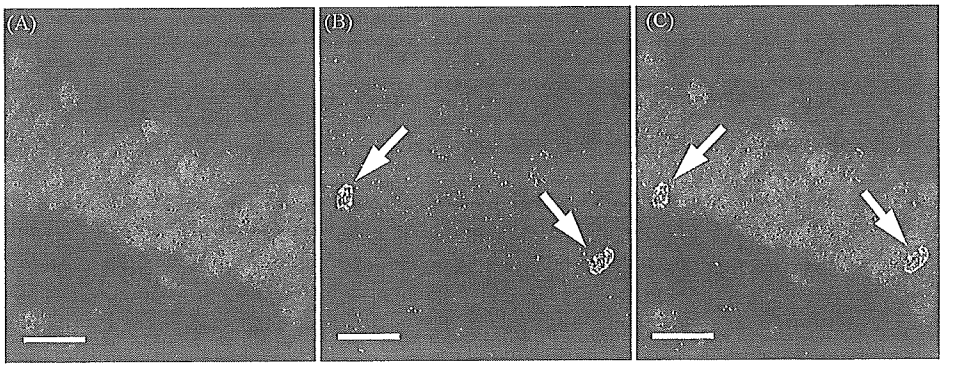

Fig. 4. Double immunolabeling of NeuN and BrdU in adult dentate gyrus of Ginseng-treated rats. Double immunolabeling of NeuN (green, A), a marker of mature neurons, and BrdU (red, B) in the adult dentate gyrus of Ginseng-treated rats surviving 4 weeks after BrdU administration. Confocal images (630× magnification) show NeuN immunoreactivity in BrdU-labeled cells (arrows, C) in both Ginseng-treated and non-treated animals. Scale bar: 20 μm.

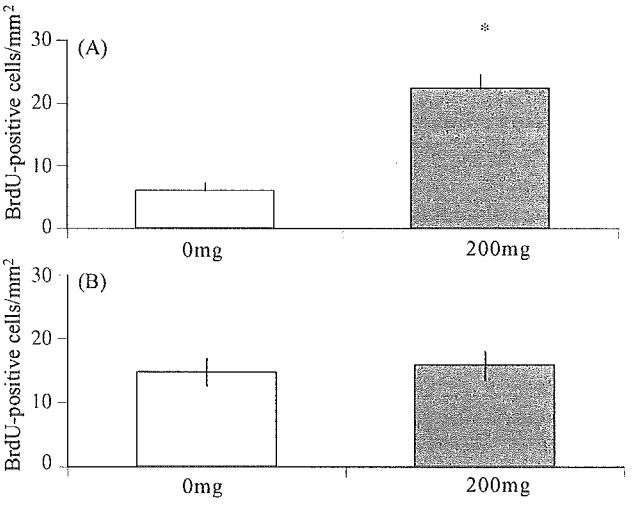

Fig. 5. Effects of Ginseng on BrdU-positive cells in rat hippocampus administration of Ginseng for five consecutive days after one treatment with BrdU significantly increased BrdU-positive cells compared to control rats ($n = 5$, $^{*}P = 0.0003$) (A). Administration of Ginseng for five consecutive days followed by one treatment with BrdU 24 h later caused no significant change in BrdU-positive cells compared to control rats ($n = 5$, $P = 0.7341$) (B).

3.6. *Effect of Ginseng on open field locomotion*

To examine whether % freezing time was influenced by the sedative effect of Ginseng, the open field test was performed. Total locomotion distance of the group treated with Ginseng 200 mg/kg/day for five consecutive days was not significantly different compared to the no Ginseng group (data not shown). There was no significant difference in grooming and rearing either (data not shown). This result indicates that Ginseng at 200 mg/kg did not cause any difference in spontaneous activity or locomotion compared with the non-treated rats.

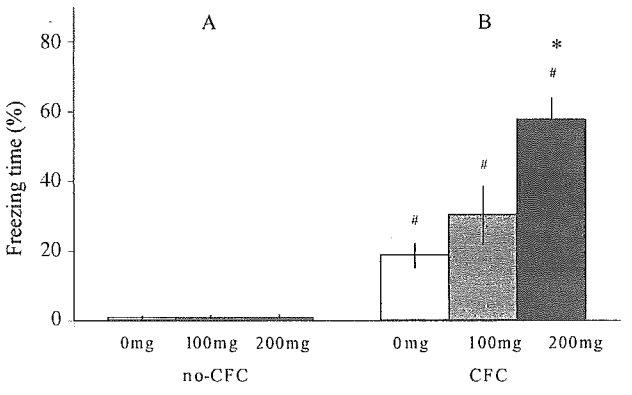

Fig. 6. Effects of Ginseng on % freezing time of rats tested in a conditioning chamber 1 day after finishing administration of Ginseng with BrdU for five consecutive days. Data are shown as mean ± S.E.M. There was a significant difference in % freezing time among the tested groups ($F_{5,24} = 25.312$, $P < 0.007$). (#) The 0, 100, and 200 mg Ginseng groups showed a significantly higher % freezing time in the CFC group than the no-CFC group, respectively. (*) The 200 mg/kg Ginseng group showed the highest % freezing time out of all the CFC groups (B). In contrast, the no-CFC groups did not show any change in % freezing time (A).

4. Discussion

Ginseng administration increased the number of BrdU-positive cells of the dentate gyrus in the no-CFC groups in a dose-dependent manner. Double staining with BrdU and NeuN suggested that the increase in BrdU-labeled cells may be mainly based on an increase in neurogenesis, and not gliogenesis.

Neurogenesis, defined as the creation of new nerve cells, consists of a series of distinct developmental steps, two of which can be examined separately; proliferation and survival/differentiation (Malberg et al., 2000). Our study suggested that the increased number of BrdU-positive cells induced by Ginseng was due to an increase in cell survival, and not cell proliferation.

We also found enhancing effects of Ginseng on performance in CFC, which is hippocampus/amygdala-dependent learning, as well as on neurogenesis in the dentate gyrus in the CFC groups. The unaltered result of the open field test in the Ginseng group shows that Ginseng causes neither a stimulating nor sedating effect at the doses employed in this study, which could potentially interfere with evaluation of CFC. Another interfering factor in evaluation of CFC is that Ginseng's effect on pain sensitivity. Since there is no previous data to suggest this effect, the possibility is low, but still remains to be examined.

Ginseng increased the CFC-related increase in the number of BrdU-positive cells in a dose-dependent manner. Although in this study we did not examine whether this CFC-related increase in the number of BrdU-positive cells was due to increased cell proliferation or cell survival, a previous study suggested that learning was associated with enhancement of cell survival but not cell proliferation (Fulder, 1981; Gould et al., 1999a), although it remains to be answered whether Ginseng and CFC have an additive or interactive effect on neurogenesis. It is reasonable to conclude that the increase in CFC-related neurogenesis may be one mechanism of Ginseng's property to enhance learning ability.

What are the molecular mechanisms underlying the regulation of hippocampal neurogenesis by Ginseng? Ginseng root consists of two major constituents: crude Ginseng saponin and crude Ginseng non-saponin fractions. To date, more than 20 saponins have been isolated from Ginseng root and identified chemically (Lim et al., 1997). Ginsenosides (the saponin constituents of Ginseng root) have been reported to have a number of actions on the CNS. These include CNS stimulation or depression (Watanabe et al., 1990), anticonvulsant activity (Gupta et al., 2001), anti-psychotic activity (Yoshimura et al., 1998), anti-fatigue (Wang et al., 1983) and anti-stress activity (Fulder, 1981; Kim et al., 1998), and improvement of performance in various memory paradigms (Jin et al., 1999; Ni et al., 1993; Nitta et al., 1995a).

The beneficial effects of Ginseng on learning and memory have often been attributed to ginsenoside Rb1

and Rg1 (Mook-Jung et al., 2001). Ginsenoside Rg1 increases cAMP level and c-fos gene expression in the rat hippocampus (Liu and Zhang, 1996). The elevation of intracellular cAMP level induces c-fos expression (Vaccarino et al., 1993). The cAMP–CREB cascade could contribute to the actions of neurotransmitters and neurotrophic factors on adult neurogenesis (Nakagawa et al., 2002a). In recent reports, CREB was shown to be necessary for both steps of neurogenesis: proliferation and cell survival (Nakagawa et al., 2002b). Since in our study, Ginseng did not enhance cell proliferation, activation of the cAMP-CREB cascade by Ginseng could not solely explain our findings.

Alternatively, the ginsenosides Rb1 and Rg1 (Mook-Jung et al., 2001) are also thought to enhance learning and memory by facilitation of cholinergic function, which is apparently essential for the functional integration of learning processes. For example, Rb1 facilitated acetylcholine (ACh) release and improved passive avoidance learning (Benishin et al., 1991). Rb1 and Rg1 increased the number of ACh receptors and improved passive avoidance learning in anisodine-treated mice (Shan et al., 2002). Rg1 improved the performance of scopolamine-injected rats in an eight-arm radial maze task. Consistent with these results, Rb1 facilitated choline uptake and increased choline acetyltransferase (Salim et al., 1997). ACh is also shown to increase neurogenesis (Ma et al., 2000). Thus, the increase of neurogenesis by Ginseng may be mediated via an increase in ACh release and ACh receptors.

In conclusion, Ginseng had an enhancing effect on CFC and increased the number of BrdU-positive cells in the dentate gyrus. The increase of neurogenesis by Ginseng was due to enhancement of cell survival, and not proliferation. Future study will be required to determine the components of Ginseng that are responsible for the enhancement of CFC and neurogenesis. Elucidation of the exact components and their mechanisms will lead to novel drugs for the treatment of memory impairment.

Acknowledgements

We thank Tsumura Pharmaceutical Company, Tokyo, Japan for donating Ginseng. We are grateful to Professor J. Arita (Department of Physiology, Yamanashi University) for his technical advice and critical reading of the manuscript. This study was supported by Target Oriented Brain Science Promotion Program supported by Japanese Ministry of Culture, Sports and Science.

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