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Consumption of Molecular Hydrogen Prevents the Stress-Induced Impairments in Hippocampus-Dependent Learning Tasks during Chronic Physical Restraint in Mice

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We have reported that hydrogen (H_2) acts as an efficient antioxidant by gaseous rapid diffusion. When water saturated with hydrogen (hydrogen water) was placed into the stomach of a rat, hydrogen was detected at several μM level in blood. Because hydrogen gas is unsuitable for continuous consumption, we investigated using mice whether drinking hydrogen water *ad libitum*, instead of inhaling hydrogen gas, prevents cognitive impairment by reducing oxidative stress. Chronic physical restraint stress to mice enhanced levels of oxidative stress markers, malondialdehyde and 4-hydroxy-2-nonenal, in the brain, and impaired learning and memory, as judged by three different methods: passive avoidance learning, object recognition task, and the Morris water maze. Consumption of hydrogen water *ad libitum* throughout the whole period suppressed the increase in the oxidative stress markers and prevented cognitive impairment, as judged by all three methods, whereas hydrogen water did not improve cognitive ability when no stress was provided. Neural proliferation in the dentate gyrus of the hippocampus was suppressed by restraint stress, as observed by 5-bromo-2′-deoxyuridine incorporation and Ki-67 immunostaining, proliferation markers. The consumption of hydrogen water ameliorated the reduced proliferation although the mechanistic link between the hydrogen-dependent changes in neurogenesis and cognitive impairments remains unclear. Thus, continuous consumption of hydrogen water reduces oxidative stress in the brain, and prevents the stress-induced decline in learning and memory caused by chronic physical restraint. Hydrogen water may be applicable for preventive use in cognitive or other neuronal disorders.

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Keywords: adult neurogenesis; cognition; hippocampus; molecular hydrogen; oxidative stress; preventive medicine

INTRODUCTION

Oxidative stress is widely accepted as a contributor to neuronal vulnerability (Langley and Ratan, 2004; Lin and Beal, 2006; Ohta and Ohsawa, 2006; Sayre *et al*, 2008). Thus, suitable antioxidants are desired to protect neural precursors and neurons against oxidative damage in the brain (Ferri *et al*, 2003); however, most antioxidants are not able to reach neurons because of the blood–brain barrier. We have recently reported that molecular hydrogen (H_2) reduces oxidative stress (Ohsawa *et al*, 2007; Fukuda *et al*, 2007), and can penetrate the blood–brain barrier to protect neurons by gaseous diffusion; however, inhalation of hydrogen gas may be unsuitable as continuous hydrogen consumption for preventive use. A brief report has suggested that consumption of water containing hydrogen at a saturated level (hydrogen water) reduces oxidative stress in rats, as shown by decreased levels of urine oxidized guanine and hepatic lipid peroxide (Yanagihara *et al*, 2005). Thus, we examined the effect of hydrogen water on cognitive decline induced by oxidative stress.

Adult hippocampal neurogenesis may be involved in cognitive functions, including learning and memory, and spatial recognition (Abrous *et al*, 2005; Bruel-Jungerman *et al*, 2007). Antidepressants increase adult neurogenesis (Dranovsky and Hen, 2006; Becker and Wojtowicz, 2007; Sahay and Hen, 2007), suggesting that suppression of adult neurogenesis may be responsible for some mental disorders. Here we show that when chronic physical stress was applied to mice continuous consumption of hydrogen water reduced oxidative stress in the brain, and prevented the decline in the proliferation of progenitor neural cells and the impairment of cognitive function.

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MATERIALS AND METHODS

Hydrogen Water

Molecular hydrogen (H_2) was dissolved in water under high pressure (0.4 MPa) to a supersaturated level using hydrogen water-producing apparatus (ver. 2) produced by Blue Mercury Inc. (Tokyo, Japan). The saturated hydrogen water (500 ml) was stored under atmospheric pressure in an aluminum bag with no dead volume. Hydrogen water was freshly prepared every week, which ensured that a concentration of more than 0.6 mM was maintained. We confirmed the hydrogen content with a hydrogen electrode (ABLE). Each day, hydrogen water from the aluminum bag was placed into a closed glass vessel (70 ml) equipped with an outlet line having two ball bearings, which kept the water from being degassed. This vessel ensured that the hydrogen concentration was more than 0.4 mM after 1 day. Hydrogen water degassed by gentle stirring was used for control animals; the complete removal of hydrogen gas was confirmed with the hydrogen electrode.

Measurement of Hydrogen in Blood

Hydrogen water (3.5 ml at 0.8 mM) was placed into the stomach of a rat (approximately 230 g) by a catheter. After 3 min, 5 ml of venous blood was collected from the heart and placed into a small aluminum bag containing 25 ml air. After hydrogen gas from blood was transferred into the air, 20 ml of the air from the aluminum bag was applied to gas chromatography to examine the content of hydrogen as described (Ohsawa *et al*, 2007).

Malondialdehyde Measurement

Brain malondialdehyde (MDA) levels were determined using a Bioxytech MDA-586 Assay Kit (OxisResearch, Oregon). Briefly, the frozen left brains were homogenized in the presence of butylated hydroxytoluene. After centrifugation, free MDA in the supernatant was converted to a stable carbocyanine dye (maximum absorption at 586 nm) by chemical reaction with *N*-methyl-2-phenylindole. MDA levels were normalized against protein concentration.

Physical Restraint Stress

This study was approved by the Animal Care and Use Committee of Nippon Medical School. We obtained ICR mice, 7 weeks of age (33–34 g body weight), from CLEA Japan Inc. (Tokyo, Japan), and divided 40 mice into four groups (each group had 10 mice) by the following combination. Stress and CTL: groups with and without restraint stress, respectively. HW(+) and HW(−): groups given water with and without hydrogen, respectively. Each mouse was placed in a $3 \times 3 \times 7.5$ cm stainless-steel cage, which completely restricted their movement, but allowed them to drink water *ad libitum*. Immobilization stress was given 10 h per day (0900–1900 hours) for 6 days each week. Each immobilized mouse was housed individually in a small $10 \times 10 \times 10$ cm compartment of a multicompartment cage for the remaining time to avoid aggression and to prevent social isolation. Hydrogen water or degassed water was available *ad libitum* throughout the whole period. Unstressed mice were housed in standard-sized cages consisting five mice per cage, and they were handled daily without stress. Schedules for providing restraint stress and performing experiments are schematically illustrated in Figure 1.

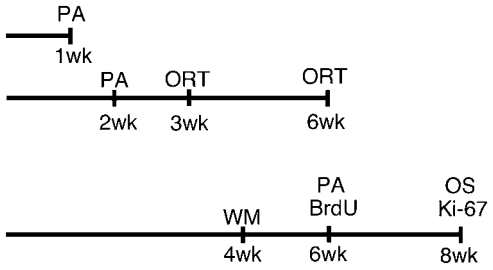

Figure 1 Schedules for subjecting mice to restraint stress and performing experiments are illustrated. Single bar indicates a series of experiments using the same mice. PA, ORT, WM, 5-bromo-2′-deoxyuridine (BrdU), Ki-67, and OS indicate the time points for passive avoidance, object recognition test, Morris water maze, injection of BrdU, and sampling for Ki-67 and oxidative stress (4-hydroxy-2-nonenal (HNE) staining and malondialdehyde (MDA) measurement). During the experiments of the Morris water maze, mice continued to be immobilized for 8 h per day.

Passive Avoidance Learning

The apparatus consisted of two compartments, one light and the other dark, separated by a vertical sliding door (O'Riordan *et al*, 2006). We initially placed a mouse in the light compartment for 20 s. After the door was opened, the mouse could enter the dark compartment (mice instinctively prefer being in the dark). After the mouse entered the dark compartment, the door was closed. After 20 s, the mouse was given a 0.3 mA electric shock for 2 s. The mouse was allowed to recover for 10 s, and was then returned to the home cage. After 24 h, the mouse was again placed in the lighted section with the door opened to allow the mouse to move into the dark section. We scored the latency time for stepping through the door. In addition, the number of mice that stayed in the light section for more than 300 s was recorded.

Object Recognition Task

The novel-object recognition and memory retention test was used to examine recognition memory (Wang *et al*, 2004; De Rosa *et al*, 2005). A mouse was habituated in a cage for 4 h, and then two different-shaped objects were presented to the mouse for 10 min as training. The number of times of explorations and/or sniffs of one object, which will be replaced with a novel one, was counted for the initial 5-min period (Training). To test memory retention after 1 day, one of the original objects was replaced with the novel one with a different shape, and then the number of times of explorations and/or sniffs of the novel one was counted for 5 min (Retention test). The recognition index was obtained as the frequencies (%) of exploring and/or sniffing the original object or the novel one.

Spatial Learning

Mice were trained on the Morris water maze (Morris *et al*, 1982; D'Hooge and De Deyn, 2001), with four trials per day

over 6 days. The water maze was a circular pool filled with water at room temperature (diameter, 125 cm; height, 55 cm; water temperature, 24 ± 1°C). A transparent platform (diameter, 10 cm) was hidden 1 cm below the surface of water that had been made opaque with white nontoxic paint. Starting points were changed every day. Each trial lasted until either the mouse had found the platform or for a maximum of 60 s. The examiner determined the time of swimming until the mouse reached the platform (latency) using a stopwatch and a video. All mice were allowed to rest on the platform for 20 s. The platform was removed for a probe trial 24 h after the last training session on day 6. Retention of the spatial training was assessed 1 h after the last training trial. The single-probe trial consisted of a 60 s free swim in the pool without the platform. Mice were placed and released opposite the site where the platform had been located and the time spent in each quadrant was recorded for the probe trial. During the experiments, mice continued to be immobilized for 8 h per day, instead of 10 h.

Immunohistochemistry

After 8-week restraint stress, mice were perfused transcardially with saline under anesthesia. The right brain hemisphere and the hemicerebellum were removed and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 24 h at room temperature. Coronal sections (40 μm) were cut rostrocaudally with a vibratome (Leica, Cambridge, UK) and immersed in PBS.

For 5-bromo-2′-deoxyuridine (BrdU) immunohistochemistry, BrdU (Sigma) was dissolved in 0.9% NaCl and sterilized by filtration. After 6-week restraint stress, the mice received one intraperitoneal injection of 50 mg/kg body weight at a concentration of 10 μg/ml per day for 5 consecutive days as described (Wolf *et al*, 2006). The sections were incubated in 2 N HCl at 37°C for 30 min to denature DNA, further incubated in 3% H_2O_2 in methanol for 30 min, and then blocked with mouse Ig blocking reagent from the M.O.M. kit (Vector Laboratories, Burlingame, CA) for 1 h. The primary antibody used was mouse monoclonal anti-BrdU (BD Pharmingen, 1 : 100).

For Ki-67 and 4-hydroxy-2-nonenal (HNE) immunoreactions, after 8-week restraint stress, sections were incubated in 10 mM citrate buffer (pH 6.0) at 90°C for 5 min, left to cool at room temperature, further incubated in 3% H_2O_2 in methanol for 30 min at 37°C, and then blocked with the M.O.M. kit and normal goat serum from the Vectastain ABC kit (Vector Laboratories), respectively. The primary antibodies used were rabbit polyclonal anti-Ki-67 antibody (Abcam, 1 : 500) and mouse monoclonal anti-HNE antibody (20 μg/ml; JaICA, Fukuroi, Japan). The HNE secondary antibody (M.O.M. biotinylated anti-mouse IgG, 1 : 250; Vector Laboratories) and the BrdU and Ki-67 secondary antibodies (biotinylated anti-rabbit IgG, 1 : 200) were applied for 2 h. The avidin/biotinylated mouse peroxidase complex (ABC kit; Vector Laboratories) was applied for 2 h, and the sections were stained with 3′3-diaminobenzidine (Sigma) for 1 min.

Wire-Hanging Test

After 6-week restraint stress, neuromuscular strength was tested by wire-hanging test (Hamann *et al*, 2003). In brief, mice were placed on wire netting, which was lightly shaken, causing the mouse to grip the wire. After the 20-s cutoff time, the wire netting was turned upside down (180°) and the time to falling was recorded.

Open-Field Test

After 7-week restraint stress, for the open-field test (Kim and Han, 2006), locomoter activity was measured in the open field of a white Plexiglas chamber (36 × 30 × 18 cm). Each mouse was individually placed in the left corner of an open field and locomotion was recorded for the indicated period. Horizontal locomotor activity was assessed according to the total rearing score for 20 min.

Statistical Analysis

All values are shown as the mean ± standard error of measurement (SEM). Differences between groups were analyzed using one- or two-way ANOVA. When statistical differences were found, Fisher's PLSD *post hoc* test was performed. Statistical significance was accepted as $P<0.05$. All the experiments were examined in a blinded fashion.

RESULTS

Physical Restraint Stress Enhanced Oxidative Stress and Hydrogen Water Decreased it

First, we examined whether hydrogen can be sufficiently incorporated into a body by drinking hydrogen water. Because it is difficult to obtain a sufficient volume of blood from mice, we used rats for the measurement of hydrogen concentration in the blood. We placed saturated hydrogen water at 3.5 ml per 230 g (15 ml/kg) directly into the stomach of a rat by a catheter. After 3 min, hydrogen was detected in the blood at the level of 5 μM (Figure 2a). Alternatively, an unpublished result suggests that the half-span of hydrogen in the muscle of rats is approximately 20 min as monitored with a needle-type hydrogen electrode, after the administration of hydrogen gas (data not shown).

We used mice for experiments of restraint stress. To examine whether mice preferably drank hydrogen water, we measured the volume of water consumed *ad libitum* by mice. The volume of water drunk by each mouse was nearly the same between groups drinking hydrogen water and degassed water (3.77 ± 0.11 *vs* 3.75 ± 0.08 ml, SEM).

Each mouse was then subjected to chronic physical restraint stress by placing it alone for 10 h per day in a very small cage, which completely restricted its movement, but allowed it to drink water *ad libitum*, and then was housed for 14 h in a small compartment as described in 'Materials and methods'. These treatments continued for 6 days per week. Water was available *ad libitum* throughout the whole period.

We examined the level of oxidative stress in the brain by immunohistologically estimating HNE, an end product of lipid peroxide (Ohsawa *et al*, 2003) after 8-week restraint stress (Figure 2b, c). Another oxidative marker, MDA, was estimated using a biochemical method (Fukuda *et al*, 2007; Figure 2d). These results revealed that chronic restraint stress enhanced oxidative stress in the brain. Notably, the

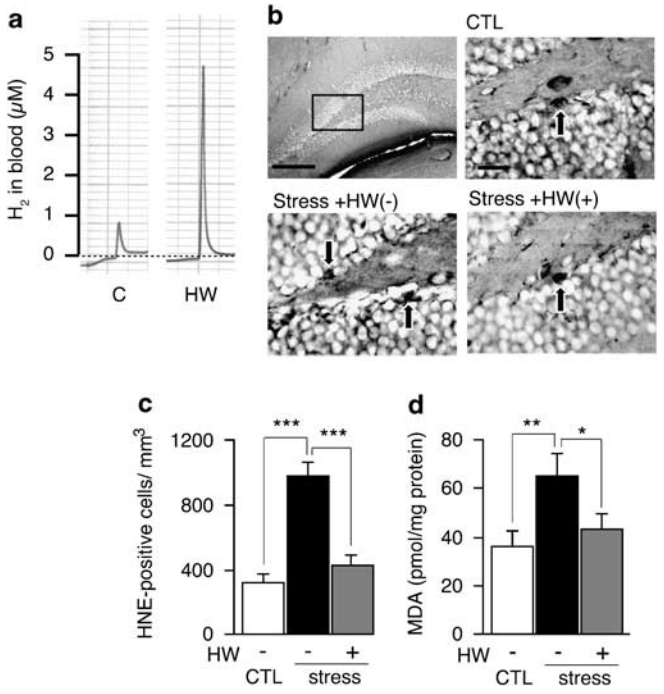

Figure 2 Consumption of hydrogen water reduced oxidative stress enhanced by restraint stress. (a) Hydrogen (H_2) in blood was measured 3 min after hydrogen water (3.5 ml) was placed directly into the stomach of a rat by a catheter. Profiles of gas chromatography are shown. (b) After 8-week exposure to restraint stress, the hippocampus region was stained with anti-4-hydroxy-2-nonenal (HNE)-conjugated peptide antibody. Representative photographs of HNE staining are shown. Arrows indicate positive cells. Scale bar: upper left panel, 100 μm; magnified panels, 25 μm. (c) HNE-positive cells in the dentate gyrus were counted using four serial sections ($F_{(2,27)} = 28.031$; $P < 0.0001$). (d) Malondialdehyde in the whole brain was biochemically measured ($F_{(2,27)} = 4.177$; $P = 0.0263$). CTL, unstressed control group; Stress, group exposed to restraint stress for 8 weeks; HW(+), group provided with hydrogen water; and HW(−), group provided with degassed water. Data are shown as the mean ± SEM (each group consisted 10 mice). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ *vs* Stress + HW(−).

consumption of hydrogen water suppressed the accumulation of the oxidative stress markers (Figure 2b–d).

Passive Avoidance Learning, Novel Recognition Test, and Morris Water Maize

We examined learning and memory ability using the passive avoidance test (O'Riordan *et al*, 2006). Mice instinctively prefer a dark compartment; however, if an electric shock is given in the dark compartment, the mice are normally reluctant to reenter it. At 1 or 2 weeks after restraint stress, the memory of the electric shock tended to be lost in mice provided with control degassed water (Figure 3a, b). On the other hand, mice that were provided with hydrogen water *ad libitum* showed a trend toward improved learning and memory (Figure 3a, b). Six-week restraint stress significantly impaired learning and memory in mice consuming no hydrogen water, whereas consuming hydrogen water *ad libitum* significantly ameliorated or prevented the cognitive impairment induced by restraint stress (Figure 3c, left panel). In particular, more mice (fourfold) stayed in the light section for more than 300 s than control group without

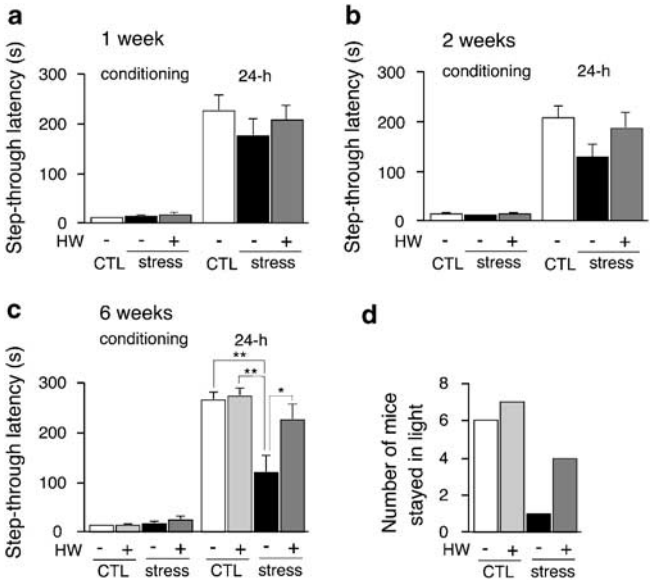

Figure 3 Passive avoidance test shows that hydrogen water prevented cognitive decline induced by restraint stress. After applying restraint stress for 1 week (a), 2 weeks (b), and 6 weeks (c) ($F_{(3,36)} = 7.661$; $P < 0.0005$), the passive avoidance test was performed by examining step-through latency time from light to dark compartments on the first day of the conditional trial (conditioning). At 24 h after an electric shock was given in the dark compartment, the latency time of mice moving from the light to dark compartment was measured (24 h). When a mouse did not enter the dark compartment for 300 s, the latency time was scored as 300 s. The number of mice that stayed in the light compartment for more than 300 s on the second day is shown (right panel). Stress and CTL, groups with or without restraint stress, respectively; HW(+) and HW(−), groups given water with and without hydrogen, respectively. Experiments in (a), (b), and (c) were performed using different mice. *$P < 0.01$ and **$P < 0.001$ *vs* Stress + HW(−). Data are the mean ± SEM (each group consisted 10 mice).

hydrogen (Figure 3c, right panel). On the other hand, consumption of hydrogen water did not improve the cognitive ability when no stress was provided (Figure 3c).

As an alternative method to confirm impaired cognitive function after restraint stress, we applied the object recognition task (Wang *et al*, 2004; De Rosa *et al*, 2005; Ohsawa *et al*, 2008). If mice remember an object, they prefer to explore and/or sniff a novel object when the original object is replaced with the novel object. After 3-week restraint stress, hydrogen water tended to prevent or restore the decline in the recognition of a novel object, observed as a decreased frequency of exploring or sniffing the novel object (Figure 4a). The mice were subjected to the second object recognition task after 6-week stress, because the second test was available if the second objects were completely different from ones used in the first examination (Mouri *et al*, 2007). When restraint stress was applied for 6 weeks, consuming hydrogen water *ad libitum* significantly prevented or restored the decline in recognition and memory (Figure 4b). It should be noted that consumption of hydrogen water could not improve the cognitive ability when no stress was provided (Figure 4b).

To test spatial recognition and learning, we subjected mice to the Morris water maze (Morris *et al*, 1982; D'Hooge and De Deyn, 2001; Ohsawa *et al*, 2008). After 4-week restraint stress, the mice took longer to reach an invisible

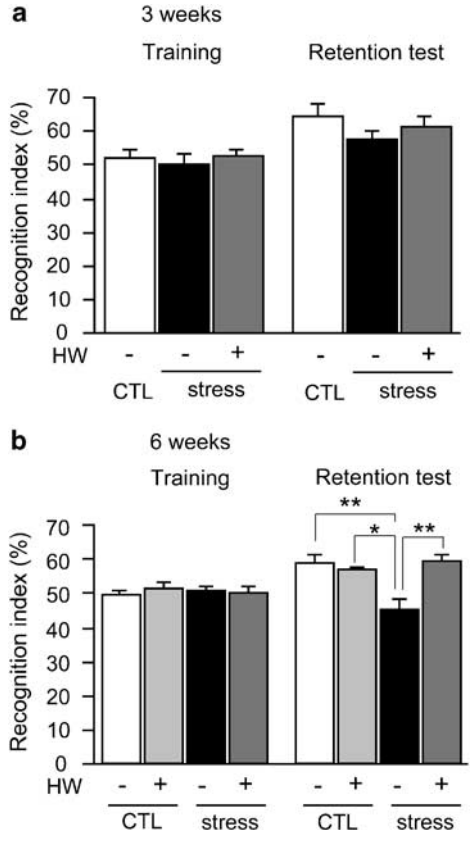

Figure 4 Object recognition task shows that hydrogen water prevented cognitive decline induced by restraint stress. For the object recognition task, after applying restraint stress for 3 (a) or 6 weeks (b) ($F_{(3, 35)} = 7.466$; $P < 0.0005$), two different objects were presented to a mouse for 10 min for visual training and the number of times of explorations and/or sniffs of an object was counted in the initial 5-min period (Training). To test memory retention after 1 day, one of the original objects was replaced with the novel one with a different shape, and then the number of times of explorations and/or sniffs of the novel one was counted for 5 min (Retention test). The recognition indexes were obtained as the frequency (%) of exploring and/or sniffing the object that will be replaced, or the novel one that had been replaced. For example, if a mouse explored and/or sniffed only the novel object, the recognition index is scored as 100%, whereas if it did only the unchanged one, the recognition index is 0%. Stress and CTL, groups with or without restraint stress, respectively; HW(+) and HW(−), groups given water with and without hydrogen, respectively. Experiments in (a) and (b) were performed using the same mice. When the second object recognition test was given to the same mice, objects that differed in shape and color were used. Data are the mean ± SEM (each group consisted 10 mice). *$P < 0.01$ and **$P < 0.001$ *vs* Stress + HW(−).

platform hidden in a pool after training than unstressed controls, indicating loss of memory of the platform location. Continuous consumption of hydrogen water shortened the time required for mice to reach the platform compared with stressed controls (Figure 5a). When the invisible platform is removed, mice should swim for longer near where the platform had previously been placed (the target quadrant) if they retain the memory of the platform location (Figure 5b, area A). Indeed, mice with chronic restraint stress swam around that location for a shorter time depending on their memory loss than unstressed controls (Figure 5c, area A). In contrast, stressed mice with consumption of hydrogen water swam in the target quadrant area (Figure 5b, area A) for a longer time than stressed controls without hydrogen water, although no group showed significant difference in three nontarget quadrant areas (Figure 5c, areas B–D). Notably, hydrogen water consumption apparently improved spatial recognition and learning that had declined by restraint stress. This experiment also indicates that hydrogen water has no potential for improving the spatial cognitive ability when no stress was applied, which agrees with the previous results.

Hydrogen Water did not Affect Stress-Induced Reductions in Muscular Strength and Movement

To examine whether hydrogen water influenced the whole body, we monitored body weight during periods of restraint stress. Body weight decreased with restraint, and hydrogen water did not overcome this decrease (Supplementary Figure S1). Wire-hanging (Hamann *et al*, 2003) and open-field tests (Kim and Han, 2006) were used to exclude the possibility that hydrogen water influenced muscular strength and movement. Wire-hanging times depended on the body weight of each mouse, and no significant difference was observed in muscular strength (Supplementary Figure S2). Although restraint stress tended to affect movement, no effect of hydrogen water consumption was observed in the locomotion test (Supplementary Figure S3). Together, the behaviors observed by the three methods used to test cognitive function were not due to changes in movement ability, but indicated a decline in learning and memory. Thus, the improvement observed in the hydrogen water group was reflected by better learning ability and memory (Figures 3–5).

Hydrogen Water Improved the Proliferation of Progenitor Cells

Cognitive function may be involved in adult neurogenesis in the hippocampus. Finally, after 8-week restraint stress, we examined the correlation between adult neurogenesis and the hippocampal function by counting proliferating progenitor cells in the dentate gyrus of the hippocampus with BrdU labeling (Kee *et al*, 2002; Ekdahl *et al*, 2003). Positive nuclei, as judged by the shape and size, were counted in the boundary region of the dentate gyrus in four serial sections. Restraint stress decreased the number of proliferating cells, and hydrogen water significantly restored them (Figure 6a, b). As an alternative method, Ki-67 was used as a marker of proliferating cells (Kee *et al*, 2002; Shidara *et al*, 2005). The results were similar to those obtained by the BrdU-labeling method (Figure 6c, d). These findings suggest that continuous consumption of hydrogen water improves the proliferation of neural progenitor cells or adult neurogenesis impaired by restraint stress.

DISCUSSION

In summary, the continuous consumption of hydrogen water throughout the whole period of physical restraint stress reduced oxidative stress, prevented the decline in the proliferation of neural progenitors, and prevented cognitive decline, all of which are induced by chronic physical restraint stress. The restraint stress applied in this study may also induce considerable psychological as well as

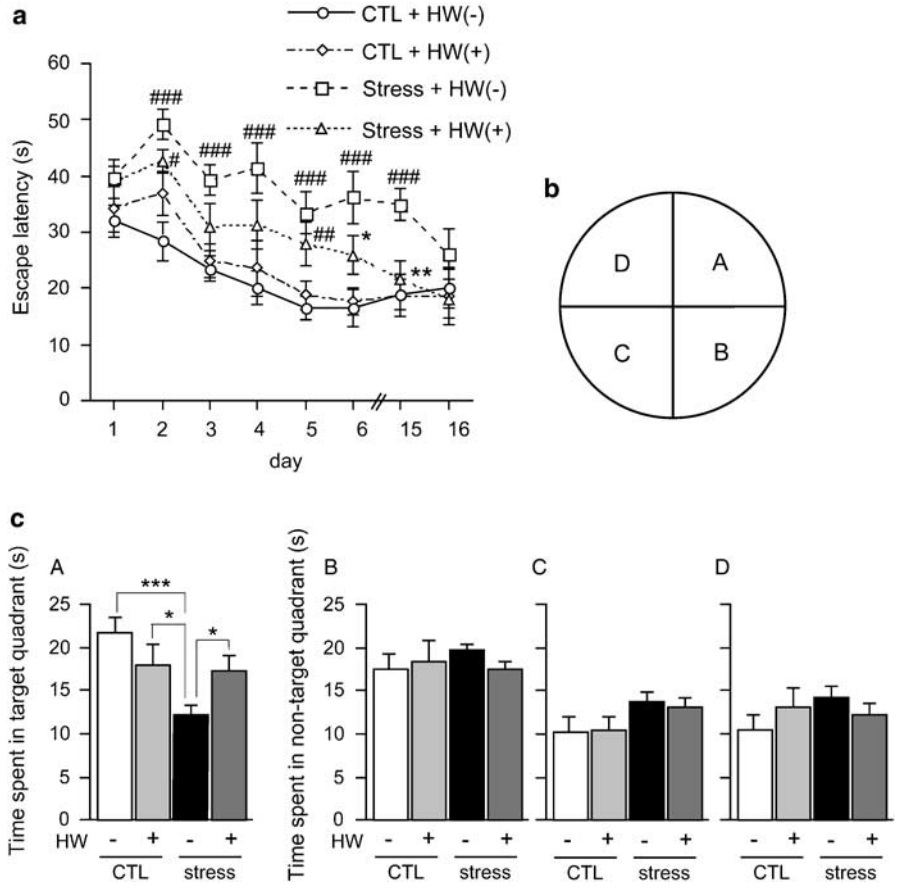

Figure 5 The Morris water maze shows that hydrogen water prevented cognitive decline induced by restraint stress. After applying restraint stress for 4 weeks, the time to reach a hidden platform was measured after four daily trials (a). During the experiments, mice continued to be immobilized for 8 h per day. (b) A single-probe trial consisted of a 60 s free swim in the pool without the platform. Quadrant areas used for the probe trial are shown, where the platform had been located in area A and mice were placed and released in region B. (c) At 6 days after daily training, after removing the platform, the time of free swimming in each area (A–D). Two parameters (stress and hydrogen) were analyzed by two-way ANOVA. Area A: $F_{(1,36)} = 4.455$; $P = 0.042$, area B: $F_{(1,36)} = 0.016$; $P = 0.901$, area C: $F_{(1,36)} = 0.933$; $P = 0.341$, and area D: $F_{(1,36)} = 3.235$; $P = 0.08$ by two-way ANOVA. Four groups were compared in area A by one-way ANOVA ($F_{(3,36)} = 5.074$; $P = 0.0049$). Stress and CTL, groups with or without restraint stress, respectively; HW(+) and HW(−), groups given water with and without hydrogen, respectively. Data are the mean ± SEM (each group consisted 10 mice). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ *vs* Stress + HW(−). $^{\#}P < 0.05$, $^{\#\#}P < 0.03$ *vs* CTL + HW(−); $^{\#\#\#}P < 0.01$ *vs* CTL + HW(−) and CTL + HW(+).

physical stress. In this study, we examined impaired learning and memory by three different methods: passive avoidance learning, object recognition task, and the Morris water maze. In these methods, successive object recognition tasks are available (Mouri *et al*, 2007) and the Morris water maze gives no influence on results of passive avoidance test (Yamada *et al*, 1999; King and Arendash, 2002). Thus, some experiments were performed using the same mice as shown in Figure 1, although a possibility cannot be ruled out that the passive avoidance test was influenced by the water-maze training.

We have recently reported that hydrogen reduced hydroxyl radicals, the most reactive oxygen species (ROS), and protected cells against oxidative stress. Inhalation of 1% hydrogen gas was enough to protect the brain and liver (Ohsawa *et al*, 2007; Fukuda *et al*, 2007), where the hydrogen in blood should be 8 μM because the saturated level of hydrogen reached 800 μM under atmospheric pressure. It is possible that continuous consumption of hydrogen defends the brain against chronic oxidative stress even at much lower concentrations than 8 μM. In this study, we showed that the incorporation of hydrogen from the stomach into blood reached 5 μM. Continuous exposure to hydrogen may change blood components toward the reductive state, and indirectly influence the oxidative state in the brain. Although it remains unclear how hydrogen reduces oxidative stress in the brain, the present study may highlight the prominent role of oxidative stress in deficits of learning and memory.

The consumption of hydrogen water ameliorated the proliferation of neural progenitors that had been declined by restraint stress although the mechanistic link between the changes in neurogenesis and cognitive impairments is at this stage correlative. However, adult neurogenesis may be involved in hippocampal functioning, including learning and memory and spatial recognition processes, and affected by multiple intrinsic and extrinsic factors. For example, adult neurogenesis is suppressed by cranial radiotherapy, stress-sensitive adrenal hormones such as glucocorticoids, and physical or psychological stress, and improved with inflammatory blockade. When we studied oxidative stress, we found growing evidence suggesting that it is involved

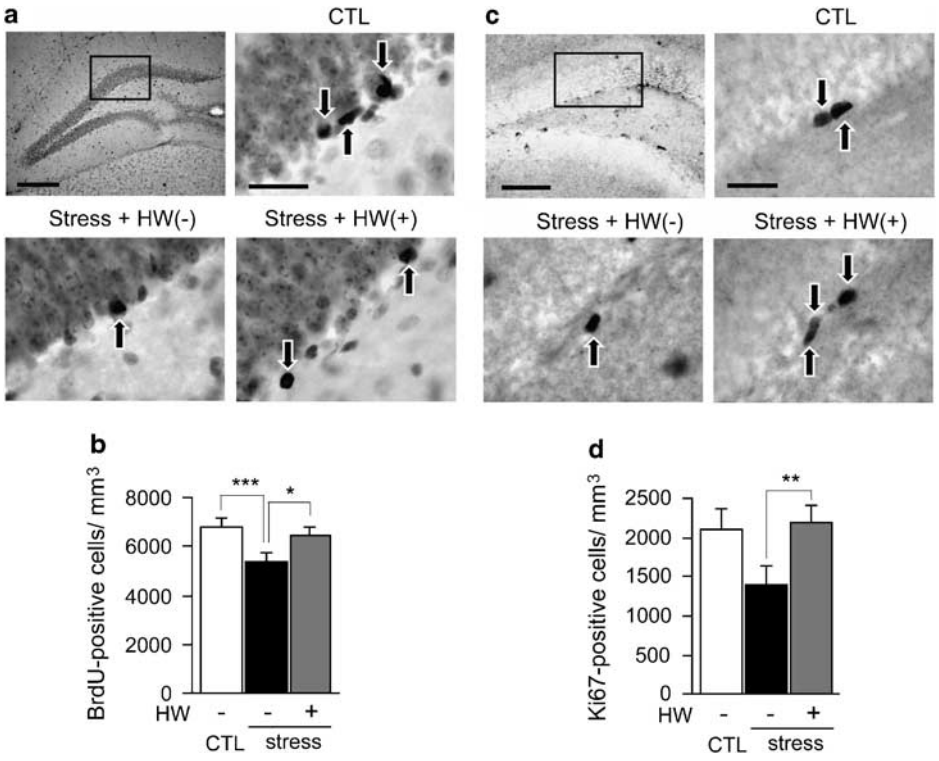

Figure 6 Hydrogen restores the proliferation of progenitor cells declined by restraint stress. (a) Mice were injected with 5-bromo-2′-deoxyuridine (BrdU) after 6-week restraint stress. Representative photographs of BrdU-positive progenitor cells in the dentate gyrus of the hippocampus are shown. Arrows indicate positive cells. Scale bar: upper left panel, 100 μm; magnified panels, 25 μm. (b) BrdU-positive nuclei of progenitor cells in the boundary region of the dentate gyrus of the hippocampus were counted in four serial sections ($F_{(2, 27)} = 4.289$; $P = 0.0241$). (c) Cell proliferation in the dentate gyrus was examined using anti-Ki-67 antibody. Representative photographs of Ki-67-positive cells in the dentate gyrus of the hippocampus are shown. Arrows indicate positive cells. Scale bar: upper left panel, 100 μm; magnified panels, 25 μm. (d) Ki-67-positive progenitor cells in the boundary region of the dentate gyrus were counted in four serial sections ($F_{(2, 27)} = 3.155$; $P = 0.0587$). CTL, unstressed control group; Stress, group exposed to restraint stress for 8 weeks; HW(+), group provided with hydrogen water; and HW(−), group provided with degassed water. Data are the mean ± SEM (each group consisted 10 mice). $^{*}P < 0.05$, $^{**}P < 0.03$, and $^{***}P < 0.01$ *vs* Stress + HW(−).

downstream of contributors that affect adult neurogenesis: (1) radiotherapy produces hydroxyl radicals of ROS (Madsen *et al*, 2003; Raber *et al*, 2004), (2) an inflammatory blockade restores adult hippocampal neurogenesis, which may be elucidated by decreasing inflammatory oxidative stress (Ekdahl *et al*, 2003; Monje *et al*, 2003), (3) glucocorticoids enhance oxidative stress-induced cell death in the hippocampus (Behl *et al*, 1997), and (4) the present study and others have shown that restraint stress itself enhances oxidative stress in the brain (Liu *et al*, 1996; Kim *et al*, 2005; Luo *et al*, 2005).

Thus, it is possible that during the exposure to physical restraint stress continuous consumption of hydrogen water reduced oxidative stress in the brain, resulting in the improvement of adult neurogenesis or the stimulation of neural proliferation, leading to the prevention of the decline in learning and memory. This is the first report showing a benefit of drinking hydrogen water. Thus, we propose that hydrogen water is applicable as preventive treatment by reducing oxidative stress.

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DISCLOSURE/CONFLICT OF INTEREST

Dr Ohta is a director of Mitos Co. Ltd (Kawasaki, Japan), and a scientific adviser to Blue Mercury Inc. (Tokyo, Japan). Blue Mercury Inc. supplied the fresh hydrogen water used in this study and has donated a research division to our institute. Other authors have no conflict of interest.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)

Supplementary Figures

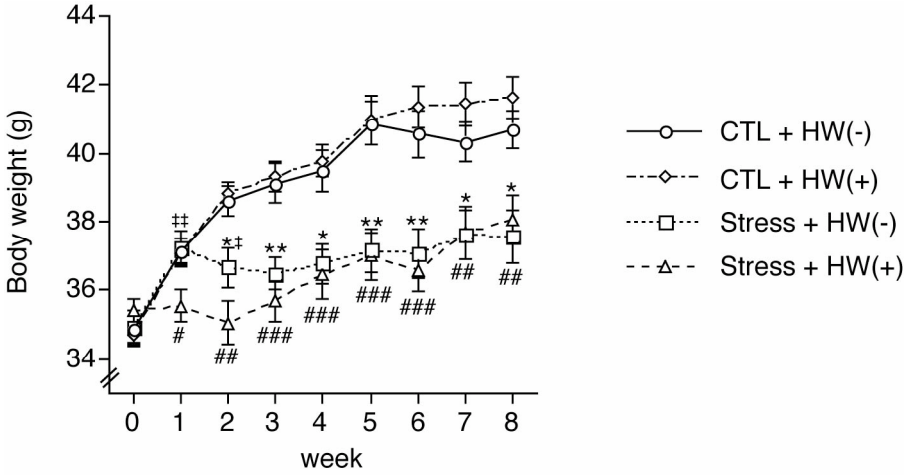

Figure S1 Body weight. Changes in body weight during the experimental period were measured. Stress and CTL: groups with or without restraint stress, respectively. HW(+) and HW(-): groups given water with and without hydrogen, respectively. Data are the mean ± s.e.m. (each group contained 10 mice. **P* < 0.01 and **P< 0.001 vs CTL + HW(-) and CTL + HW(+). #*P* < 0.03, ##*P*<0.01 and ###*P*<0.001 vs CTL + HW(-) and CTL + HW(+). ‡ *P* < 0.03 and ‡ ‡ *P*<0.01vs. Stress + HW(+).

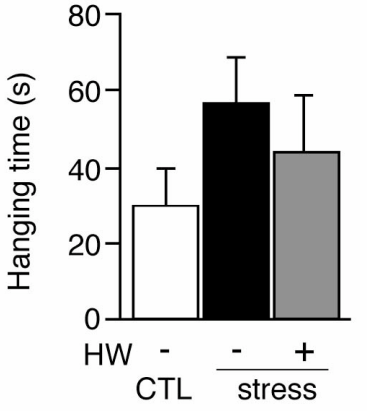

Figure S2 Wire hanging test. After 6-week restraint stress, wire-hanging test to determine neuromuscular strength was performed by measuring the hanging time. Stress and CTL: groups with or without restraint stress, respectively. HW(+) and HW(-): groups given water with and without hydrogen, respectively. Data are the means ± s. e. m. (each group contained 10 mice).

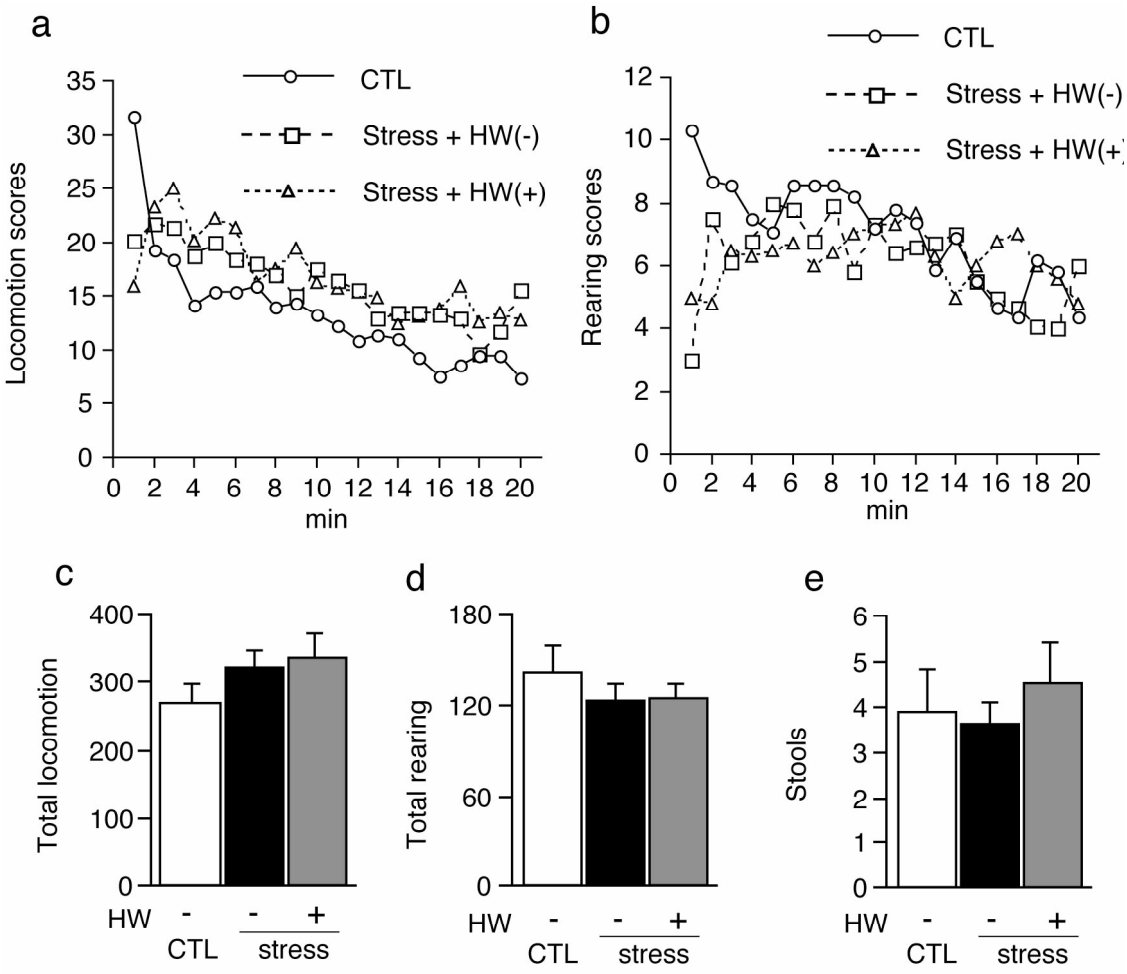

Figure S3 Open field test. After 7-week restraint stress, the open field test was performed to examine the change in movement ability. (**a**) locomotion score, (**b**) total locomotion score, (**c**) rearing score, (**d**) total rearing score, and (**e**) stools found at the end of the session were also counted. Stress and CTL: groups with or without restraint stress, respectively. HW(+) and HW(-): groups given water with and without hydrogen, respectively. Data are the mean ± s. e. m. (each group contained 10 mice).