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γ-Oryzanol, a unique ingredient specific to brown rice, effectively restores mild cognitive impairment (MCI) in obese aged mice by ameliorating microglial inflammation and promoting neurogenesis in hippocampus: Novel therapeutic insight into obesity-associated MCI

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Abbreviations: CI, cognitive impairment; Orz, γ-oryzanol; Nano-Orz, nanoparticulated γ-oryzanol; FITC, fluorescein isothiocyanate; LCD, lab chow diet; HFD, high-fat diet; PO, per os

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Abstract

Obesity-diabetes syndrome poses a considerable risk for mild cognitive impairment (MCI). Our study was designed to explore therapeutic potential of γ-oryzanol (Orz), a brown rice-specific oil composed of ferulic acid ester with several phytosterols, on MCI in high-fat diet (HFD) induced obese aged mice. After being housed on an HFD for 4 months, regular Orz or the nanoparticulated form of Orz (Nano-Orz), which markedly enhances its intestinal absorption, was administered to aged mice. Mice treated with regular Orz for 4 months exhibited significant improvement in spatial cognitive function. Impressively, mice treated with Nano-Orz demonstrated cognitive improvement as early as 1 month, with substantial recovery by 3 months. In the hippocampus, treatment with both regular Orz and Nano-Orz upregulated genes associated with neurogenesis and stem cell function accompanied by a significant increase in gene expressions of anti-inflammatory cytokines. Our data highlight a therapeutic potential of Orz for obesity-associated MCI.

Keywords: γ-Oryzanol; Mild cognitive impairment (MCI); Hippocampus; Neurogenesis; Obesity.

1. Introduction

Mild cognitive impairment (MCI) stems from a variety of factors, including aging, chronic inflammation, and beta-amyloid accumulation in the hippocampus. Of note, obesity has recently been recognized as a considerable risk for MCI, highlighting that chronic over-ingestion of a high-fat diet (HFD) substantially impairs hippocampus-dependent memory in both rodents and humans (Kanoski and Davidson, 2010; Kanoski et al., 2007; Nyaradi et al., 2014; Sharma, 2021). γ-Oryzanol (Orz), a brown rice-specific oil composed of ferulic acid ester with several phytosterols, is known to act preferentially on lipophilic organs and tissues (Masuzaki et al., 2019). A line of our studies showed that orally administered Orz accumulated considerably in the brain and pancreas, thereby reducing exaggerated endoplasmic reticulum (ER) stress by suppressing mRNA expression of ER stress-associated genes (*Chop, ERdj4, Xβp1*) in both hypothalamus and pancreatic β-cells in HFD-induced obese diabetic mice (Kozuka et al., 2015; Kozuka, Shimizu-Okabe, et al., 2017). We also demonstrated in mouse experiments that Orz potently inhibited DNA methyltransferases, thereby reducing animal fat preference via epigenetic modulation of dopamine receptor gene in brain reward system (Kozuka, Kaname, et al., 2017). Moreover, a rat model of streptozotocin-induced sporadic Alzheimer's disease showed that Orz was potent to delay the onset of MCI (Jha and Panchal, 2017).

To date, detailed molecular mechanisms whereby Orz would improve MCI have been poorly elucidated. Furthermore, due to extremely poor solubility in water of Orz, oral administration of Orz in mice provided a weak impact on some effects (Kozuka et al., 2013). To overcome such a hazard, we previously reported that oral administration of nanoparticulated γ-oryzanol (Nano-Orz) in mice markedly enhanced absorption efficiency from intestine by more than 1,000-fold (Kozuka, Shimizu-Okabe, et al., 2017). In this context, we here provide evidence that oral administration of Nano-Orz potently mitigates hippocampal dysfunction via novel mechanisms, thereby improving MCI in obese aged mice.

2. Materials and methods

2.1. Animals, diets, and nanoparticulated agent

All animal studies were approved by the Animal Experiment Ethics Committee of the University of the Ryukyus (A2019041) and conducted according to animal care guidelines. Fifty-week-old geriatric male C57BL/6J mice from Nihon SLC (Hamamatsu, Shizuoka, Japan) were individually housed at 24 °C under a 12-h/12-h light/dark cycle. Mice were provided with water and experimental diet (Lab chow diet: (LCD) D12450J, Research Diets Inc., New Brunswick, NJ, USA) ad libitum. The mice were then divided into two groups. One group was fed γ-oryzanol (Orz) -containing feed immediately after the start of the experiment to investigate its beneficial effects on the onset of cognitive impairment (CI). The other group was orally administered Orz-containing nanoparticles after cognitive function decline to verify whether symptom alleviation was possible. To explore potentially-preventive impact of Orz on CI, mice were fed an LCD as a control, a 1% Orz-containing high-fat diet (HFD+Orz, Wako Pure Chemical Industries, Ltd., Osaka, Japan, and Research Diet Inc.) or an HFD (D12492, Research Diets Inc.) *ad libitum* for 4 months, based on the notion demonstrating that HFD feeding for sixteen weeks does reduce cognitive function in mice (Morrison et al., 2010; Pistell et al., 2010). For research on therapeutic impact, fifty-week-old mice were fed an LCD as a control, an HFD for 4 months to induce obesity, and were divided into three groups.

To substantially improve the absorption efficiency of Orz, we used a nanoparticulate carrier made of hydroxypropyl cellulose (HPC) (SENTAN Pharma Inc., Fukuoka, Japan), a widely used food additive (Food and Drug Administration, 2025). Nanoparticles containing 42 % Orz were adjusted to two concentrations (50 or 100 mg/ml) and orally administered (per os: PO) in a 250 µl water suspension twice a week for 4–12 weeks. Detailed information on diet is provided in Table S1.

2.2. Preparation of nanoparticles containing Orz or FITC

Nanoparticles containing Orz (Nano-Orz, for short) (SENTAN Pharma Inc.) were prepared using an emulsion solvent diffusion method (Kozuka, Shimizu-Okabe, et al., 2017). Briefly, solutions A and B were prepared using CLEARMIX® (M TECHNIQUE, Osaka, Japan). Liquid A was an aqueous solution containing 23.4% maltitol, 34.2% hydroxypropyl cellulose (HPC), and 0.3% sodium bicarbonate. Liquid B contained brown rice germ extract (containing 90 % or more of Orz, Okayasu Co., Ltd., Koshigagya, Saitama, JAPAN) and HPC in ethanol. Solutions were mixed in the ULREA® microreactor (M TECHNIQUE). After collecting the discharge liquid for the proper period, ethanol was removed by distillation *via* an evaporator. Resulting nanoparticle aqueous dispersion was freeze-dried, preparing a nanoparticle agent containing γ-oryzanol at a final concentration of 42 %. The nanoparticle agent used in the present study were measured for particle size using Nanotrac Wave (EX), with a D50 value of 116 nm. Regarding nanoparticle containing fluorescein isothiocyanate (FITC) as an oral control, liquid A was an aqueous solution containing polyvinyl alcohol, and liquid B was a solution containing polylactide-co-glycolide acid (PLGA), FITC, acetone, and ethanol. Solutions were mixed and fed into the ULREA® microreactor (M TECHNIQUE). After collecting the discharge liquid for the proper period, organic solvents were removed by distillation using an evaporator. Subsequently, FITC-PLGA-Nanoparticles (NPs) were washed with ultrapure water and freeze-dried.

2.3. Behavioral test

To evaluate cognitive function in mice, two behavioral analysis experiments were performed.

2.3.1. Y-maze test

First, we assessed working and spatial memories in mice using a three-armed horizontal maze (each arm 10 cm × 40 cm) made of gray plastic walls (Figure 1b, upper, right). This test is well-established for spontaneous behavioral monitoring, particularly the willingness of rodents to explore new environments (Landmann et al., 2019). Basically, mice prefer to seek a novel arm in the Y-maze

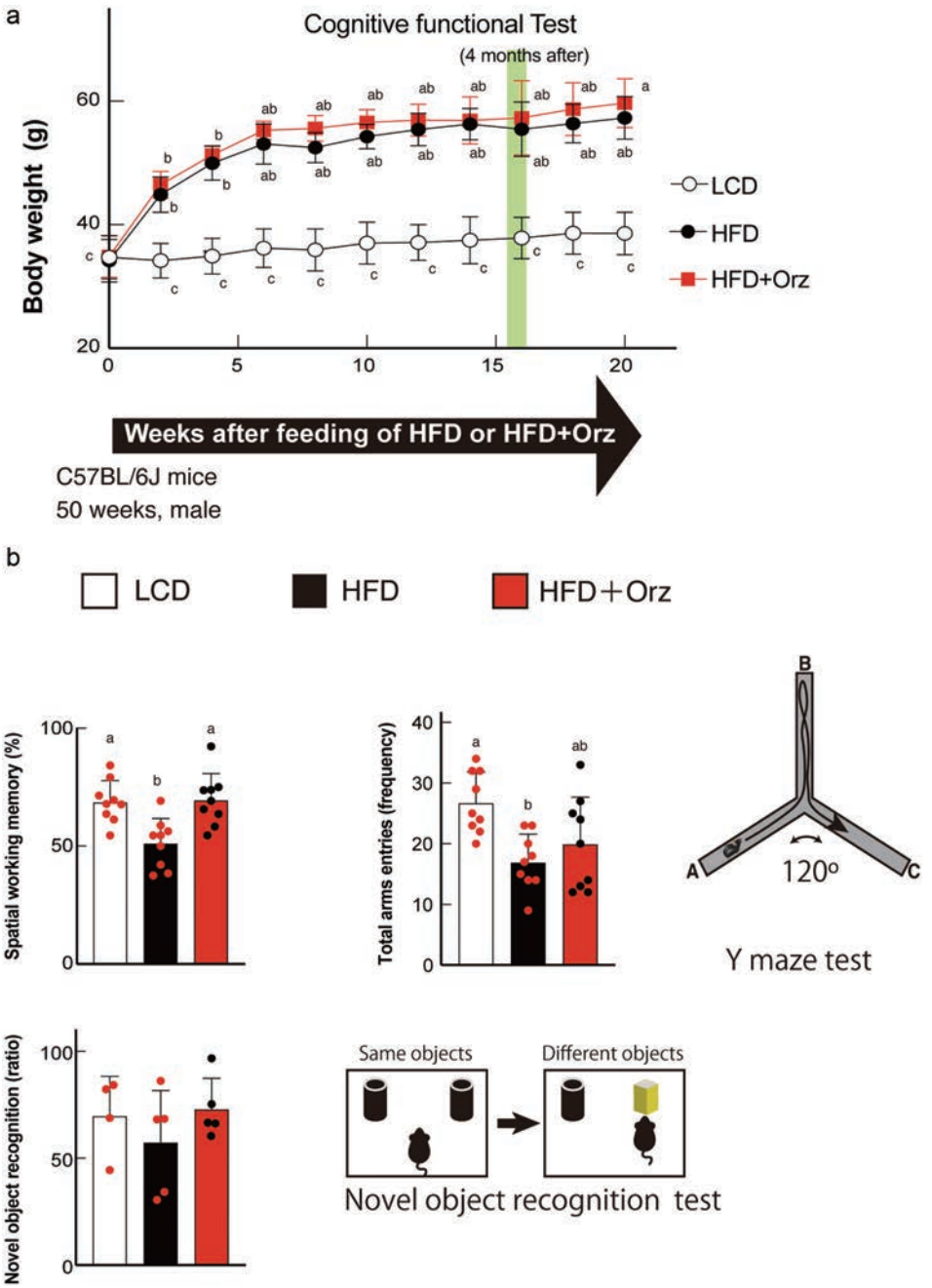

Figure 1. Body weight change and results of cognitive functional tests in obese aged mice treated with regular Orz. (a) Body weight changes among 3 groups consisting of aged mice fed lab chow diet (LCD), fed high-fat diet (HFD), and fed high-fat diet with 1% γ-oryzanol (HFD+Orz). Data are expressed as means ± SD (n = 6–8). Feeding Time: F(2, 187) = 657.0, $P < 0.0001$, Agents: F(10, 187) = 48.69, $P < 0.0001$, Interaction: F(20, 187) = 7.998, $P < 0.0001$, a–c: Means with different letters differ significantly at $P < 0.05$. (b) The upper panels demonstrate the percentage of spontaneous alternation in behavior (spatial working memory: SWM) (left) and the number of entries in the arms of the Y maze (middle) in aged mice fed lab chow diet (LCD) or after a 4-month fed HFD with or without Orz (HFD+Orz, HFD, respectively). A schematic diagram of a Y-maze is shown on the right. Mice prefer to search for a new arm in the Y-maze rather than returning to one previously visited. The lower panel demonstrates the percentage of time spent for exploring the novel objects. Mice memorize familiar object and prefer to examine novel object. Data are expressed as means ± SD (n = 4–9). a, b: Means with different letters differ significantly at $P < 0.05$.

rather than returning to one previously visited. Therefore, normal mice chose a different arm, considering a correct response. In contrast, returning to the previous arm was considered an incorrect response. After the video-tracing for the number of consecutive entries into another arm within 8 minutes, the rate of spatial working memory was automatically calculated to estimate cognitive function. Details are available in Landmann et al (Landmann et al., 2019). The total number of arm entries and the sequence of entries

were recorded to calculate the percentage of alternations (Magen et al., 2012). The percentage of spontaneous alternations between arms was calculated as follows:

$$\left[\frac{\text{number of alternations}}{\text{total number of arm entries}} - 2\right] \times 100.$$

2.3.2. Novel object recognition (NOR) test

Second, we performed the novel object recognition (NOR) test, also widely used for estimating memory alterations in mice (Leger et al., 2013). The NOR task is based on rodents' natural curiosity to explore their immediate environment. The test consists of a sample phase and a test phase. In the sample phase, the mouse is given 10 min to freely explore two identical objects (object A), after which it is returned to the home cage (Figure 1b, lower, right). After 3 h of resting, the mouse is returned to the test arena (50 cm × 50 cm), where one of the sample objects has been replaced by a new one (object B) of different luminosity, shape, and material. During this test phase, the time in contact with the objects is measured, and a preference index is calculated. Theoretically, the mouse spends longer time exploring the novel object than the familiar one, while equal time on the exploration of both objects suggests the inability to remember the sample object.

2.4. RNA extraction and quantitative real-time PCR

Brain tissue samplings after behavioral tests were performed at Zeitgeber Time 4 under *ad libitum* feeding conditions. Immediately after the removal of the brain, sagittal sections with a thickness of 1 mm were separated from the midline to obtain both mRNA and protein samples from the same individual as described (Okamoto et al., 2018). According to the atlas of Paxinos and Franklin (1997), the hippocampus from one side of the section was harvested. Total RNA was isolated with the TRIzol reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA). The cDNA was synthesized using an oligo $(dT)_{18}$ primer and avian myeloblastosis virus reverse transcriptase (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Levels of mRNA in the hippocampus were determined by real-time qPCR analyses with Power SYBR Green PCR Master Mix, respectively. Rn18s (18S rRNA) mRNA was used as an internal standard for mouse cDNA. Specific primer sequences are shown in Table S2.

2.5. Protein extraction and western blotting

Samples of hippocampus from another side of the brain section for mRNA analyses were assigned to immunoblot analyses. Homogenates of tissue samples cleared of debris (10 µg of protein) were subjected to 4–12 % SDS-polyacrylamide gel electrophoresis (PAGE). The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and probed with rabbit polyclonal antibodies to doublecortin (Dcx) or Tet methylcytosine dioxygenase 2 (Tet2) as primary antibodies in Table S3 in Supporting Information. Immune complexes were detected with horseradish peroxidase–conjugated secondary antibodies, enhanced with chemiluminescence reagents, and relative values were estimated by Amersham Imager 600 and Quant TL software. The expression level in each sample was expressed as a ratio to the expression level of actin, probed with a mouse monoclonal antibody to beta-actin as the primary antibody. Specific antibodies and detailed protocols are shown in Table S3.

2.6. Immunohistochemistry

Mice were anesthetized with a combination of medetomidine (0.6 mg/kg), midazolam (8.0 mg/kg), and butorphanol (10 mg/kg), then perfused with phosphate-buffered saline followed by 4% paraformaldehyde. Brain tissue was embedded in paraffin, and 6-µm sections were prepared. Sections were incubated at 4 °C with rabbit polyclonal Ab to Dcx (1:500 dilution, Table S3) in TBST containing 5% normal goat serum for 24 h and then for 2 h at room temperature with biotinylated secondary Ab (Vector Laboratories, Burlingame, CA, USA). Immune complexes were detected with streptavidin-conjugated horseradish peroxidase and diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO, USA).

2.7. Statistical analyses

Data are expressed as means ± standard deviation and compared by One-way or Two-way ANOVA, followed by Tukey-Kramer's post hoc test using GraphPad PRISM (version 9.0). A p-value of <0.05 was considered statistically significant.

3. Results

3.1. Regular Orz apparently delays the onset of MCI induced by long-term consumption of an HFD in obese aged mice

Body weights of mice in both HFD and HFD + Orz (HFD+Orz) groups were substantially increased compared to LCD group, but there was no significant difference in the value between HFD and HFD+Orz groups (Figure 1a). Comparing to HFD group, HFD+Orz group for 4 months showed a marked improvement in spatial working memory (SWM) in Y-maze tests at 66 weeks of age (Figure 1b, upper, left), accompanied by a trend to increase in total arms entries (Figure 1b, upper, right). Furthermore, HFD+Orz group showed a trend to improve recognition for novel objects (Figure 1b, lower, left) . On the other hand, it should be noted that there was no significant improvement at the one month after the HFD+Orz feeding (unpublished observation).

3.2. Nano-Orz potently ameliorates phenotypes of MCI in obese aged mice

To further evaluate therapeutic potential of Orz for MCI, HFD-fed obese mice were administered Nano-Orz in higher or lower doses (HFD + high Orz PO or HFD + low Orz PO, for short) or nanoparticulated FITC as a control twice a week for up to 12 weeks. Expectedly, doses of Nano-Orz did not affect body weight compared to FITC group (Figure 2a). In accordance with our data above, rates of spatial working memory (SWM) were apparently decreased in control mice fed HFD with nanoparticulated FITC (Figure 2b, left). On the other hand, decreased rates of SWM of control mice were significantly improved by administration of Nano-Orz at 3 months (Figure 2b, lower, left). Such an improvement was comparable to that in mice fed HFD with regular Orz for 4 months (Figure 1b, upper, left). In parallel, total arms entries were apparently decreased in control mice fed HFD with nanoparticulated FITC at 3 months (Figure 2b, lower, middle). In contrast, the value showed an apparent trend to increase in mice fed HFD with high Orz PO (Figure 2b, lower, middle). At 3 months, levels of novel object recognition (NOR) also showed a trend to improve

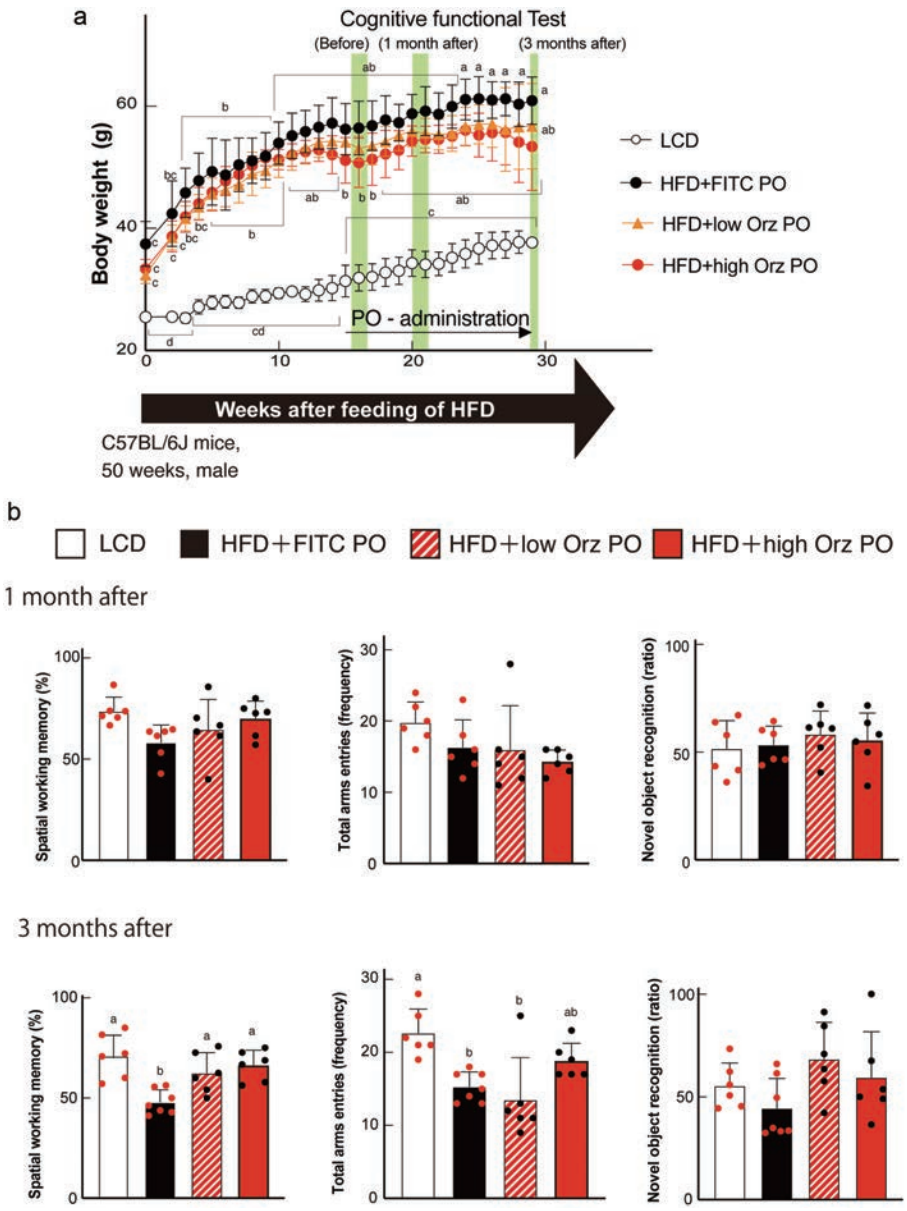

Figure 2. Nano-Orz potently ameliorated MCI in HFD-induced obese aged mice. (a) Body weight changes among 4 groups consisting of aged mice fed lab chow diet (LCD), fed high-fat diet (HFD) treated with control fluorescein isothiocyanate (FITC) (HFD+FITC PO), and fed HFD treated with two different doses of Nanoparticles containing γ-oryzanol (Nano-Orz) (HFD+low Orz PO, HFD+high Orz PO, respectively) (n = 6–7 in each group). Data represent means ± SD. Feeding Time: $F(3, 635) = 2{,}123$, $P < 0.0001$, Agents: $F(28, 635) = 68.47$, $P < 0.0001$, Interaction: $F(84, 635) = 2.026$, $P < 0.0001$, a-d: Means with different letters differ significantly at $P < 0.05$. (b) The upper panels demonstrate the percentage of spontaneous alternation in behaviors (spatial working memory: SWM) (left), the number of entries in the arms of the Y maze (middle) as well as the percentage of time spent for exploring the novel and familiar objects (right) in mice fed lab chow diet (LCD), fed high-fat diet (HFD) treated with FITC (HFD+FITC PO), or fed HFD with two different doses of Nano-Orz (HFD + low Orz PO, HFD + high Orz PO, respectively) for 1 month. The lower panels demonstrate a line of the data after 3 months. Data are expressed as means ± SD (n = 6–7). a, b: Means with different letters differ significantly at $P < 0.05$.

in HFD + Orz PO mice as compared to control mice, but did not reach statistical significance (Figure 2b, lower, right).

3.3. Impact of supplementation of regular Orz on microglial inflammation and neurogenesis in obese aged mice

We initially confirmed that mRNA level of ionized calcium binding adapter molecule 1 (Iba1), an authentic marker for microglia, was considerably increased in hippocampus of HFD mice. In contrast, the level in HFD+Orz mice was markedly decreased comparable to that in the LCD mice (Figure 3a, upper, left), suggesting a beneficial impact of Orz on microglial inflammation in hippocampus. On the other hand, levels of representative pro-inflammatory cytokines such as tumor necrosis factor α(TNF-α) and interleukin 6 (IL-6) were not significantly changed (Figure 3a, upper, middle).

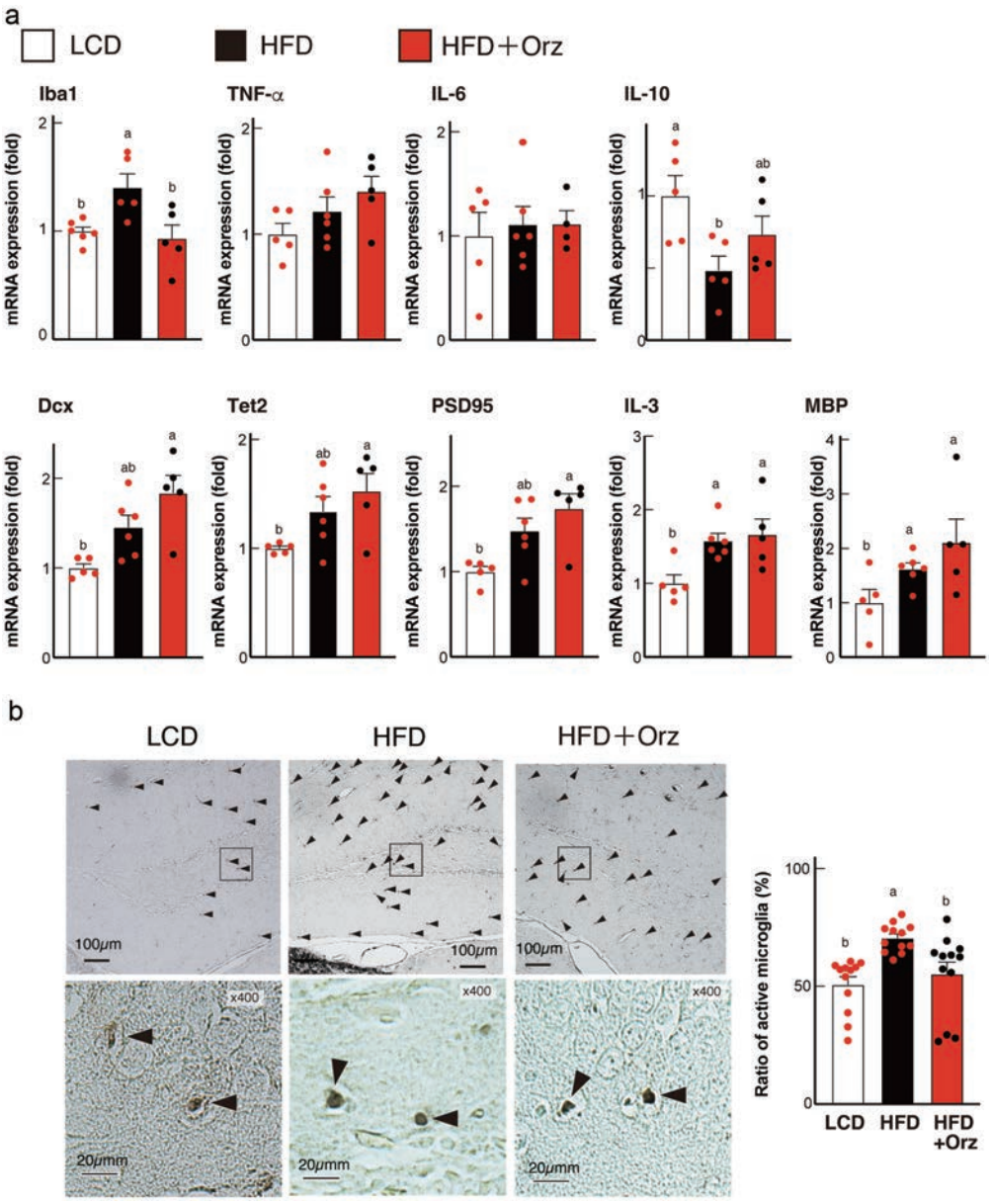

Figure 3. Regular Orz ameliorates microglial inflammation and promotes neurogenesis in hippocampus of obese aged mice. (a) Levels of mRNA for ionized calcium binding adapter molecule 1 (Iba1), tumor necrosis factor α (TNF-α) , interleukin 6 (IL-6), interleukin 10 (IL-10), doublecortin (Dcx), Tet methylcytosine dioxygenase 2 (Tet2), postsynaptic membrane-scaffolding protein 95 (PSD95), interleukin 3 (IL-3) and myelin basic protein (MBP) in hippocampus from aged mice fed lab chow diet (LCD) or fed HFD with or without regular γ-oryzanol (HFD+Orz and HFD, respectively) after 4 months. Data are expressed as means ± SD (n = 5–6). a, b: Means with different letters differ significantly at $P < 0.05$. (b) Percentage of activated microglia in hippocampus after 4 months of HFD feeding with or without regular Orz. Typical histological images of hippocampus in each group and the percentage of activated macroglia in three hippocampal regions in each mouse are shown. The lower boxes show images magnified 400 times. Closed arrowheads correspond to typical morphologies of activated microglia. Right bar graphs illustrate the data analyzed. Data are expressed as means ± SD (n = 12). a, b: Means with different letters differ significantly at $P < 0.05$.

In contrast, mRNA level of representative anti-inflammatory cytokine, IL-10 was substantially decreased in HFD mice, but was apparently increased in HFD+Orz mice (Figure 3a, upper, right). Of note, comparing to HFD mice, level of mRNA of Dcx, an authentic marker of neurogenesis, as well as that of Tet2, a representative marker of neural stem cell showed a clear trend to increase in HFD+Orz mice (Figure 3a, lower, left). On the other hand, mRNA levels of postsynaptic membrane-scaffolding protein PSD95, a major regulator of synaptic maturation implicated in cognitive impairment, as well as astrocyte-derived microglial activation marker IL-3 were not changed by Orz supplementation (Figure 3a, lower, middle). The level of myelin basic protein (MBP), a key protein of nerve myelination, was also not changed (Figure 3a, lower, right).

Immunohistochemical images of hippocampus also showed an apparent increase in number of Iba1-positive, activated microglia

in HFD mice as compared to LCD (Figure 3b, left, left and middle, upper), while the number was decreased in HFD+Orz mice as compared to HFD (Figure 3b, left, middle and right, upper), where closed arrowheads correspond to typical morphologies of activated microglia (Figure 3b, left, lower). Indeed, cell count analyses confirmed that there was a significant accumulation of large amoeboid-activated microglia in HFD mice, while significantly reduced in HFD+Orz mice (Figure 3b right, bar graphs).

3.4. Nano-Orz potently promotes neurogenesis in hippocampus in obese aged mice

After 3 months of oral Nano-Orz administration, contrary to our results in mice treated with regular Orz (Figure 3), mRNA level of Iba1 in hippocampus was unexpectedly elevated in both HFD+low Orz PO and HFD+high Orz PO mice compared to HFD+ FITC control mice (Figure 4a, upper, left). Under the administration of Nano-Orz, it is possible that the polarity (pro-inflammatory M1 *vs.* anti-inflammatory M2 phenotypes) of microglia in hippocampus would be changed irrespective of the total amount of microglia. However, we have not yet clarified mechanisms to explain such a discrepancy.

On the other hand, mRNA level of IL-6 was not significantly changed among 4 groups (Figure 4a, upper, right), as in the regular Orz experiments (Figure 3). Similarly, IL-10 mRNA level was significantly elevated in HFD+high Orz PO mice compared to HFD+FITC PO mice (Figure 4a, middle, left), as in the regular Orz experiments (Figure 3).

Levels of mRNA for Dcx and Tet2 in HFD+low Orz PO and HFD+high Orz PO mice were apparently increased as compared to HFD+FITC PO mice in a dose-dependent fashion (Figure 4a, middle, middle and right). In accordance with this finding, western blot analyses verified that level of Dcx protein was substantially decreased to approximately 30% in HFD+FITC PO as compared to LCD, whereas it was apparently increased in Nano-Orz mice as compared to HFD+FITC PO mice (Figure 4b, left). Tet2 protein level in HFD+high Orz PO mice also showed a trend to increase as compared to HFD+FITC PO mice (Figure 4b, right).

4. Discussion

The present study is the first to demonstrate that γ-oryzanol (Orz), a unique component specific to brown rice, promotes neurogenesis and ameliorates microglial inflammation in hippocampus of obese aged mice, thereby offering therapeutic potential for obesity-related mild cognitive impairment (MCI). Furthermore, many parts of beneficial effects provided by the regular Orz were much exaggerated by Nano-Orz. Based on our previous report demonstrating that oral administration of Nano-Orz in mice markedly enhanced absorption efficiency from intestine by approximately 1,000-fold, thereby resulting in substantial attenuation of ER stress throughout the brain (Kozuka, Shimizu-Okabe, et al., 2017), the present study suggests that Nano-Orz would be a promising tool to improve obesity-related MCI also in humans.

We previously demonstrated that a high-fat diet containing 0.4% Orz for 12 weeks considerably reduced the overconsumption of animal fats in obese mice via epigenetic modification of type 2 dopamine receptor in striatum (Kozuka, Kaname, et al., 2017). Based on this finding, in the present study, a roughly comparable amount of Orz in total was provided to mice for 4 months via a high-fat diet supplemented with 1 % Orz.

Arithmetically, feeding an 1% Orz-containing diet for 4 months results in a total dose of 112×10^3 mg/kg of Orz. Using body surface area normalization methods (Reagan-Shaw et al., 2008), the human equivalent dose for Orz was approximately calculated as 9.08 g/kg, which equates to a 544.8 g dose for a person with 60kg. Orz is exclusively contained in brown rice at a concentration of 0.045 % (Tsuzuki et al., 2019), suggesting a total of 1,211 kg of brown rice would be required to improve MCI in humans. Given the standard serving size for an adult male is 1/2 cup of cooked rice (approximately 150g) (Foley, 2023), this equates to roughly 7.3 years of daily consumption. While the intake seems to be undoubtedly substantial, this dosage is considered entirely achievable through the routine consumption of rice. Because Orz is water-insoluble, but also its absorption efficiency from the intestine is extremely low (Kozuka, Shimizu-Okabe, et al., 2017), human evidence on optimal doses of γ- oryzanol in terms of improvement in metabolic or cognitive dysfunction has not yet been established.

To enhance the absorption efficiency of Orz, a maximum concentration of 100 mg/mL nanoparticle solution containing 42% Orz was orally administered in a 250 μL water suspension twice a week for 4-12 weeks in mice. The total dose of Nano-Orz was 140×10^3 mg/kg, equivalent to 121.35 g/kg in humans. This translates to a dose of 681 g for a human with 60 kg. To consume such an amount, a person would need to eat a total of 1,513 kg of brown rice. In case a person consumes rice daily, it would take 9.2 years. In fact, we previously reported that orally-administered Nano-Orz in mice was rapidly absorbed as an aqueous solution, remained in the body for longer than a day without adverse effects such as diarrhea and dysmetabolism under careful observation, and, noticeably, at least 1/1,000 of the dosage of Nano-Orz reproduced equivalent metabolically-beneficial effects to those in regular Orz (Kozuka, Shimizu-Okabe, et al., 2017). In the present study, administration of Nano-Orz accelerated cognitive improvement roughly one month earlier as compared to Regular Orz ingested with feed. Based on these findings, it is tempting to speculate that Nano-Orz would be beneficial for humans with diet-related CI. However, a line of clinical trials is warranted to test these hypotheses in humans in the near future.

Obesity is known to promote inflammatory responses in various organs, particularly in the brain (Woo et al., 2022). It has been documented that excessive and long-term intake of an animal fat diet robustly activates microglia via Toll-like-4 receptor (Wang et al., 2012), thereby causing microglial inflammation in the hippocampus and resultant MCI in mice (Miller and Spencer, 2014). In agreement with this notion, the mRNA level of Iba1, a representative marker for microglia, was considerably elevated in hippocampus of mice fed a HFD for 4 months (Figure 3a, upper, left). Although the HFD+Orz mice did not show a significant reduction in body weight gain compared to the LCD mice (Figure 1a), their cognitive decline was apparently improved (Figure 1b). In the brain where Orz accumulates in large amounts (Kozuka et al., 2015), the hippocampal Iba1 mRNA level in HFD+Orz mice was comparable to that in LCD mice, indicating that Orz potently suppresses microglial inflammation and contributes to prevent cognitive impairment. (Figure 3a and 3b). Furthermore, the ratio of ameboid-shaped, large, activated microglia was considerably reduced in the hippocampus of HFD+Orz mice as compared to HFD mice (Figure 3b). Of note, mRNA level of IL-10, an authentic anti-inflammatory cytokine, was decreased in HFD mice, whereas it was significantly elevated in HFD+Orz mice (Figure 3a, upper, right) as well as HFD + high Orz PO mice (Figure 4a, middle, left) as compared to HFD control mice, raising a possibility that anti-inflammatory impact is concomitantly reinforced by Orz, thereby ameliorating chronic inflammation in hippocampus of obese aged

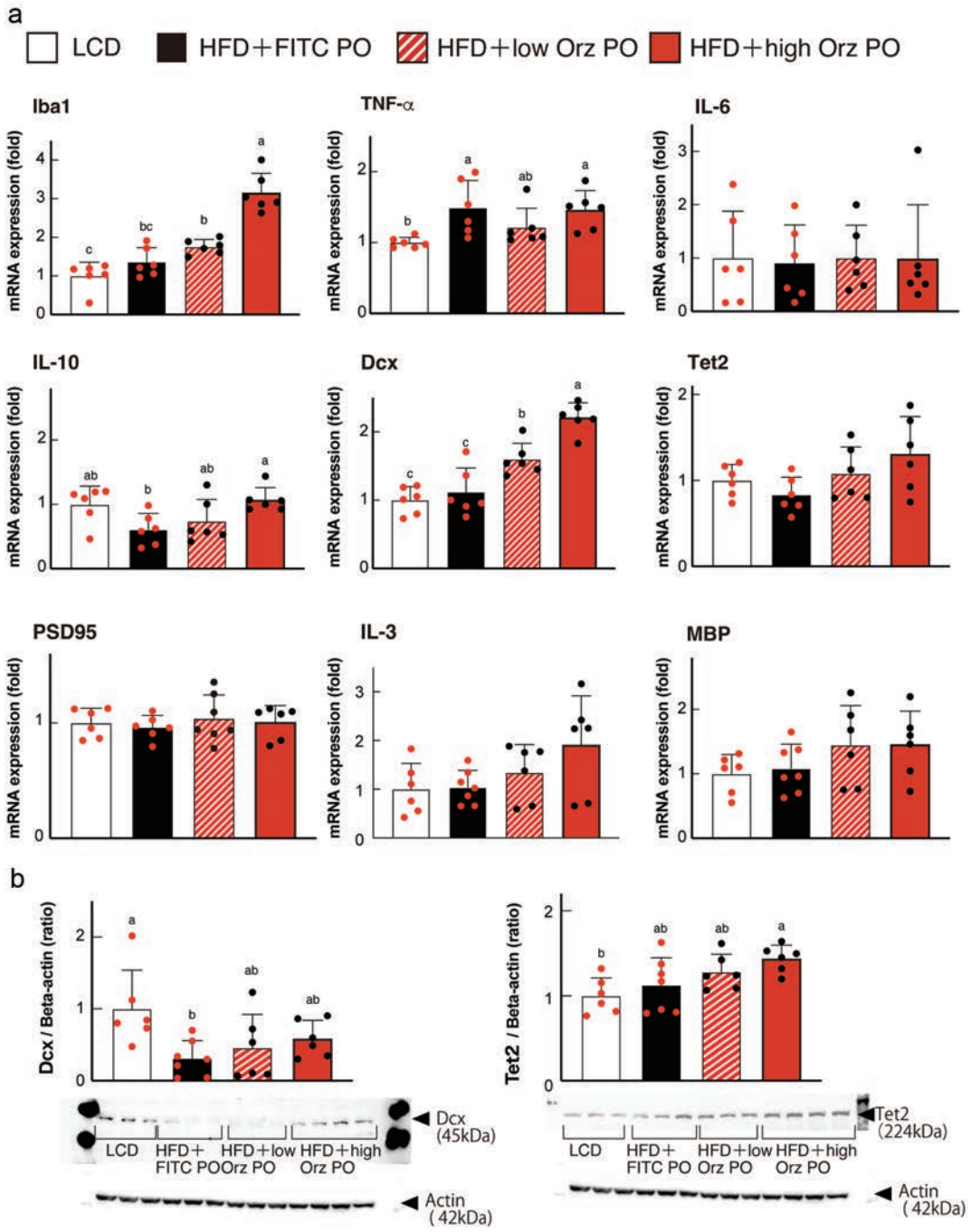

Figure 4. Nano-Orz is potent to promote neurogenesis in hippocampus from obese aged mice. (a) Levels of mRNA for ionized calcium binding adapter molecule 1 (Iba1), tumor necrosis factor α (TNF-α) , interleukin 6 (IL-6), interleukin 10 (IL-10), doublecortin (Dcx), Tet methylcytosine dioxygenase 2 (Tet2), postsynaptic membrane-scaffolding protein 95 (PSD95), interleukin 3 (IL-3) and myelin basic protein (MBP) in hippocampus from aged mice fed lab chow diet (LCD), fed high-fat diet (HFD) after 3 months treated with FITC (HFD+FITC PO) or fed HFD with two different doses of Nano-Orz (HFD+low Orz PO, HFD+high Orz PO, respectively). Data are expressed as means ± SD (n = 6–7). a, b: Means with different letters differ significantly at $P < 0.05$. (b) Protein levels of Dcx and Tet2 in hippocampus after 3 months treated with FITC (HFD+FITC PO) or two different doses of Nano-Orz (HFD+low Orz PO, HFD+high Orz PO), respectively. Data are expressed as means ± SD (n = 6–7). a, b: Means with different letters differ significantly at $P < 0.05$.

mice.

It has also been shown that hippocampal neurogenesis is suppressed by HFD and worsened by obesity and type 2 diabetes in mice (Robison et al., 2020). In the present study, regular Orz feeding as well as Nano-Orz administration considerably increased the expression of Dcx and Tet2, both of which are representative markers for neurogenesis in obese aged mice. This result suggests that Orz would promote neurogenesis in hippocampus (Figure 3a,

lower, left, Figure 4a, middle, middle, and right). Indeed, microglial cells are crucial for both waste removal and reconstruction of the neural network in the brain (Kalakh and Mouihate, 2017). Furthermore, enhanced expression of Tet2 is known to restore stem cell function, thereby mitigating decline in neurogenesis and MCI in aged mice (Gontier et al., 2018). We therefore consider that intake of Orz suppressed microglial inflammation in the hippocampus, thereby promoting neurogenesis from neural stem cells and preventing cognitive impairment exacerbated by diet-induced obesity. In this context, further studies are warranted to identify cell populations activated by Orz in hippocampus.

It would be intriguing that administration of Nano-Orz showed an apparent trend to increase the mRNA level of astrocyte-derived cytokine IL-3 in hippocampus (Figure 4a, lower, middle). IL-3 is known to promote neuronal proliferation and increase brain volume (Luo et al., 2012). It has also been demonstrated that oral administration of 100 mg/kg of γ-oryzanol to old mice for 21 days increased α-synuclein expression, contributing to enhance synaptic plasticity and glutathione-S-transferase level in hippocampus (Rungratanawanich et al., 2019). Similarly, the same kind of forced administration of Orz in mice augmented the expression of phase II antioxidant enzymes such as heme oxygenase-1 (HO-1) and NADPH-dehydrogenase-quinone-1 (NQO1), and suppressed the expression of a line of inflammatory cytokines in the hippocampus, thereby alleviating neuroinflammation and cognitive impairment induced by the bacterial endotoxin LPS (Mastinu et al., 2019). It has also been reported that consuming 0.5% Orz-containing diet from younger age in mice augmented mRNA level of brain-derived neurotrophic factor (BDNF) but also decreased mRNA level of IL-1β in the hippocampus, thereby reducing anxiety-like behaviors that is exacerbated by adolescent alcohol exposure (Akter et al., 2020). These reports did demonstrate that γ-oryzanol absorbed from the digestive tract acts on the brain to reduce hippocampal inflammation, and experimental consequences are in accordance well with our findings. The present study demonstrated that the expressions of Dcx, a marker of potent hippocampal neurogenesis as well as Tet2, an established marker of neural stem cells, were considerably augmented by the intake of Orz in mice (Figure 3a, lower, left, Figure 4a, middle, middle, and right). We also found that the beneficial impact of cognitive impairment by Orz was not limited to a prevention when administered before obesity develops, but was highlighted as a therapeutics when administered after MCI was established (Figure 1b, upper, left, and right, Figure 2b, lower, left, and right). Taken together, it is likely to speculate that Orz may enhance cognitive function in a multi-faceted fashion.

We do acknowledge there are a couple of limitations in the present study. As we described in the manuscript, regular Orz markedly decreased the mRNA level of Iba1 as well as the number of Iba1-positive, activated microglia in hippocampus, suggesting a beneficial impact of Orz on microglial inflammation. However, the mRNA level of Iba1 in hippocampus was unexpectedly elevated under the supplementation of Nano-Orz. Currently, we have not yet clarified underlying mechanisms to explain such a discrepancy, and to answer this enigma, a couple of further studies will be warranted, including precise analyses of the polarity (pro-inflammatory M1 *vs.* anti-inflammatory M2 phenotypes) of microglia in hippocampus of obese aged mice under the administration of Nano-Orz. Second, we focused our attention in the present study on potential impact of regular Orz and Nano-Orz on MCI in obese aged mice. Therefore, the present study did not include any experimental paradigm focused on diabetes mellitus *per se.* Third, the present study did not evaluate the impact of Orz on the real neurogenesis in hippocampus but just assessed the expression of convincing surrogate markers, including Dcx and Tet2. We acknowledge this issue is also our future challenge.

5. Conclusions

Collectively, our results highlight a versatile potential of Orz on both reducing aging-associated and HFD-driven microglial inflammation and promoting neurogenesis in hippocampus in aged obese mouse models. In particular, a line of beneficial impact of Orz on cognitive function is much exaggerated under the administration of Nano-Orz. Considering that natural food-based prophylactic approach toward MCI is attracting broad interest from academic, clinical, and industrial fields, nanoparticulated Orz is worth being one of the prototypes for such an approach.

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Declaration of Generative AI and AI-assisted technologies in the writing process

The authors declare that neither generative AI nor Ai-assisted technologies were used in the writing of this manuscript.

Data availability statement

Data for this article are available at https://data.mendeley.com/datasets/zm6498pc95/2 in Mendeley Data. The additional data sets generated are available from the corresponding author upon reasonable request.

Conflict of interest

This research is covered by the following patent: Japan Patent Office. Japan Patent No. 7426036 (24 January 2024).

Author contributions

Conceptualization, S.O., C.K., M.S., C.T., M.M., K.A. and H.M.; methodology, S.O., I. N., A.K., Y. M., C.H. and T.T.; investigation, S.O., I. N., A. K., and Y. M.; resources, T.N.; writing-original draft preparation, S.O and H.M.; writing-review, editing and guarantors, S.O., T.T., T.U. and H.M.; supervision, C.K., M.S., C.T., M.M., K.A.; funding acquisition, S.O. and H.M. All authors have read and agreed to the published version of the manuscript.

Supplementary material

Table S1. Comparison of nutritional composition among Lab chow diet, high fat diet and γ-oryzanol (Orz)-containing high fat diet.

Table S2. Primer sequences used in the present study oligonucleotides.

Table S3. Antibody information in the present study.

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