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Trehalose intake and exercise upregulate a glucose transporter, GLUT8, in the brain

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ABSTRACT

Physical exercise influences cognitive function through a cascade of cellular processes that promote angiogenesis and neurogenesis. Autophagy is a cellular degradation system that is capable of producing energy in response to various conditions such as starvation, physical exercise and several treatments. Our previous report demonstrated that a disaccharide, trehalose, induced autophagy in the brain and reduced the levels of potentially toxic proteins. To achieve more efficient induction of autophagy in the brain, in this study, we examined the effect of disaccharide intake combined with exercise on autophagy in vivo. Consistent with the results of previous studies, our biochemical analyses demonstrated that trehalose increased the level of lipidated LC3 (LC3II) in the brain and liver of adult mice. However, contrary to our expectation, treadmill exercise reduced the level of LC3II in the brain and liver. Interestingly, glycogen storage was preserved in the liver of trehalose-intake mice even after exercise. Moreover, the trehalose transporter GLUT8 was increased in the liver by trehalose or in the brain by trehalose together with exercise. In contrast, the level of GLUT4 remained stable in the liver and brain even after exercise. These findings suggest that trehalose and GLUT8 coordinately contribute to energy supply in the brain.

1. Introduction

Physical exercise is essential for maintaining physical function and health and appears to be important for maintaining cognitive performance in humans. In fact, epidemiological studies demonstrated a reduced risk of mild cognitive impairment and dementia in older adults who maintain higher levels of physical activity [1,2]. Aerobic exercise changes the structure and function of brain regions vulnerable to age-related and disease-related atrophy [3,4], suggesting that physical exercise may improve cognitive performance and could be a therapeutic strategy for neurodegenerative diseases such as dementia.

There has been no treatment to halt neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, and dementia with Lewy bodies, to date. Accumulating evidence has revealed that the autophagic system is disrupted and that autophagic substrates, including p62, Keap1 and NBR1, accumulate in the brains of these patients [5–7]. Autophagy is a cellular degradation system that is capable of producing nutrients and energy in response to various conditions, such as nutrient starvation and exercise [8,9]. Because autophagy also serves as a way of efficiently degrading abnormal proteins [10], activated autophagy could represent a potential therapeutic strategy for diseases that involve abnormally aggregated proteins, including neurodegenerative diseases and lysosomal diseases.

Indeed, we previously reported that oral intake of trehalose for a relatively short period (up to 1 week) is sufficient to activate autophagy in the mouse brain. Furthermore, activation of autophagy in the brain can ameliorate neurodegenerative pathology and phenotypes [11]. Therefore, we aimed to more efficiently induce autophagy in the brain and examined the effect of exercise together with trehalose intake on autophagic flow in the mouse brain.

2. Materials and methods

2.1. Animals

All studies and procedures were carried out with the approval of the Committee of Medical Ethics of Hirosaki University Graduate School of Medicine, Hirosaki, Japan. Animals were housed under

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standard conditions (12 h light, 12 h dark; food and water available ad libitum). Normal and transgenic (Tg) mice (n = 36, 12–48 weeks of age) were orally subjected to 2% (w/v) trehalose (n = 10), maltose (n = 10), sucrose (n = 8) or water (n = 8). All disaccharides were purchased from Wako (Osaka, Japan) and dissolved in drinking water, and these solutions were changed every two days. A subgroup of 18 mice were exercised on a rodent treadmill (Columbus Instruments, Columbus, Ohio, USA). The treadmill exercise training was performed on the last 3 days (the 5th, 6th and 7th day). Each training session started with a speed of 8 m/min for 5 min and was then increased by 1 m/min every 5 min until a maximum speed of 12 m/min was reached: 5 min on the first day, 10 min on the second day, and 40 min on the last day. GFP-LC3 Tg mice were purchased from RIKEN BioResource Center (Wako, Japan). GFP-LC3 Tg mice were confirmed by conventional PCR [12].

2.2. Antibodies and immunohistochemistry

Mice were transcardially perfused with phosphate-buffered saline (PBS). The brain was removed, and the right hemisphere was fixed with 4% paraformaldehyde for 48 h. After dehydrating through a graded ethanol series, the right hemisphere was embedded in paraffin and cut into 4-μm-thick sections. The left hemisphere was frozen at −80 °C for subsequent biochemical analyses. The sections were dehydrated and pretreated with heat retrieval using an autoclave for 10 min in 10 mM citrate buffer (pH 6.0) for primary antibodies. The sections were then subjected to immunohistochemical processing using the avidin-biotin-peroxidase complex method with diaminobenzidine (Sigma, St. Louis, MO, USA). In addition, the sections were counterstained with hematoxylin. The following primary antibodies were used: rabbit antibodies against AMPK (Cell Signaling Technology, Inc., Danvers, MA, USA; 1:2,000), phosphorylated AMPK at The173 (Cell Signaling Technology, Inc.; 1:1,000), actin (Sigma; 1:2,000), LC3 (Sigma; 1:4,000), mTOR (Cell Signaling Technology, Inc.; 1:1,000), P-mTOR (CST, 1:1,000), glucose transporter 4 (GLUT4; ProteinTech Group, Inc., Chicago, IL, USA; 1:2,000), and GLUT8 (Abcam, Cambridge, UK; 1:2,000).

2.3. Periodic acid-Schiff stain

Periodic acid-Schiff (PAS) staining was performed to detect glycogen in the liver according to standard protocols. Succinic dehydrogenase staining on deparaffinized sections was carried out using 0.2 M phosphate buffer at pH 7.6. For staining, 270 mg succinic acid and 10 mg nitro blue tetrazolium were freshly dissolved in 10 ml phosphate buffer, and the sections were incubated for 1 h. The sections were washed several times. To confirm that the positive staining is glycogen, we pretreated the sections with 1% amylase for 30 min at 37 °C followed by PAS staining.

2.4. Immunoblot analysis

Western blot analysis was performed as previously described [13]. For total cell lysate, we used a lysis buffer with 4% sodium dodecyl sulfate (SDS; 75 mM Tris-HCl, pH 6.8, 4% SDS, 25% glycerol, 5% β-mercaptoethanol) and passed the sample through a 21-gauge needle attached to a 1-ml syringe. Signal detection was performed according to the protocol provided with the ECL or ECL prime detection systems (Amersham Pharmacia Biotech, Piscataway, NJ, USA). We performed each immunoblot analysis a minimum of three times, and all data were quantified and collected.

2.5. Tissue clearing and light sheet microscopy

To detect LC3 in three dimensions (3D), we used whole-brain imaging with single-cell resolution using the SCALE method [14], which enables the visualization of fluorescent-labeled proteins and cells deep within the brain. The brain and liver were harvested from GFP-LC3 Tg mice and made transparent with the SCALE method. Fluorescence images were acquired with a light sheet microscope (Zeiss Light-sheet Z.1 Microscope, Carl Zeiss, Jena, Germany) using the same process described above. 3D-rendered images were captured with ZEN software (Version 7.6.4).

2.6. Quantitative analysis and statistical analysis

A semiquantitative analysis of protein levels was performed using ImageJ software provided by the NIH. All data were represented as the mean ± standard deviation. The statistical significance was evaluated using one-way ANOVA with Bonferroni's post hoc test to analyze four or eight groups and Student's *t*-test to analyze two groups. A probability value of less than 0.05 ($p < 0.05$) was considered to be significant.

3. Results

3.1. Alteration of autophagy-related molecules in the brain after exercise with or without disaccharide

To initially examine the effect of disaccharide on autophagic molecules in the brain, mice were orally subjected to 2% (w/v) trehalose, maltose, sucrose or water for 7 days. Consistent with previous studies [15], Western blot analysis demonstrated that LC3 levels were increased in mice with trehalose compared with others (Fig. 1A and B). Next, to investigate the combined effect of disaccharides and exercise on autophagic molecules in the brain, mice fed as described above were subjected to a series of treadmill exercises. Contrary to our expectation, the LC3II level was decreased in all groups after the treadmill exercise. Nevertheless, one of the autophagic pathways, AMPK, changed its phosphorylation level. Thus, the rate of phosphorylated AMPK was significantly higher in trehalose-intake mice after exercise compared to controls. Despite our extensive attempts to find any differences between mice with and without exercise, LC3 immunostaining (Fig. 1C) and morphological studies (Fig. 1D) demonstrated no changes in the distribution and staining intensity of LC3 in the brain.

3.2. Alteration of glycogen and autophagy-related molecules in the liver after exercise with or without disaccharide

In general, exercise reduces glycogen storage in the liver, which is a major organ that regulates energy sources in the whole body [16]. To confirm the effect of exercise on metabolic responses in mice, we examined glycogen storage in the liver. PAS staining revealed purple granular staining in maltose-, trehalose-, and sucrose-intake mice (Fig. 2A). Pretreatment with amylase diminished PAS-positive staining (data not shown), regardless of the kind of disaccharide, suggesting that glycogen storage occurs in the liver of these mice. After exercise, the staining clearly disappeared in maltose- and sucrose-intake mice, whereas it remained in trehalose-intake mice (lower panels of Fig. 2A). Western blot analysis showed that the rate of phosphorylated AMPK was higher in the liver of trehalose-intake mice, regardless of the presence or

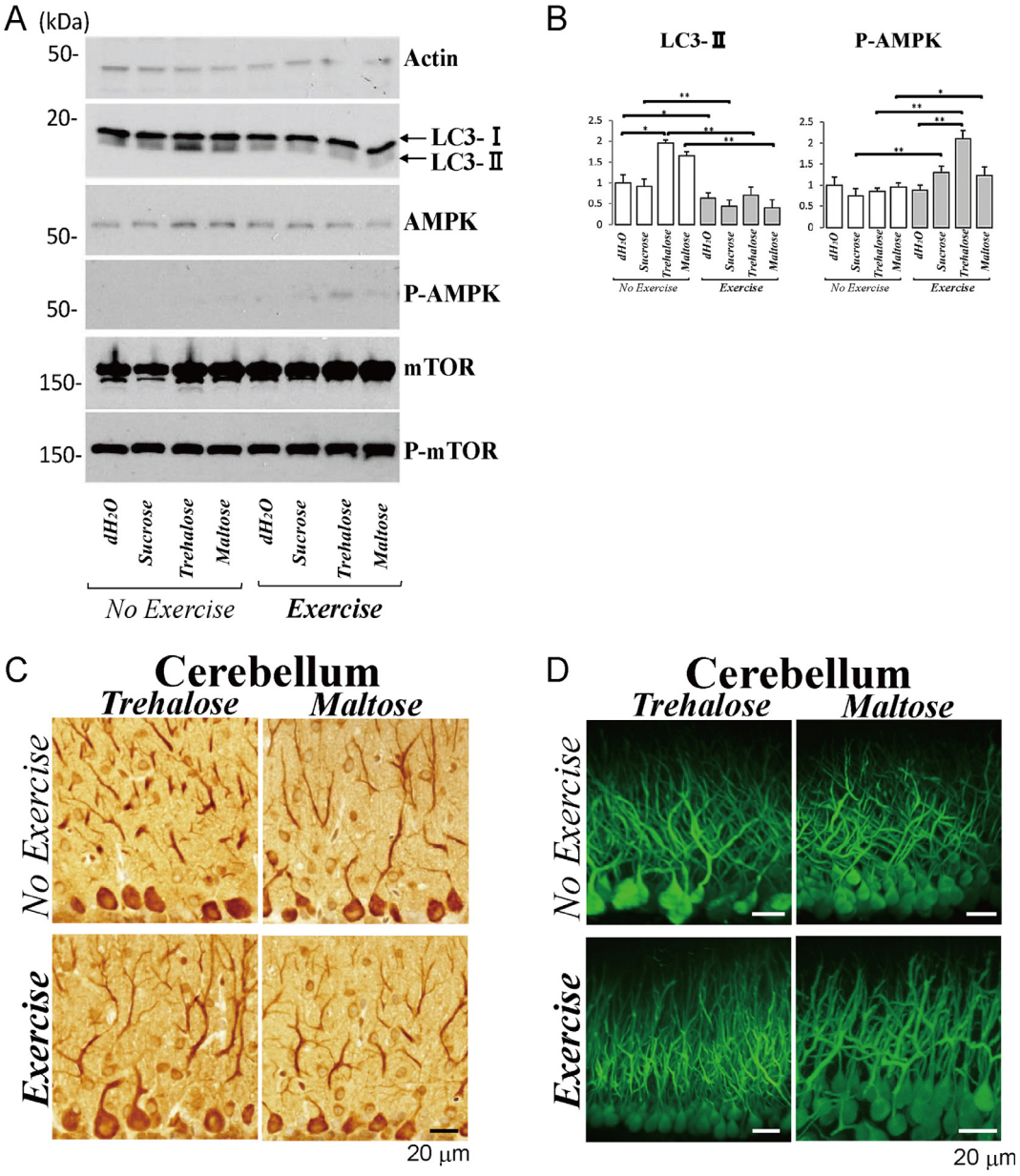

Fig. 1. Alteration of autophagy-related molecules in the brain after exercise with or without disaccharide. Mice were divided into 8 groups: distilled water (dH_2O, n = 4, n = 4 with exercise), sucrose (n = 4, n = 4 with exercise), trehalose (n = 5, n = 5 with exercise) or maltose (n = 5, n = 5 with exercise) for 7 days. Four of 8 groups were subjected to treadmill exercise (5 min on the first day, 10 min on the second day, and 40 min on the last day at 15°). All mice were sacrificed immediately after the last exercise. (A) Western blot analysis showing that LC3II levels are significantly increased in trehalose intake mice (p <0.01) but decreased after exercise (p <0.01). P-AMPK signaling is significantly activated in trehalose-intake mice after exercise (p <0.01). Molecular mass is indicated on the left side of the panel. (B) Quantitative analysis of the data shown in A. LC3 or P-AMPK levels are normalized by the level of actin or AMPK protein in each sample, respectively, and indicated as the mean + SD. The control value is defined as 1.0. *p <0.05, **p <0.01. (C) LC3 immunohistochemistry staining of the cerebellum demonstrating that LC3-positive signals are densely found in the cytoplasm, as well as the dendrites, of Purkinje cells. There was no change in the staining intensity of LC3 between wild-type mice with and without exercise. (D) Light-sheet microscopy showing that LC3 signals are detected in the cerebellum of GFP-LC3 Tg mice. There were no differences in GFP-LC3 localization among the 4 groups.

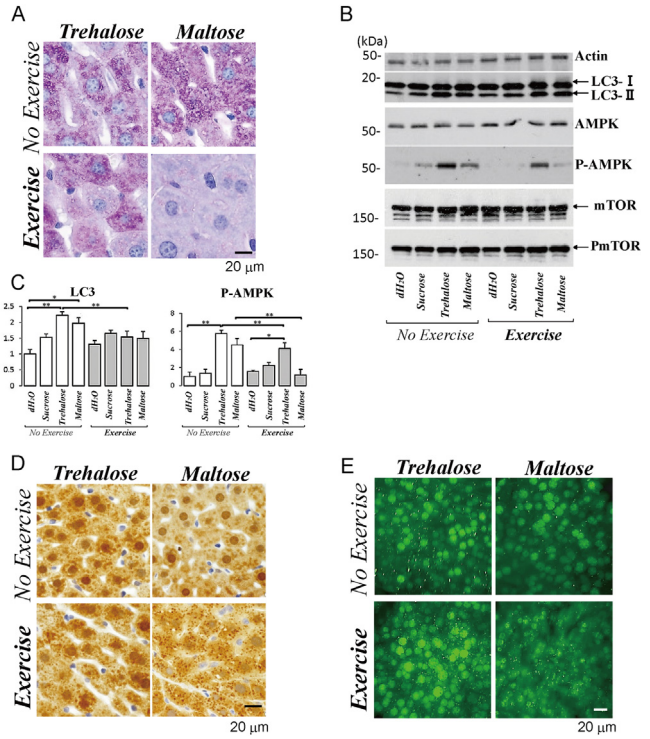

Fig. 2. Alteration of glycogen (A) and autophagy-related molecules (B, C) in the liver after exercise with or without disaccharide. (A) Periodic acid-Schiff (PAS) staining showing glycogen deposits in the liver of trehalose- and maltose-intake mice. Exercise reduces glycogen storage in maltose-intake mice but not in trehalose-intake mice. (B) Western blot analysis showing that LC3II levels are significantly increased in trehalose- and maltose-intake mice, whereas LC3II levels are decreased after exercise (p <0.01). AMPK signaling is activated in trehalose-intake mice after exercise. (C) Quantitative analysis of the data shown in A. $^{*}p$ <0.05, $^{**}p$ <0.01. (D) LC3 immunohistochemistry showing that nuclear staining is apparent in the hepatocytes of trehalose- or maltose-fed mice. After exercise, cytoplasmic and nuclear signals are observed in trehalose-intake mice. Dot-like signals appear instead of nuclear staining weaken in maltose-intake mice. (E) In the GFP-LC3 Tg mice, the nuclear signals are apparent in the hepatocytes in trehalose- or maltose-intake mice. Cytoplasmic signals appear in addition to the nuclear signals in both trehalose- and maltose-intake mice.

absence of exercise (Fig. 2B and C). In addition, LC3II levels were significantly higher in trehalose-intake mice than in other mice. These results suggest that trehalose has some roles in metabolic homeostasis. LC3 immunohistochemistry revealed that cytoplasmic staining was apparent in the hepatocytes after exercise (lower panels of Fig. 2D). In support of immunostaining data, light sheet microscopy demonstrated that GFP-LC3 signals appeared in the cytoplasm of the hepatocytes after exercise. Notably, nuclear GFP-LC3 signals remained in mice after exercise with trehalose (Fig. 2E).

3.3. Increase of GLUT8 in the brain after exercise with trehalose

To further investigate how trehalose exhibits unique properties, such as activation of the AMPK pathway and glycogen preservation in the liver, we focused on GLUT8, which was recently identified as a trehalose transporter [17]. Immunoblotting showed that GLUT8 levels were significantly increased in the brains of trehalose-intake mice after exercise, whereas one of the other GLUTs, GLUT4, which regulates metabolic homeostasis in general organs, was not altered among all groups (Fig. 3A and B). Similarly, GLUT8 levels were significantly increased in the livers of trehalose-intake mice (Fig. 3C and D). Immunohistochemical staining demonstrated that GLUT8-positive signals were mainly observed in the cerebral cortex in all groups: controls and sucrose-, maltose- and trehalose-intake mice (Fig. 4A). A more intense staining pattern of GLUT8 occurred in the brain and liver of trehalose-intake mice. In detail, GLUT8-positive staining spread to the hippocampus and striatum in trehalose-intake mice (Fig. 4B). Further analysis showed that GLUT8-positive signals were partly immunolabeled with anti-GFAP antibody but not with anti-synaptophysin or CNPase antibodies, suggesting that increased GLUT8 protein is partly expressed in astrocytes (Fig. 4C).

4. Discussion

Autophagic flux is crucial to maintain physiological functions in various organs and cells and is activated by several factors, such as fasting, and several treatments [18]. We examined the effect of disaccharide and/or exercise on autophagy in this study. Consistent with these lines of evidence [10,19,20], immunoblotting and immunohistochemical analyses demonstrated that a natural saccharide, trehalose, can activate autophagy in the brain, as well as in the liver. Since it is well-known that exercise is also able to activate autophagy, we expected that the synergistic effect of trehalose and exercise on autophagy in the brain. However, contrary to our expectations, immunoblot analysis showed that LC3II levels were decreased in the brain, even in mice fed disaccharides, such as sucrose, maltose or trehalose. Furthermore, despite our extensive efforts to find any effects during exercise, we could not demonstrate any changes in the distribution and staining intensity of LC3 in the brains of mice after exercise. Consistently, a previous study described that autophagy was not induced in the brain, even after 48 h of starvation [12]. Although autophagy is thought to be required for normal turnover of cellular components, particularly in urgent response to starvation and exercise, we have to pay more attention to differences in autophagic response depending on organs and/or cell types.

Nevertheless, in the brain, we found that trehalose phosphorylates AMPK, which is a metabolic sensor. AMPK is known to be transiently activated in muscle by acute exercise [21,22]. Bayod et al. also reported that AMPK was significantly activated in both the cerebral cortex and hippocampus in exercised rats [23]. Consistently, our results demonstrated that exercise significantly induced phosphorylated AMPK in the brains of mice with sucrose, maltose or trehalose. However, we could not find increased LC3II levels in mice after exercise. He et al. could also not demonstrate increased LC3II levels in the whole brain and the cerebrum [24]. Although we do not have a clear answer as to why AMPK did not activate LC3 lipidation, our results indicate that disaccharides, especially trehalose, can cause differences in metabolic responses in the brain after exercise.

To further investigate the effects of trehalose and exercise on metabolic responses outside the brain, we examined the liver, which plays a well-established role in governing body energy metabolism. Immunoblot analysis showed that LC3II levels were significantly higher in the livers of trehalose- or maltose-fed mice than in controls. Similar to the response found in the brain, LC3II levels were decreased in trehalose- or maltose-intake mice after exercise, and PAS staining revealed that glycogen storage occurred in mice with disaccharides. After exercise, these staining effects diminished, while surprisingly, PAS-positive staining remained in mice with trehalose. After pretreatment with amylase, these staining effects disappeared, indicating that glycogen storage was

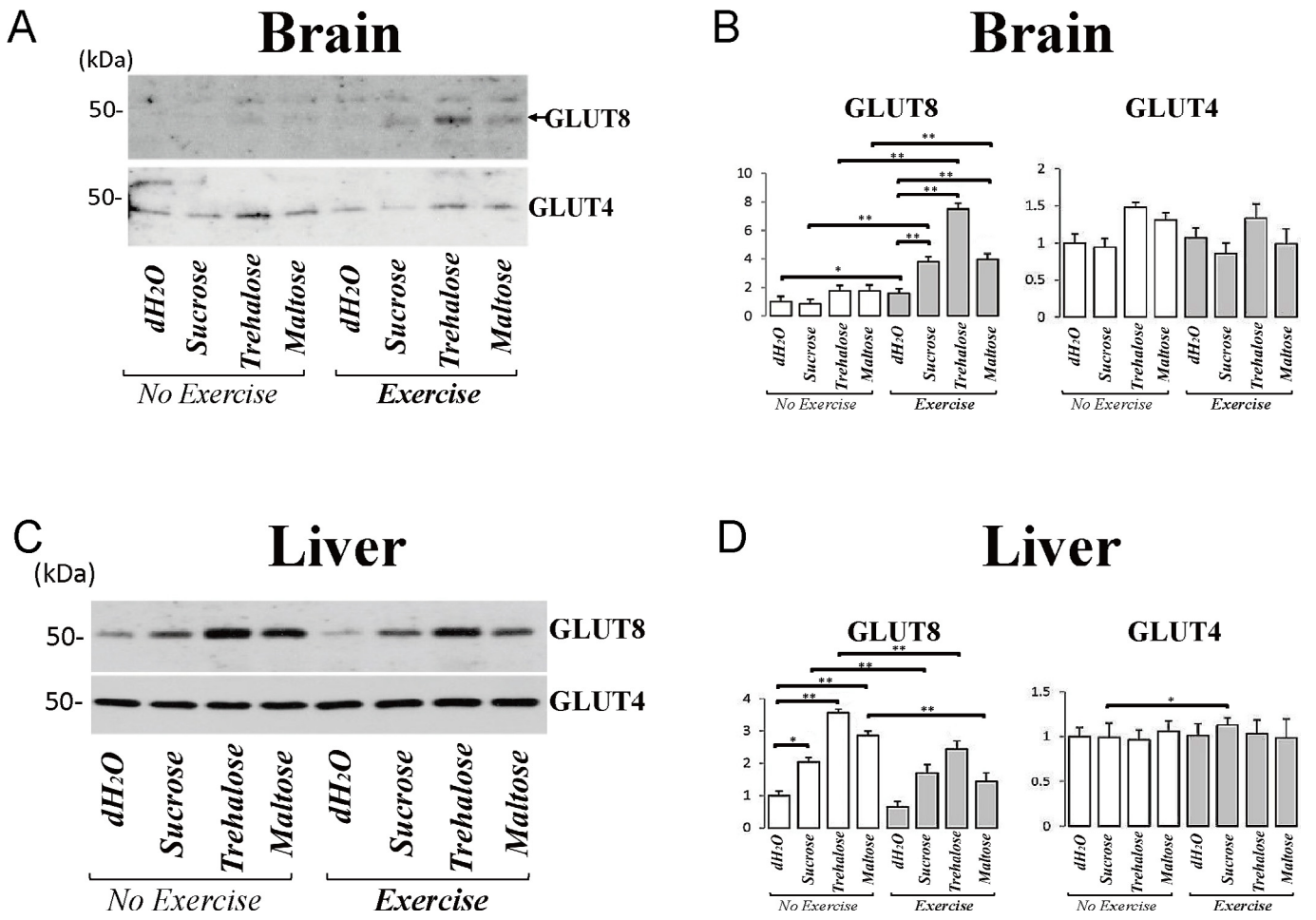

Fig. 3. Increase of GLUT8 in the brain after exercise with trehalose. (A) Western blot analysis showing that GLUT8 levels are increased in the brains of trehalose-intake mice after exercise, whereas GLUT4 levels are not significantly altered. (B) Quantitative analysis of the data shown in A. (C) Immunoblot analysis of the liver showing that GLUT8 levels are increased in trehalose- or maltose-fed mice. After exercise, the GLUT8 level is altered similar to that in mice without exercise. GLUT4 levels are not significantly altered. (D) Quantitative analysis of the data shown in C. Data are normalized by the level of actin protein in each sample and indicated as the mean + SD.

preserved in the liver of mice with trehalose even after exercise. Based on this finding, we hypothesized that trehalose can be used as an alternative energy source instead of glycogen. Recently, Mayer et al. reported that GLUT8 is a trehalose transporter [25], and trehalose induced autophagy through GLUT8. The GLUT family is categorized into 3 groups regarding the homology of amino acid sequences: Class I (GLUT1, 2, 3, 4), Class II (GLUT5, 7, 9, 11) and Class III (GLUT6, 8, 10, 12). Of these isoforms, GLUT4 and GLUT8 are reported to be insulin-responsive, and both exist in the brain [26,27]. Indeed, we detected GLUT4 and GLUT8 signals in the brains and demonstrated that trehalose intake significantly increased GLUT8 levels, while GLUT4 levels remained constant even after trehalose intake together with exercise. An immunohistochemical study showed that both GLUT4 and GLUT8 are expressed in neuronal cells. In addition, GLUT8-positive signals were detected as dot-like structures in certain regions, such as the cerebral cortex. In particular, GLUT8-positive signals spread to the hippocampus and striatum in mice after exercise with trehalose. We attempted to identify whether GLUT8-positive signals are located in neurons or glial cells. A double immunofluorescence study demonstrated that some of these signals exist along with astrocytes, which were immunolabeled with anti-GFAP antibody. GLUT8 signals clearly did not associate with synaptophysin or CNPase, indicating that GLUT8 is unlikely to be involved in neurons or oligodendrocytes. Taken together, these results indicate that neuronal cells require more energy to maintain brain functions during exercise, and astrocytes support this demand through the induction of GLUT8 in the presence of trehalose in serum.

In summary, we found that disaccharide can cause differences in metabolic responses in the brain. Exercise is also believed to work on neuronal and glial functions, as well as physiological brain functions. Further studies will be needed to determine how much exercise maximizes the metabolic benefits, which may lead to efficient therapeutic strategies for diseases in which abnormal proteins aggregate.

Conflicts of interest

The authors declare no competing interests.

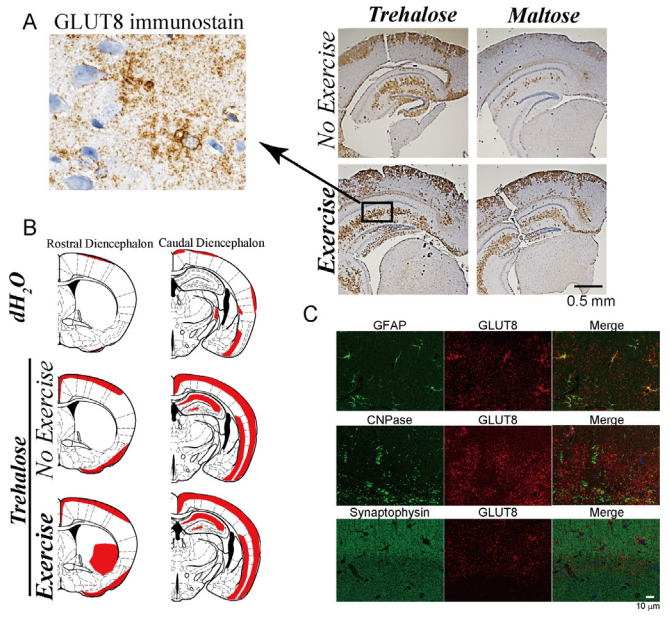

Fig. 4. Distribution of GLUT8 immunoreactivity in the mouse brain. (A) GLUT8 expression is found in certain regions of the central nervous system, mainly in the cerebral cortex and hippocampus. (B) Schematic distribution of GLUT8-immunoreactive structures in the mouse brain with water- or trehalose-intake mice with or without exercise. (C) Double-immunofluorescence labeling demonstrating the colocalization of GLUT8 and GFAP in the hippocampus (upper panel). GFAP appears green, GLUT8 appears red, and colocalization of GFAP and GLUT8 appears yellow. GLUT8 is not colocalized with synaptophysin or CNPase in the hippocampus in trehalose-intake mice after exercise (lower panel). Bars = 20 μm.

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