The Journal of Veterinary Medical Science

FULL PAPER

Laboratory Animal Science

Pathophysiological abnormalities in the brains of Spontaneously Diabetic Torii-*Lepr*^{fa} (SDT fatty) rats, a novel type 2 diabetic model

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J. Vet. Med. Sci.
80(9): 1385–1391, 2018
doi: 10.1292/jvms.18-0296

Received: 30 May 2018
Accepted: 9 July 2018
Published online in J-STAGE: 17 July 2018

ABSTRACT. In recent years, a relationship between diabetes and neurodegenerative diseases, such as Parkinson's disease, Alzheimer disease or depression, has been proposed. In this study, pathophysiological changes in the brain, especially in the hippocampus, of male SDT fatty rats with obesity and hyperglycemia were investigated. Brains of SD rats and SDT fatty rats were collected at 32 and 58 weeks of age, and parietal cortical thickness and number of pyramidal cells in the hippocampal cornu ammonis 1 and 3 (CA1 and CA3) regions were measured. At 58 weeks of age, the parietal cortical thickness and number of pyramidal cells in the hippocampal CA1 and CA3 regions were lower in SDT fatty rats than in age-matched SD rats. Measurements of mRNA in rat brains at 58 weeks of age showed that the expression of genes related to inflammatory responses (S100a9, TNFα, NF-κB) was elevated in SDT fatty rats. From the aforementioned results, changes suggestive of brain atrophy and impairment in cognitive function were observed in male SDT fatty rat brains.

KEY WORDS: hippocampus, neurodegenerative disease, SDT fatty rat

There is some evidence of a relationship between diabetes and neurodegenerative diseases, such as Parkinson's disease [14], Alzheimer disease (AD) [19], depression [1] or cognitive dysfunction [36], in patients. Diabetes-related cognitive dysfunction is complicatedly intertwined with long-term hyperglycemia, insulin deficiency and genetic/environmental factors [37] and is also associated with increased risk of dementia and AD [41]. Although the mechanism by which diabetes reduces cognitive function is not clear, several factors such as oxidative stress, neuroinflammation, and neuronal apoptosis have been shown to be involved in the impairment of brain structure and function [37, 40].

In AD patients, a typical neurodegenerative disease, cognitive decline, and morphological abnormalities such as cerebral atrophy and cell death have also been reported from MRI and postmortem brain studies [9, 27]. Cortical volume and cortical thickness have also been reported as being decreased in type 2 diabetes mellitus (T2DM) patients without AD [4, 7]. Furthermore, in preclinical studies, AD pathogenesis, cognitive decline, and morphological abnormalities in the brain have been reported in diabetic model animals. For example, forebrain cortex and hippocampal volume reduction, neurodegeneration, and increases in amyloid β42 have been observed in streptozotocin (STZ)-induced diabetic rats [39]. Impairments in both maze performance and hippocampal long-term potentiation (LTP) have been observed in Otsuka Long–Evans Tokushima Fatty rats (OLETF) and Zucker Diabetic Fatty (ZDF) rats [2, 30]. Many clinical and preclinical studies suggest that diabetes is closely related to cognitive dysfunction such as AD.

Spontaneously Diabetic Torii (SDT) fatty rats have been reported to be a useful animal model for investigating diabetic complications associated with DM in the kidneys, eyes, and peripheral nerves [16, 18, 23, 26]. In addition, SDT fatty rats have also been shown to be a feasible model for depression [34]. Since diabetes is observed from a young age in this model, the expectation is that the animals may exhibit neurodegenerative diseases that are found in other diabetic model animals. However, there have been no reports to date on neurodegenerative diseases of the brain in this model. In this study, we investigated the pathophysiological changes in the brains of male SDT fatty rats.

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MATERIALS AND METHODS

Animals

This experiment was conducted in strict compliance with our own Laboratory Guidelines for Animal Experimentation and was approved by the Institutional Animal Care and Use Committee of Central Pharmaceutical Research Institute of Japan Tobacco Inc. A total of 15 male SDT $^{fa/fa}$ (SDT fatty) rats (Clea Japan, Tokyo, Japan) were used in the study. Fifteen age-matched male Sprague-Dawley (SD) rats (Clea Japan) were used as control animals. Animals were housed in a climate-controlled room (temperature 23 ± 3°C, humidity 55 ± 15%, 12 hr lighting cycle) and allowed free access to a basal diet (CRF-1, Oriental Yeast, Tokyo, Japan) and sterilized water.

Measurement of biophysiological parameters

Body weights and biochemical parameters, such as plasma glucose, insulin, and blood hemoglobin A1c (HbA1c), were measured at 32 and 58 weeks of age in a non-fasting state. Blood samples were collected from the subclavian vein of rats. Plasma glucose, and blood HbA1c were measured using commercial kits (Roche Diagnostics, Basel, Switzerland) and an automatic analyzer (HITACHI Clinical analyzer 7180; Hitachi, Tokyo, Japan). Plasma insulin levels were measured using rat insulin enzyme-linked immunosorbent assay (ELISA) kits (Morinaga Institute of Biological Science, Yokohama, Japan).

Tissue sampling

Necropsies were conducted at 32 and 58 weeks of age and brains were collected from all animals. For the histopathological examination, rats were anesthetized with isoflurane, and then subjected to transcardiac perfusion with 0.1 M Phosphate buffered saline (PBS) and 4% paraformaldehyde. For the mRNA analysis, designated rats at 58 weeks of age were also subjected to transcardiac perfusion with 0.1 M PBS under isoflurane anesthesia, and brain samples were stored at −80°C until analysis.

Morphometric examination

The tissues were paraffin-embedded using standard techniques and were thin-sectioned (5 μm) from approximately −3.30 mm from the bregma. The sections were stained with hematoxylin and eosin (HE) and Nissl. Each stained section was photographed under an optical microscope and images were digitally saved. HE-stained sections were used to measure left and right parietal cortical thicknesses, and left and right mean values were calculated. Nissl stained sections were used to measure pyramidal cells in the left and right hippocampal cornu ammonis 1 and 3 (CA1 and CA3) regions. Using image processing software, Image J (NIH), the number of pyramidal cells in each of the 3 left and right locations (6 locations in total) per unit area was measured for each section using a blinded method. The unit area was set to 50 × 150 μm for both the CA1 and CA3 regions of the hippocampus. The number of pyramidal cells was taken as the average value of 6-unit areas. In this experiment, only cells with clear nuclear borders and boundaries were counted.

mRNA from real-time reverse-transcriptase-polymerase chain reactions

Total RNA was extracted from the brains at 58 weeks of age using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using a High-Capacity cDNA Reverse Transcription Kit with an RNase Inhibitor (Applied Biosystems, Foster City, CA, U.S.A.). The reaction mixture was incubated for 10 min at 25°C, 2 hr at 37°C, and 5 min at 85°C. Real-time PCR quantification was performed in a 10 μl reaction mixture on a QuantStudio 7 Real-Time PCR system (Applied Biosystems). The reaction mixture contained 1× TaqMan Universal PCR Master Mix II (Applied Biosystems), 20 *n*g of synthesized cDNA, and 0.9 μM primers/0.25 μM probes or TaqMan primers/probe mix (TaqMan Gene Expression Assays, Applied Biosystems). Cycle parameters were 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. The expression of the following genes was confirmed using TaqMan Gene Expression Assays: β-actin (Rn00667869_m1), S100 calcium binding protein A9 (S100a9) (Rn00585879_m1), heat shock 70kD protein 1A (HSP70-1a) (Rn04224718_u1), nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NF-κB) (Rn01399572_m1), and tumor necrosis factor (TNF)-α (Rn99999017_m1). Each relative change in gene expression level was calculated using the $2^{-\Delta\Delta Ct}$ method [22].

Statistical analyses

Results were expressed as means ± standard deviations. Statistical analyses of differences between mean values in SD rats and SDT fatty rats were performed using the F-test, followed by Student's *t*-test or Aspin-Welch's *t*-test. Differences were defined as significant when $P<0.05$.

RESULTS

Body weights and biophysiological parameters

The body weights of SDT fatty rats were significantly ($P<0.01$) lower than those of age-matched SD rats at 32 and 58 weeks of age (Fig. 1A). The plasma glucose levels and blood HbA1c levels of SDT fatty rats were obviously higher than that of SD rats at both ages (Fig. 1B and 1C). The fluctuations in HbA1c levels reflected the changes in blood glucose levels. The plasma insulin levels of SDT fatty rats were significantly ($P<0.01$) lower than those of SD rats after 32 weeks of age (Fig 1D).

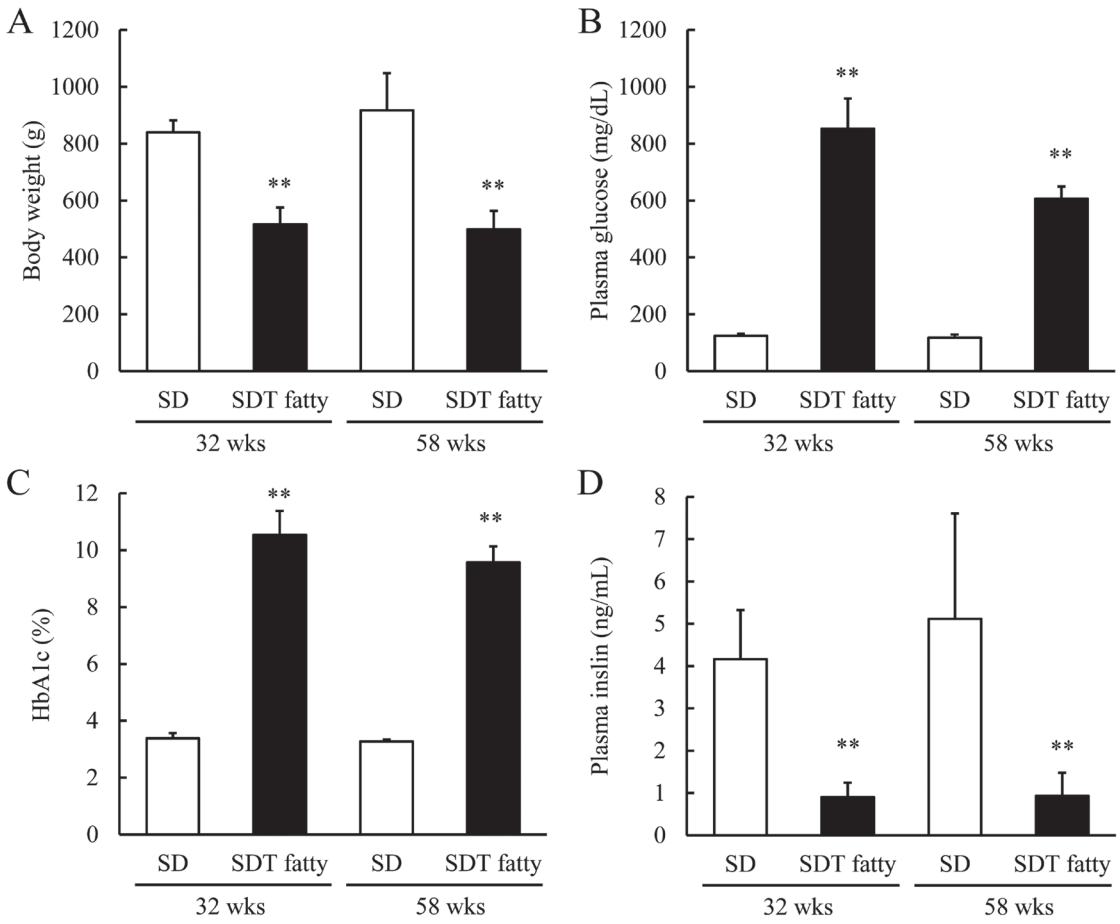

Fig. 1. Changes in biochemical parameters in SDT fatty rats. Changes in body weight (A), plasma glucose levels (B), blood HbA1c levels (C), and plasma insulin levels (D). Data represent means ± standard deviations (n=5). ***P*<0.01; significantly different from the SD group.

Morphometric analysis

The parietal cortical thickness of SDT fatty rats was significantly ($P<0.05$) lower than that of age-matched SD rats at 58 weeks of age, but not at 32 weeks of age (Fig. 2). At 58 weeks of age, the thickness in SD rats and SDT fatty rats was 1.05 ± 0.08 mm (n=5) and 0.94 ± 0.04 mm (n=5), respectively. The number of cells in the CA1 and CA3 regions of the hippocampus of SDT fatty rats was significantly ($P<0.01$) lower than that of age-matched SD rats at 58 weeks of age, but not at 32 weeks of age (Fig. 3). The number of pyramidal cells in the CA1 region was 27.4 ± 1.4 in SD rats (n=5) and 22.3 ± 1.1 in SDT fatty rats (n=5) at 58 weeks of age. The number in the CA3 region was 21.0 ± 0.4 in SD rats (n=5) and 16.6 ± 1.4 in SDT fatty rats (n=5) at 58 weeks of age.

mRNA analysis

Changes in mRNA expression related to inflammation in the brain at 58 weeks of age were determined for each group. In SDT fatty rats (n=4), the mRNA expression of S100a9, a calcium binding protein, and TNFα, a cytokine involved in inflammation and NF-κB, a transcription factor, in the brain significantly ($P<0.01$, 0.01 and 0.05, respectively) increased compared with those in SD rats (n=5), and the mRNA expression of HSP70-1a, a molecular chaperone, tended to increase (Fig. 4).

DISCUSSION

In the present study, the morphological changes in the brains of SDT fatty rats that developed obesity and diabetes were investigated. The parietal cortical thickness and hippocampal pyramidal cells in the CA1 and CA3 regions decreased in SDT fatty rats. The relationship between cerebral cortical thickness and DM has been reported in clinical practice and animal models. Diabetic patients have been reported to have a cerebral cortex thickness of 0.03 mm, which is lower than the thickness observed in those without DM regardless of cognitive impairment [27]. Similarly, in db/db mice in a T2DM model, reductions in cortical thickness have been reported compared with control [33]. Therefore, the decrease in cortical thickness observed in SDT fatty rats in this study is considered to contribute to the hyperglycemic state. In addition, Moran *et al*. mentioned that "cortical atrophy

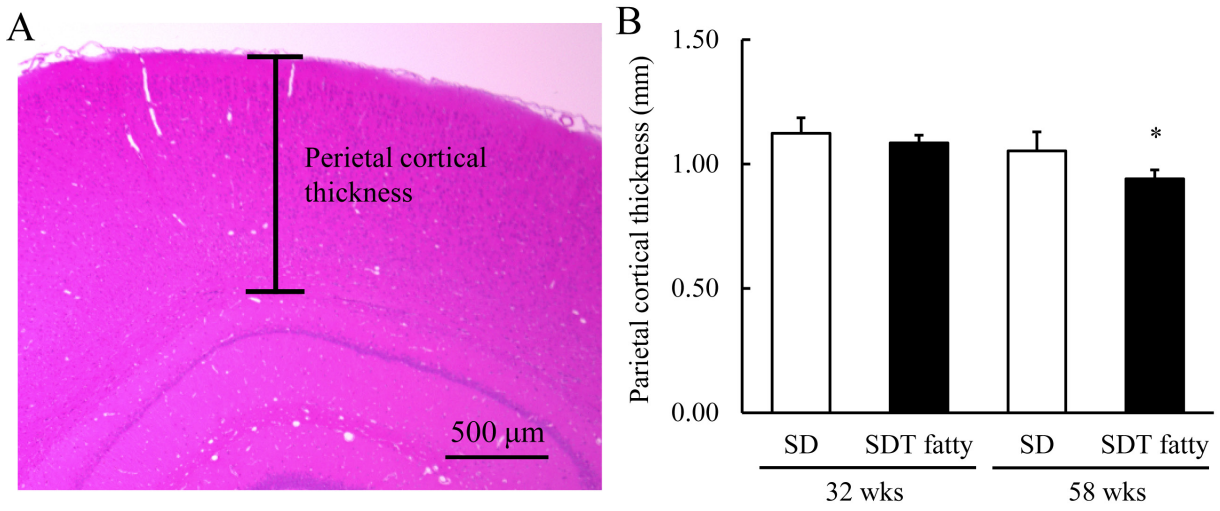

Fig. 2. Brain atrophy in SDT fatty rats at 58 weeks of age. Illustrative example of cortical thickness (A). Thickness measurement of the parietal cortex in male SDT fatty rats at 32 and 58 weeks of age (B). Data represent means ± standard deviations (n=5). **P*<0.05; significantly different from the age-matched SD group.

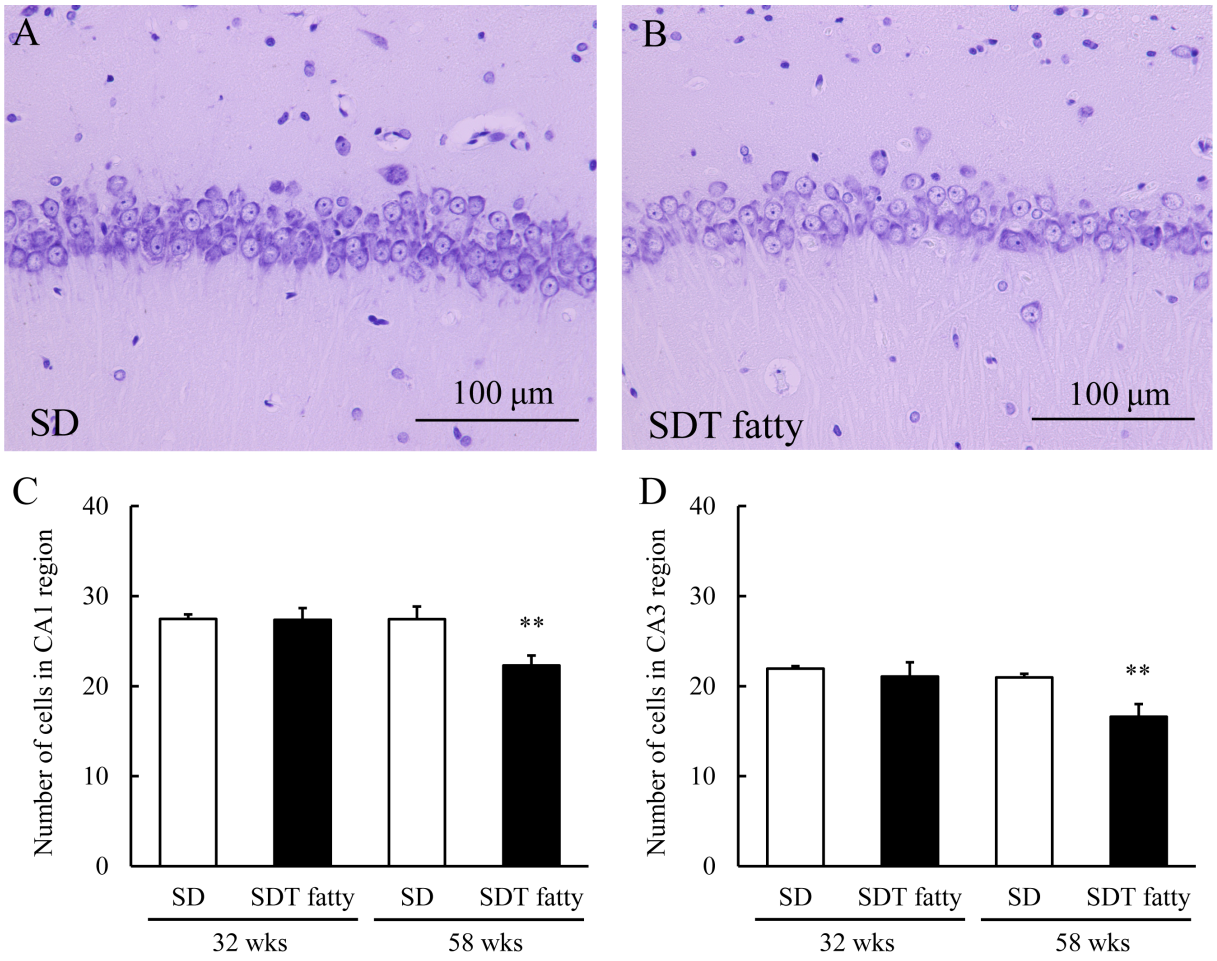

Fig. 3. Number of cells in hippocampal CA1 and CA3 regions of SDT fatty rats at 32 and 58 weeks of age. Illustrative example of the CA1 region in SD rats (A) and SDT fatty rats (B). Number of cells in the CA1 (C) and CA3 regions (D). Data represent means ± standard deviations (n=5). ***P*<0.01; significantly different from the age-matched SD group.

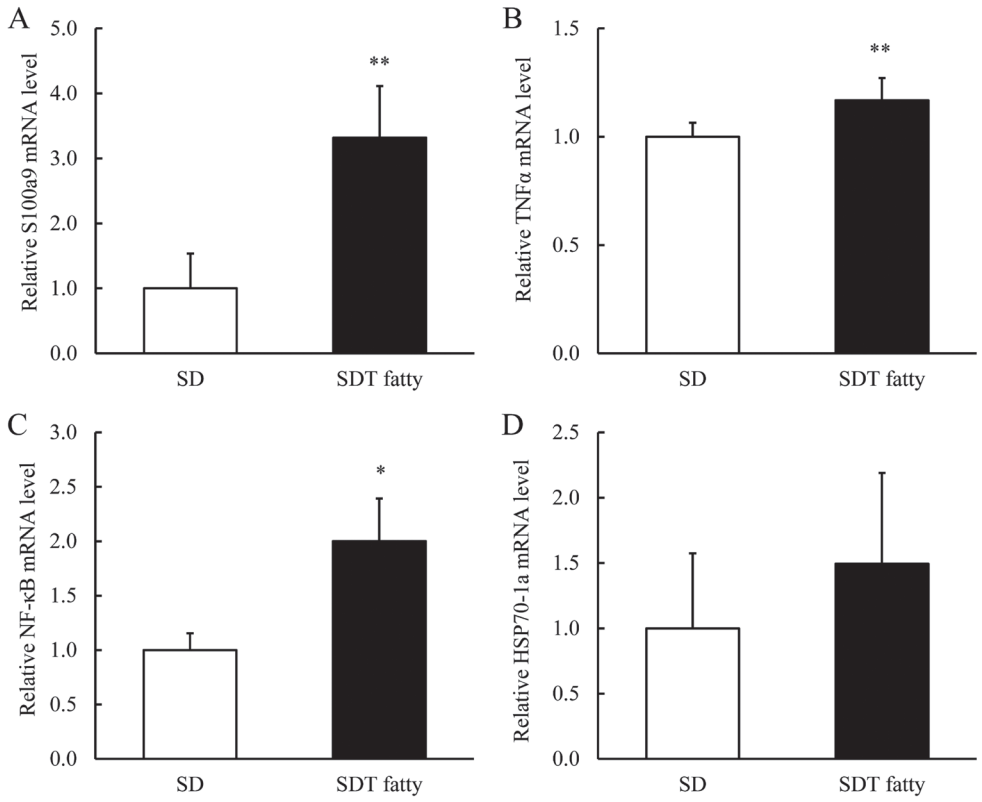

Fig. 4. Changes in mRNA levels in SDT fatty rat brains at 58 weeks of age. Changes in S100a9 mRNA levels (A), TNFα mRNA levels (B), NF-κB mRNA levels (C), and HSP70-1a mRNA levels (D). Data represent means ± standard deviations (n=4 to 5). **P*<0.05, ***P*<0.01; significantly different from the SD group.

in T2DM is similar to that seen in preclinical AD, and neurodegeneration may play a key role in cognitive deficits associated with T2DM" [28]. Therefore, changes in cortical thickness in SDT fatty rats are suggested as being a possible change related to cognitive impairment. The number of pyramidal cells in the hippocampal CA1 and CA3 regions was low in SDT fatty rats. The CA1 region of the hippocampus is reportedly a site in which CA1 neuronal density volume is reduced in patients with dementia and AD post-stroke, or ischemic vascular disease [10]. Furthermore, the CA3 region is reportedly weak against aging, and the number of cells per unit area decreases due to aging [38]. Reductions in nerve density in the hippocampal region of BB/W rats in a type 1 DM model [21] and reductions in nerve density of the prefrontal cortex in BBZDR/Wor rats in a T2DM model [20] have been reported. In this study, the number of pyramidal cells in the hippocampus did not change with age in SD rats; however, SDT fatty rats showed a decrease in the number of pyramidal cells. This result suggests that the sustained hyperglycemia may contribute to these morphological changes. In the preliminary study, the brain weights of SDT fatty rats at 32 and 58 weeks of age were measured. At 32 weeks of age, the absolute brain weights decreased and the relative brain weights increased in SDT fatty rats (absolute weights; 2,031 ± 63 mg, relative weights; 4.2 ± 0.5 mg/g body weight) as compared with the age-matched SD rats (absolute weights; 2,233 ± 49 mg, relative weights; 2.8 ± 0.3 mg/g body weight). Changes in the brain weights at 58 weeks of age (SD rats: absolute weights; 2,251 ± 87 mg, relative weights; 2.2 ± 0.3 mg/g body weight, SDT fatty rats: absolute weights; 2,033 ± 56 mg, relative weights; 5.0 ± 0.4 mg/g body weight) were similar to those at 32 weeks of age. Since changes in the brain weights were observed before the morphological changes occurred, it is necessary to investigate the relationship between the brain weights and the pathophysiological changes in other brain regions.

In the present study, the expression of inflammation-related genes was observed in the brains of SDT fatty rats. S100a9 reportedly participates in the inflammation of AD pathogenesis [35]. Furthermore, the expression of S100a9 is also recognized in AD patients and in genetically modified AD animal models, and the expression of S100a9 is suggested as possibly being involved in AD pathology [11]. Neuroinflammation is known as a crucial factor in the mechanism that associates T2DM with AD. Increased interleukin-1 and TNF-α mRNA in the hippocampus of *db/db* mice [8] and TNF-α may elicit insulin resistance in the hippocampus [3], and increased expression of NF-κB that promotes the production of inflammatory cytokines in the brain of high-fat diet and STZ-induced diabetic mice [17, 32] were reported. In addition, the upregulation of S100a9 has been reported to activate the p38 mitogen-activated protein kinase cascade and NF-κB [12].Therefore, neuroinflammation was considered as being involved in the brain abnormality observed in this model. It has been reported that HSP70-1a is induced by various stress and it has anti-inflammatory and cytoprotective effects [5, 25]. On the other hand, lipopolysaccharides, which induce inflammation, reportedly induced HSP70-1a expression [24]. Since SDT fatty rat is a hyperglycemic and obese model, it may be exposed to chronic

inflammation and stress by those factors. In this study, HSP70-1a tended to be increased in the brains of SDT fatty rats, suggesting the involvement of inflammation and stress.

It has been reported that insulin resistance, advanced glycation end-products (AGEs), oxidative stress and inflammatory response are involved in cognitive dysfunction of human DM patients [29]. SDT fatty rats have also been reported to represent insulin resistance and inflammatory responses [15]. Elevated expression of inflammation-related gene has also been observed in this study, and neuroinflammation with the sustained hyperglycemia may cause organic changes in the brain. In addition, female SDT fatty rats represent an obvious hyperinsulinemia as compared with male SDT fatty rats [31], and a severe insulin resistance may be induced in the brain of female SDT fatty rats. To investigate the pathophysiological changes in the brain of female SDT fatty rats is worthful as a future plan.

In this study, histological analyses revealed that SDT fatty rats showed brain atrophy and a decreased number of hippocampal cells. The behavioral evaluation is often used in the evaluation of cognitive functions of animals [13]. SDT fatty rats reportedly show a depression-like behavior [34] as one of behavioral features, and the evaluation of learning function is under consideration. Although the neurotransmitter such as serotonin, γ-aminobutyric acid and glutamate in the brain were impaired even in SDT fatty rats [34], histological changes in the brain developed in aged SDT fatty rats. Moreover, the survival rate of male SDT fatty rats at 50 weeks of age was approximately 70–80% in the preliminary study. From the viewpoint of versatility as a model animal, the early development of the brain pathological changes of SDT fatty rats is a future subject. In conclusion, this model rat showed the possibility of developing not only peripheral neuropathy [23] but also central nervous disorders. The expectation is that this model can be used to elucidate the pathologic pathway in AD which is recently recognized as new type of DM (or type 3 DM) [6].

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