



# Pathophysiological features in the brains of female Spontaneously Diabetic Torii (SDT) fatty rats

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**ABSTRACT.** Diabetes mellitus (DM) and obesity are associated with neurodegenerative diseases such as Alzheimer's disease and psychiatric disorders such as major depression. In this study, we investigated pathophysiological changes in the brains of female Spontaneously Diabetic Torii (SDT) fatty rats with diabetes and obesity. Brains of Sprague-Dawley (SD), SDT and SDT fatty rats were collected at 58 weeks of age. The parietal cortical thickness was measured and the number of pyramidal cells in the hippocampal cornu ammonis 1 and 3 (CA1 and CA3) and the number of granule cells in the dentate gyrus (DG) regions were counted. The area of glial fibrillary acidic protein (GFAP) positivity in CA1, CA3 and DG regions were measured. The parietal cortical thickness and the number of cells in CA3 and DG regions of SDT and SDT fatty rats did not show obvious changes. On the other hand, in the CA1 region, the number of cells in SDT rats and SDT fatty rats was significantly lower than that in SD rats, and that in SDT fatty rats was significantly lower than that in SDT rats. The GFAP-positive area in SDT fatty rats was significantly reduced compared to that in SD rats only in the DG region. Preliminary result showed that the expression of S100a9, an inflammation-related gene, was increased in the brains of SDT fatty rats. These results suggest that female SDT fatty rat may exhibit central nervous system diseases due to obesity and DM.

**KEY WORDS:** female Spontaneously Diabetic Torii (SDT) fatty rat, hippocampus, neurodegenerative disease

*J. Vet. Med. Sci.*  
84(3): 330–337, 2022  
doi: 10.1292/jvms.21-0654

Received: 16 December 2021  
Accepted: 17 January 2022  
Advanced Epub:  
27 January 2022

Diabetes mellitus (DM) and obesity are one of the risk factors for neurodegenerative diseases, in which neurological and mental functions are impaired due to degeneration and shedding of the central nervous system (CNS), and for psychiatric diseases, in which social functioning is impaired due to significant bias in emotions and behavior based on them [3, 6, 22, 35]. Impairment of brain structure and function has been shown to be related to several factors, including oxidative stress, neuroinflammation, and neuronal apoptosis in diabetes [44, 47]. Morphological abnormalities such as brain atrophy and cell death in Alzheimer's disease (AD) patients have been reported in several studies using MRI and postmortem brain studies [10, 33]. In addition, preclinical studies have observed volume loss in the forebrain cortex and hippocampus, neurodegeneration, and increased amyloid  $\beta$ 42 in streptozotocin (STZ)-induced diabetic rats [46]. Moreover, the expression of amyloid precursor protein and phosphorylated tau has been reported in a rat model of type 2 DM in which diabetes induced by STZ after rearing on high fat, glucose, and protein diet [48]. Turning to psychiatric disorders, the prevalence of DM is increased in patients with schizophrenia [15], while the incidence of depression is higher in diabetic patients [36]. Epidemiological studies have also reported a bidirectional association between depression and diabetes [12]. In animal models, immobility time, a marker of depression, was increased in STZ-induced diabetic rats in a forced swimming test [5], and in Flinders Sensitive rats, a genetic model of depression, a high-fat diet caused abnormal behavior related to depression [1]. Thus, numerous clinical and preclinical studies suggest that diabetes is closely associated with cognitive dysfunction and mental disorders.

Spontaneously Diabetic Torii (SDT) fatty rat is a model for the development of the three major complications of diabetes: nephropathy, retinopathy, and neuropathy [17, 20, 27, 30]. Recently, SDT fatty rats have been shown to be a potential model for depression [38]. Since diabetes is observed from a young age in this model, it is expected that this model may exhibit neurodegenerative and psychiatric diseases seen in other animal models of diabetes. Previously, we reported that parietal cortical

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thickness and hippocampal pyramidal cells in cornu ammonis 1 and 3 (CA1 and CA3) regions were reduced in the brain at 58 weeks of age in obese and diabetic male SDT fatty rats [26]. Female SDT fatty rats showed obvious hyperinsulinemia compared with male SDT fatty rats [37], and we thought that severe insulin resistance might cause some changes in the brain of female SDT fatty rats, so we investigated the pathophysiological changes in the brain of female SDT fatty rats in the present study.

## MATERIALS AND METHODS

### *Animals*

In this study, 10 female SDT rats and 10 female SDT fatty rats (both from Clea Japan, Tokyo, Japan) were used. SDT rat is a spontaneously diabetic strain of the Sprague-Dawley (SD) rats [42], and loci for glucose intolerance have been identified [28]. The SDT fatty rat was established by inserting the leptin gene into the genome of the SDT rat [29]. Moreover, age-matched 10 female SD rats (Clea Japan) were also used as control animals. The 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. Rats were housed in a climate-controlled room (temperature  $23 \pm 3^\circ\text{C}$ , humidity  $55 \pm 15\%$ , 12 hr/12 hr light-dark cycle). A basal diet (CRF-1, Oriental Yeast, Tokyo, Japan) and sterilized water were provided *ad libitum*. Initially, 10 SDT fatty rats were provided, but 4 out of 10 died by 58 weeks of age. Therefore, the remaining 6 rats were assigned to 4 rats for biochemical parameter measurements and morphological studies, and 2 rats for mRNA analysis. In the previous study using male rats, morphological changes in the brain were observed at 58 weeks but not at 32 weeks [26], so the following measurements were performed at 58 weeks of age.

### *Measurement of biochemical parameters*

At 58 weeks of age, body weights and biochemical parameters such as plasma glucose, and blood hemoglobin A1c (HbA1c), insulin, triglyceride (TG), total cholesterol (TC) were measured. Blood samples were collected from the right subclavian vein under unanesthetized, non-fasting and overnight fasting conditions. Plasma glucose, HbA1c, TG, and TC were measured using the commercial kits (Roche Diagnostics, Basel, Switzerland) and an automatic analyzer (HITACHI Clinical analyzer 7180; Hitachi, Tokyo, Japan). Plasma insulin levels were measured using the rat insulin enzyme-linked immunosorbent assay (ELISA) kits (Morinaga Institute of Biological Science, Yokohama, Japan).

### *Tissue sampling*

For pathological specimen preparation, brains were collected from all rats for the morphological analyses at 58 weeks of age. Three days after blood sampling, rats were anesthetized with isoflurane and transcardially perfused with 0.1 M phosphate-buffered saline (PBS) and 4% paraformaldehyde using a perfusion device, and the brain was collected. For mRNA analysis, remaining rats were transcardially perfused with 0.1 M PBS under isoflurane anesthesia, and brain samples collected were stored at  $-80^\circ\text{C}$  until analysis.

### *Preparation of tissue specimen*

Brain blocks at approximately  $-1.50$  to  $-3.00$  mm from the bregma were created using a rodent brain matrix (Neuroscience, Tokyo, Japan), and paraffin-embedded brains using standard techniques were sliced ( $5 \mu\text{m}$ ) at approximately  $-3.30$  mm from the bregma while referring to the brain map. Brain sections were stained with hematoxylin-eosin (HE) and Nissl. In addition, immunostaining for a glial fibrillary acidic protein (GFAP) was performed. After deparaffinization and rehydration, sections were treated with L.A.B. solution (Polyscience, Niles, IL, USA) for 20 min at room temperature to retrieve antigen. Sections were incubated with 0.3% hydrogen peroxide for 15 min at room temperature to block endogenous peroxidase activity, and then incubated with primary antibody (anti-GFAP rabbit monoclonal antibody, 1:100, Abcam, Cambridge, UK) for 2 hr at room temperature. The sections were incubated with a secondary antibody labeled with horseradish peroxidase for 30 min at room temperature and immunoreaction was developed with the StayBlack/HRP reaction solution (Abcam).

### *Morphometric analyses*

HE- and Nissl-stained sections were digitally photographed using an optical microscope and images were saved. Measurements of parietal cortex thickness, hippocampal pyramidal cell count in CA1 and CA3 regions, and granule cell count in dentate gyrus (DG) region were performed according to the previously described method [26]. Left and right parietal cortical thicknesses were measured using HE-stained sections and left and right mean values were calculated. Nissl-stained sections were used to count pyramidal cells in the left and right CA1 and CA3 regions and granule cells in DG region. Using image processing software, Image J (NIH; <https://imagej.nih.gov/ij/>), the number of cells with clear nuclear borders and boundaries in each of the 3 locations per unit area (6 locations on each side) was counted for each section using a blinded method. The unit area was set to  $50 \times 150 \mu\text{m}$  for both the CA1, CA3 and DG regions. The number of cells was taken as the average value of 6-unit areas. GFAP immunostained sections were used to measure GFAP positive areas. In hippocampal CA1, CA3, and DG, the percentage of GFAP positive areas in a single field of view (approximately  $0.54 \text{ mm} \times 0.72 \text{ mm}$ ) was automatically measured by Image J. 2 sections per animal, left and right area per 1 section, were acquired and averaged (4 locations in total per animal).

**Table 1.** Biochemical parameters during non-fasting and overnight fasting in Sprague-Dawley (SD), Spontaneously Diabetic Torii (SDT) and SDT fatty rats at 58 weeks of age

Condition	Strain	N	Body weight (g)	Plasma glucose (mg/dl)	Plasma insulin (ng/ml)	HbA1c (%)	Triglyceride (mg/dl)	Total cholesterol (mg/dl)
Non-fast	SD	5	567.1 ± 55.3	121.6 ± 5.9	2.5 ± 1.0	3.2 ± 0.1	502 ± 338	131 ± 37
	SDT	5	399.8 ± 77.9	523.6 ± 204.2**	1.7 ± 2.1	7.1 ± 2.4**	343 ± 115	84 ± 5
	SDT fatty	4	607.6 ± 174.3 <sup>#</sup>	455.5 ± 232.1*	4.1 ± 2.8	6.9 ± 2.2**	2,413 ± 1,842* <sup>#</sup>	542 ± 462 <sup>#</sup>
Fast	SD	5	–	139.6 ± 12.8	1.5 ± 0.5	2.1 ± 0.2	331 ± 314	122 ± 37
	SDT	5	–	163.6 ± 43.1	0.6 ± 0.3	7.5 ± 3.5*	113 ± 33	66 ± 6
	SDT fatty	4	–	158.0 ± 31.5	2.0 ± 1.1 <sup>#</sup>	7.2 ± 3.4*	2,323 ± 2,415	508 ± 382* <sup>#</sup>

Data represent means ± standard deviation (n=4 to 5). Body weights after fasting were not measured. Non-fast: normal breeding condition, Fast: overnight fasting condition. \* $P < 0.05$ , \*\* $P < 0.01$  vs. SD rats, <sup>#</sup> $P < 0.05$  vs. SDT rats.

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

The mRNA measurement was performed in the same way as our previous report [26]. Total RNA was extracted from whole brain of rats 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, USA). 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 ng 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), *HSP70-1b* (Rn02532795\_s1), nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (*NF-κB*) (Rn01399572\_m1), tumor necrosis factor (*TNF*)-α (Rn99999017\_m1), insulin-like growth factor-1 (*IGF-1*) (Rn00710306\_m1), and *caspase 3* (Rn00563902\_m1). Each relative change in gene expression level was calculated using the  $2^{-\Delta\Delta Ct}$  method [25].

### Statistical analyses

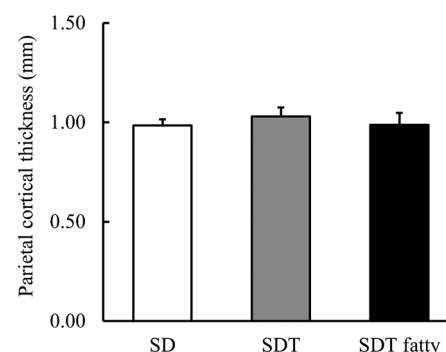
Results were expressed as means ± standard deviations. Statistical analyses of differences between groups were performed using an one-way ANOVA with Tukey *post hoc* test. Differences were defined as significant when  $P < 0.05$ .

## RESULTS

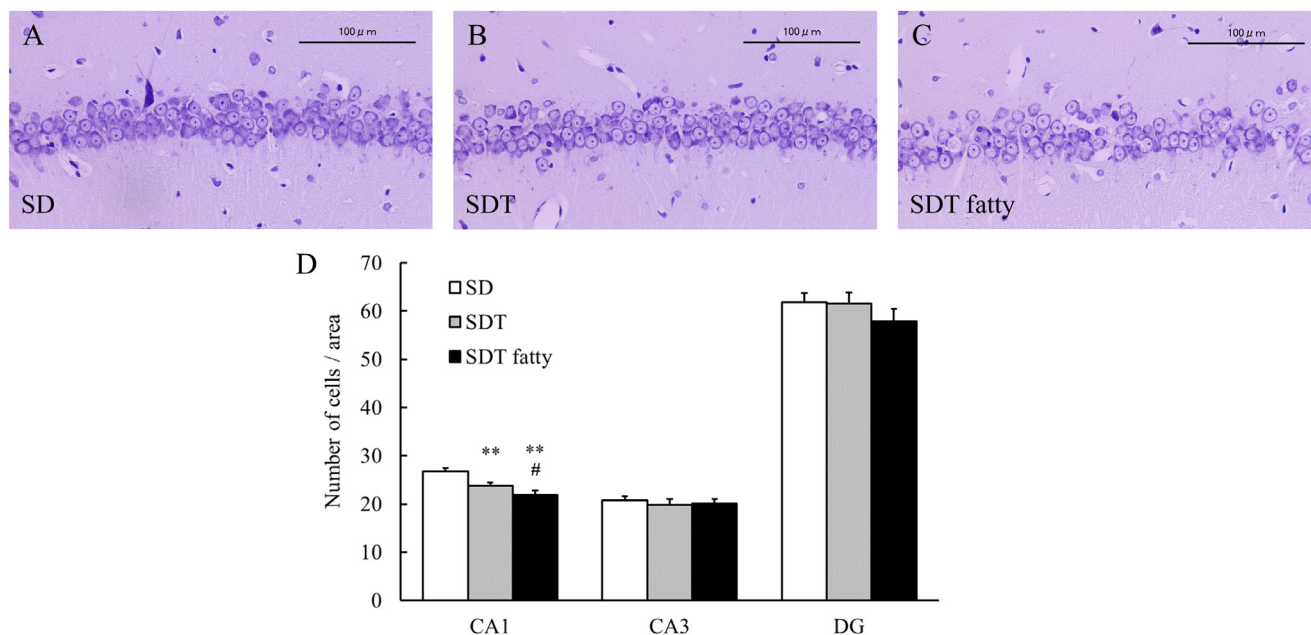
As mentioned above, in this experiment, 10 SD, SDT and SDT fatty rats were provided each, but 4 out of 10 SDT fatty rats died by the age of 58 weeks, so the remaining six rats were assigned to 4 rats for biochemical parameter measurements and morphological studies, and 2 rats for mRNA analysis, respectively.

At 58 weeks of age, the mean body weights of SD, SDT, and SDT-fat rats were 567.1 g (n=5), 399.8 g (n=5), and 607.6 g (n=4), respectively. Plasma glucose levels in SDT rats and SDT fatty rats were significantly higher than in SD rats under non-fasting conditions, but not overnight fasting condition. HbA1c was significantly higher level in SDT rats and SDT fatty rats compared with SD rats, with or without overnight fasting. Insulin levels were significantly higher in SDT fatty rats than in SDT rats, but it was similar among the three groups; both SDT rats and SDT fatty rats showed hyperglycemia but no obvious increase in insulin levels. The TG and TC levels were higher in SDT fatty rats than in SD and SDT rats, although the differences may or may not be significant due to the large inter-individual variability (Table 1).

There was no difference in the thickness of the parietal cortex between the groups (Fig. 1); the thickness of SD rats, SDT rats, and SDT fatty rats was 0.984 mm (n=5), 1.030 mm (n=5), and 0.988 mm (n=4), respectively. The number of cells in CA1 region of SDT and SDT fatty rats was significantly ( $P < 0.01$ ) lower than that of SD rats, and even significantly ( $P < 0.01$ ) lower in SDT fatty rats than in SDT rats. There were no statistically significance in the number of cells in CA3 and DG region between the groups, but the number of cells in the DG region of SDT fatty rats tended to be lower ( $P = 0.051$ ) than that of SD rats (Fig. 2). The number of cells in the CA1, CA3, and DG regions was  $26.8 \pm 0.7$ ,  $20.8 \pm 0.8$  and  $61.9 \pm 1.9$  in SD rats (n=5),  $23.8 \pm 0.7$ ,  $19.9 \pm 1.2$  and  $61.6 \pm 2.3$  in SDT rats (n=5), and



**Fig. 1.** Parietal cortical thickness in female Sprague-Dawley (SD) rats, Spontaneously Diabetic Torii (SDT) rats, and SDT fatty rats at 58 weeks of age. Data represent means ± standard deviations (n=4 to 5). There were no significant differences between the groups.



**Fig. 2.** The number of cells in hippocampal cornu ammonis (CA) 1, CA3, and dentate gyrus (DG) regions of female Sprague-Dawley (SD) rats, Spontaneously Diabetic Torii (SDT) rats, and SDT fatty rats at 58 weeks of age. Illustrative example of the CA1 region in SD rats (A), SDT rats (B) and SDT fatty rats (C). Number of cells in the CA1, CA3 and DG regions (D). Data represent means  $\pm$  standard deviations (n=4 to 5). \*\* $P < 0.01$  and # $P < 0.05$ ; significantly different from SD rats group and SDT rats group, respectively.

$21.9 \pm 0.9$ ,  $20.1 \pm 0.9$ , and  $57.8 \pm 2.6$  in SDT fatty rats (n=4), respectively.

The sites of measurement of GFAP positivity in the hippocampal CA1, CA3, and DG regions ( $0.54 \text{ mm} \times 0.72 \text{ mm}$ ) were shown in Fig. 3B. In the CA1 and CA3 regions, there was no obvious difference between the groups. On the other hand, in the DG region, the GFAP-positive area in SDT fatty rats was significantly ( $P < 0.05$ ) reduced compared to SD rats, and the GFAP-positive rate in the DG region was  $6.9 \pm 1.6\%$  in SD rats (n=5),  $5.8 \pm 1.5\%$  in SDT rats (n=5), and  $3.5 \pm 1.0\%$  in SDT fatty rats (n=4) (Fig. 3A).

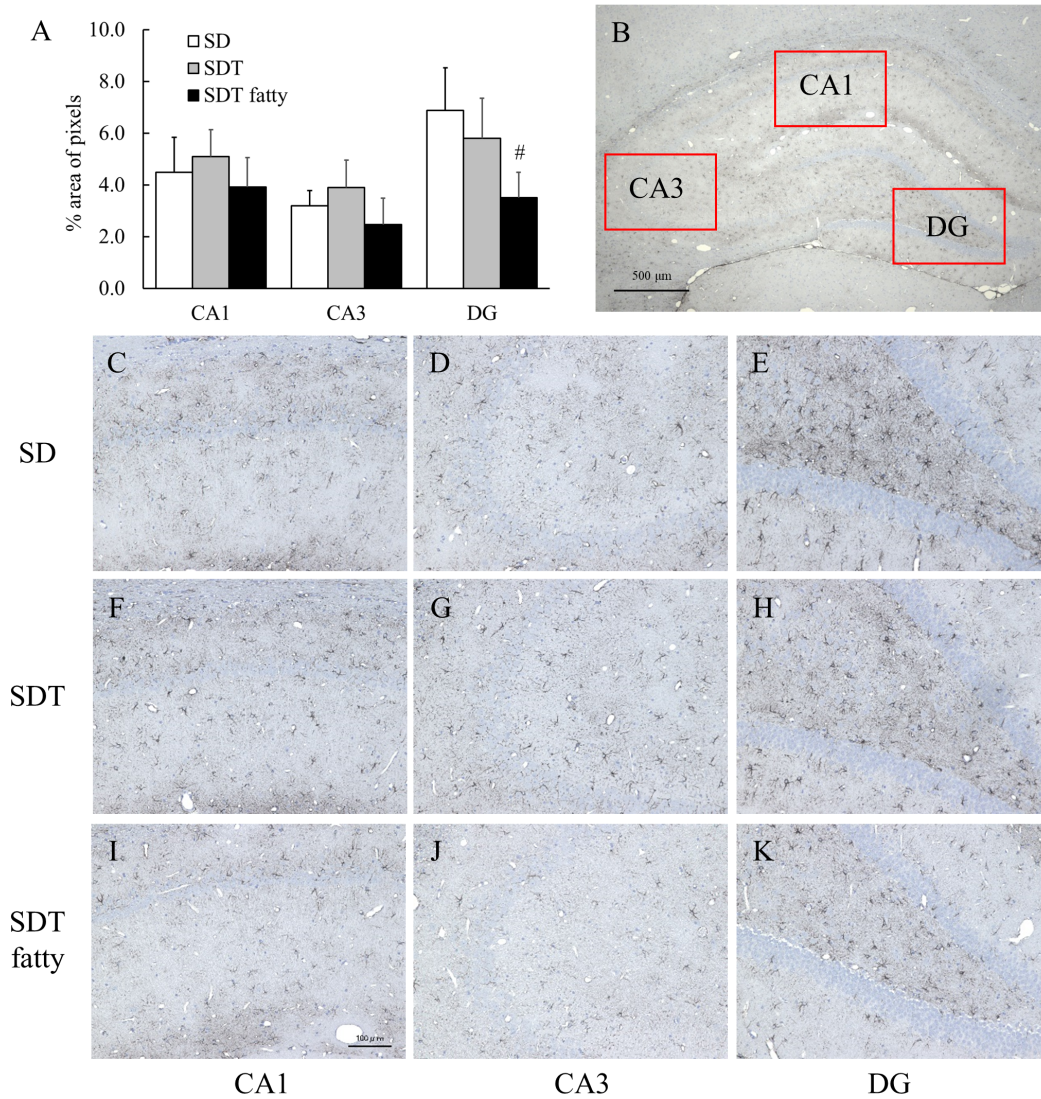
Since there were only two SDT fatty rats, the data can only be supplementary, but we measured changes in mRNA expression related to inflammation in the brain at 58 weeks of age in each group. In SDT fatty rats (n=2), mRNA expression of S100a9, a calcium-binding protein, was increased in the brain compared to SD or SDT rats (n=5) (statistical analysis not performed) (Fig. 4). SDT rats also showed a slight upward trend of S100a9.

## DISCUSSION

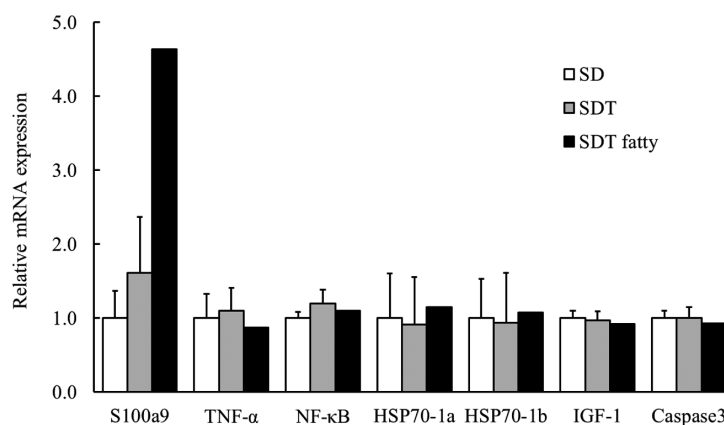
Previously, we reported that parietal cortical thickness and hippocampal pyramidal cells in CA1 and CA3 regions were reduced in the brain at 58 weeks of age in obese and diabetic male SDT fatty rats [26]. Since female SDT fatty rats show obvious hyperinsulinemia compared to male SDT fatty rats [37], we hypothesized that severe insulin resistance could be induced in the brain of female SDT fatty rats, and we investigated the pathophysiological changes of the brain in non-obese SDT rats in addition to female SDT fatty rats.

In male rats, a decrease in the thickness of the cerebral cortex, decreased the number of neurons in the CA1 and CA3 regions, and an increase in inflammation-related genes have been observed in male SDT fatty rats compared to male SD rats [26]. For instance, the number of neurons in the CA1 region was  $27.4 \pm 1.4$  in SD rats and  $22.3 \pm 1.1$  in SDT fatty rats (Data from reference No. [26]). However, no changes other than a decrease in the number of neurons in the CA1 region of the hippocampus were observed in female SDT fatty rats, suggesting that there may be sex differences in the effects on the brain. Clinical studies have reported that the volume of the hippocampus in males is smaller than in females and is particularly pronounced after 60 years old [18]. The male brain may be more susceptible to various influences such as diabetes and stress, while the female brain may be more robust than the male brain. On the other hand, women are said to have a higher incidence of AD, although there may be the influence of a very complex system of endocrine and susceptibility differences [31]. The CA1 region of the hippocampus has been reported to have a reduced density of CA1 neurons in AD patients after dementia and stroke [11]. Furthermore, the CA3 region has been reported to be vulnerable to aging, and the number of cells per unit area decreases with aging [45]. In addition, a decrease in the number of granule cells in the DG has been reported in an animal model of diabetes [8]. In the present study, there was no apparent effect on the number of pyramidal cells in the CA3 region or granule cells in the DG region in SDT rats and SDT fatty rats. However, the number of pyramidal cells in the CA1 region, which has been associated with dementia as described above, was decreased in SDT rats and SDT fatty rats, suggesting that there may be some effect on the hippocampus related to memory.





**Fig. 3.** Glial fibrillary acidic protein (GFAP) positive area in hippocampal cornu ammonis (CA) 1, CA3, and dentate gyrus (DG) regions of Sprague-Dawley (SD), Spontaneously Diabetic Torii (SDT), and SDT fatty rats at 58 weeks of age. The % of GFAP-positive area (A) and the site used for analysis (B). Data represent means  $\pm$  standard deviations ( $n=4$  to  $5$ ).  $^{\#}P<0.05$ ; significantly different from SD rats group. Illustrative example in CA1 (C, F, I), CA3 (D, G, J), and DG region (E, H, K) in SD rats (C–E), SDT rats (F–H), and SDT fatty rats (I–K). Bar in (B)=500  $\mu\text{m}$ , bar in (C) to (K)=100  $\mu\text{m}$ .



**Fig. 4.** Changes in mRNA levels in Sprague-Dawley (SD), Spontaneously Diabetic Torii (SDT), and SDT fatty rat brains at 58 weeks of age. Data represent means  $\pm$  standard deviations ( $n=2$  to  $5$ ). Statistical analyses were not performed due to the small number of cases.

It has been reported that female SDT fatty rats at a young age show insulin resistance [37]. Although insulin resistance in these rats at higher weeks of age was less pronounced, the hyperglycemic state was maintained. Since a significant neuronal decrease was also observed in the CA1 region of non-obese SDT rats, sustained hyperglycemia may contribute to the decrease in neurons in the CA1 region. It has been reported that neuronal loss by apoptosis may be related to the duration of diabetes in BB/W rats with spontaneous type 1 diabetes [24]. In addition, TG and TC tended to be higher in female SDT fatty rats. While increased TG and TC have been reported to be associated with AD [2], there is also a report that the causal relationship between high TC and AD has not been confirmed [32]. The relationship between dyslipidemia and dementia is not certain and controversial. However, dyslipidemia is a risk factor for the development of cerebral infarction [21] and may cause vascular dementia [4]. In the present study, we did not observe any decrease in cortical thickness, which may indicate cerebral atrophy. However, future detailed studies are needed to investigate the relationship between organic changes in cerebral blood vessels, the presence of infarct foci, and TG and TC metabolism in blood and the brain.

In the present study, the results of the expression of GFAP, a brain astrocyte marker, suggest that astrocytes in the DG region may be reduced in SDT fatty rats. It has been reported that astrocytes in the DG are increased in streptozotocin-induced diabetic rats and NOD mice [19, 40]. In addition, in *ob/ob* mice with knock-in  $\beta$ -amyloid, GFAP is decreased at 6 months of age but increased at 18 months of age, indicating that astrocytes may be activated by obesity and diabetes in addition to aging [43]. On the other hand, a decrease in GFAP-positive cells has been reported in the hypothalamus of STZ rats and the suprachiasmatic nucleus of the anterior hypothalamus of type 2 diabetic patients [14, 23]. These findings indicate that the pathogenesis of diabetes is related to neurosecretion and circadian rhythm. It has also been shown that SDT fatty rats have impaired circadian rhythms and dysregulated melatonin secretion [39] and that GFAP-responsive astrocytes in the hippocampus are reduced in the postmortem brains of patients with major depression [7]. Therefore, the reduction of GFAP in the DG observed in SDT fatty rats may be related to circadian rhythms, depression, and other CNS disorders other than cognitive function.

In this study, although the results are preliminary, the expression of S100a9, an inflammation-related gene, was observed in the brains of female SDT fatty rats as well as male SDT fatty rats [26]. S100a9 has been reported to be involved in AD and depression [34, 41]. Furthermore, S100a9 expression has been found in AD patients and genetically modified AD animal models, suggesting that S100a9 expression may be involved in the pathology of AD [13]. It has been reported that S100a9 is also expressed in animal models of major depressive disorder [16] and is significantly higher in the dorsolateral prefrontal cortex of schizophrenia patients [9], suggesting that S100a9 is associated not only with dementia but also with psychiatric disorders such as depression. Although SDT fatty rats are hyperglycemic and obese models and thus may be exposed to chronic inflammation and stress, no increase in TNF- $\alpha$  or HSP70 was observed in this study. Since the present results are preliminary due to the small number of cases, further studies on gene expression changes in the brain are needed.

Although the results of this study suggest that SDT fatty rats may be used as a model animal for various CNS diseases, many issues remain to be addressed. A more detailed study of the characteristics of this rat brain, including sex differences, may lead to future applications in CNS diseases.

POTENTIAL CONFLICTS OF INTEREST. The authors declare no conflict of interest.

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