

# Differential effects of type 2 diabetes on brain glycometabolism in rats: focus on glycogen and monocarboxylate transporter 2

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**Abstract** Astrocyte-neuron lactate shuttle (ANLS) is a pathway that supplies glycogen-derived lactate to active neurons via monocarboxylate transporter 2 (MCT2), and is important for maintaining brain functions. Our study revealed alterations of ANLS with hippocampal hyperglycogen levels and downregulated MCT2 protein levels underlying hippocampal dysfunctions as a complication in type 2 diabetic (T2DM) animals. Since T2DM rats exhibit brain dysfunctions involving several brain regions, we examined whether there might also be T2DM effects on ANLS's disturbances in other brain loci. OLETF rats exhibited significantly higher glycogen levels in the hippocampus, hypothalamus, and cerebral cortex than did LETO rats. MCT2 protein levels in OLETF rats decreased significantly in the hippocampus and hypothalamus compared to their controls, but a significant correlation with glycogen levels was only observed in the hippocampus. This suggests that the hippocampus may be more vulnerable to T2DM compared to other brain regions in the context of ANLS disruption.

**Keywords** Astrocyte-neuron lactate shuttle · Brain glycogen · Hippocampus · Monocarboxylate transporter · Type 2 diabetes mellitus

## Introduction

The brain uses lactate as an energy source [1, 2] and for neuro-modulation [3, 4]. Lactate is sourced by the brain in two ways: (a) externally, through blood circulation via the blood–brain barrier and (b) locally, through astrocytes via glycolysis and/or glycogenolysis. Indeed, glycogen stored in astrocytes is an important source of lactate production in the brain [2]. Glycogen-derived lactate provided by astrocytes is released into the extracellular fluid via monocarboxylate transporters (MCT) 1 and 4, and is then taken up by neurons via MCT2 [5]. This metabolic pathway is called the astrocyte-neuron lactate shuttle (ANLS), and as stated above, it is the most dominant pathway for lactate supply and is critical for neuronal activity [6]. A number of studies have shown that downregulated protein levels and function of MCT2 in the hippocampus [3, 7, 8] and cerebellum [9] cause the impairment of specific brain functions in these regions. Hence, MCT2 is regarded as a crucial component of the ANLS system [3, 7–9]; therefore, alterations in ANLS, such as disruption of lactate transport via downregulated MCT2 in the brain, may lead to the impairment of brain function.

A growing body of evidence shows that adverse alterations in glycometabolic pathways in peripheral organs is a common and frequent feature of type 2 diabetes mellitus (T2DM), which in turn leads to further progression of organ complications. T2DM-induced alterations in glycometabolism has been observed in the brain [8], as well as in peripheral organs [10]. Recently, we revealed that

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T2DM rats with memory dysfunction exhibit alterations in ANLS-related glycometabolism in the hippocampus accompanied by increased glycogen levels and decreased MCT2 protein levels [8]. This elevated glycogen deposition observed in the T2DM hippocampus [8] could be a metabolic adaptation to compensate for diabetes-induced decreased lactate utilization through downregulated MCT2. Indeed, a similar metabolic adaptation takes place in the heart of diabetic patients [10]. However, it is not yet clear whether the observed alterations in ANLS-related glycometabolism via glycogen levels and associated MCT2 are region-specific within the brain, whether they are only confined to the hippocampus [8], or do similar types of ANLS-related glycometabolism alterations prevail in other brain regions.

Of note, the hippocampus is not the only brain region with a specific brain function; other brain regions also have distinct and specific functions, for example, the hypothalamus regulates feeding behavior [11] and sympathoadrenal response [12, 13], and the frontal cortex performs executive function [14]. Interestingly, the functions of these brain regions are also impaired in T2DM, as demonstrated by past studies [13, 14]. Collectively, based on these facts, we hypothesized here that there might be other brain regions prone to be affected by T2DM as is the hippocampus [8] in the context of alterations in the crucial components of ANLS-related glycometabolism, such as local glycogen and MCT2 levels.

In the current study, we investigated whether alterations of ANLS-related glycometabolism, such as increased glycogen levels and decreased MCT-series protein levels as observed in the hippocampus [8] in a T2DM rat model, is widespread in other brain regions. For this, we used a 10-kW microwave irradiation method, the gold standard for detecting brain glycometabolism *in vivo*, to euthanize the animals [15]. To test the stated hypothesis, we used Otsuka Long Evans Tokushima Fatty (OLETF) rats as the T2DM animal model because they exhibit hyperphagia and dysregulated sympathoadrenal response and executive function [16], and also because they exhibit similarities with human T2DM, in terms of late onset compared to other T2DM model animals [17].

## Materials and methods

### Animals

Four-week-old male OLETF rats and their genetic counterpart control Long-Evans Tokushima (LETO) rats obtained from Hoshino Laboratory Animals Inc. (Ibaraki, Japan) were housed and cared for in an animal facility, and used in the present study. The rats were fed a standard

pellet diet (MF, Oriental Yeast Co., Ltd, Tokyo, Japan) and given water *ad libitum*, and maintained in temperature-controlled rooms (between 22 and 24 °C) under a 12 h light/dark cycle (lights on: 7:00–19:00). All experimental protocols were pre-approved and conducted in accordance with the University of Tsukuba Animal Experiment Committee guidelines.

### Food intake measurements

For assessments of the animals food intake, amounts of food pellets were measured immediately before giving to experimental animals and 24 h after with the replacement of fresh food pellets.

### Tissue preparation

At 30 weeks of age, the rats were anesthetized with isoflurane (a mixture of 30% v/v isoflurane in propylene glycol, Dainippon Sumitomo Pharma Co., Ltd, Osaka, Japan) after a 2-h period of fasting and then sacrificed using high-power microwave irradiation (NJE-2603, New Japan Radio Co., Ltd., Tokyo, Japan; 10-kW, 1.4 s), which momentarily inactivates glycogenolytic and glycosynthetic enzymes in the brain. Then, the hippocampus, the hypothalamus, the whole cerebral cortex, the cerebellum, and the brainstem were collected or harvested using a method modified from the Hirano et al. study [18]. The fat around the kidneys of each animals was also taken and measured.

### Blood glucose and HbA<sub>1C</sub> assays

Glucose levels in the blood collected from the jugular vein were measured using an automated analyzer (2300 Stat Plus, Yellow Springs Instruments, USA). Measurement of circulatory HbA<sub>1C</sub> levels was consigned to Oriental Yeast Co. (Shiga, Japan).

### Glycogen assays

Glycogen assays were determined as described by previous studies [8, 15, 19]. Brain tissues from the left hemisphere were homogenized (MS-100R, TOMY, Tokyo, Japan; 30 s, three times) in 6% perchloric acid (PCA) with 1 mM EDTA. For detection of glycogen content in tissue samples, glycogen was hydrolyzed to glucose in 100 µl of homogenate incubated for 3 h at 37 °C with 1 ml of 0.2 M sodium acetate, 20 µl of 1.0 M KHCO<sub>3</sub>, and 20 U/ml of amyloglucosidase. Then, 0.5 ml of PCA solution was added. After centrifugation (14,000 rcf, 10 min, 4 °C), supernatants were collected and neutralized with KOH solution containing 3 M KOH, 0.3 M imidazole, and 0.4 M

KCl. The neutralized supernatants were centrifuged (16,000 rcf, 10 min, 4 °C) and used to determine glucose content. To measure endogenous glucose levels in tissue samples, non-hydrolyzed samples were obtained by centrifuging homogenates (14,000 rcf, 10 min, 4 °C), and then the pH of supernatants was adjusted to pH 6–8 with KOH solution. Neutralized supernatants were centrifuged (16,000 rcf, 10 min, 4 °C) and used for assaying endogenous glucose levels. The assay of glucose content was performed in 96-well plates using a coupled enzyme assay method modified from Passonneau and Lauderdale [20]. Two hundred  $\mu$ l of a reaction solution (containing 50 mM Tris–HCl (pH 8.1), 0.5 mM ATP, 0.5 mM NADP<sup>+</sup>, 5 mM MgSO<sub>4</sub> and 0.1 U/ml glucose-6-phosphate dehydrogenase), 30  $\mu$ l of a sample and 20  $\mu$ l of hexokinase (0.3 U) were added to each well. After the addition of hexokinase to each well, the plate was then shaken in a fluorescence plate reader (Arvo, Perkin-Elmer, Groningen, The Netherlands) and measurements were taken after 30 min of incubation. The measurements of NADPH generated from glucose in brain tissues via enzyme reaction were taken at 350-nm excitation and 450-nm emission. Tissue glycogen levels, indicated as glucose units, were calculated by subtracting the final micromole concentration of glucose per gram of wet weight in the non-hydrolyzed tissue sample.

### Western-blot analysis

Immunoblot analysis was taken as described previously [8, 21]. Brain tissues were powdered by grinding tissue from the right hemisphere in liquid nitrogen. Subsequently, these powdered samples were homogenized in 1.0 ml of LB-TT containing 7.0 M urea, 2.0 M thiourea, 4.0% CHAPS, 18 mM, 1.0 M of Tris–HCl buffer (pH 8.0), 14 mM Tris-Base, 1% protease inhibitors cocktails, 0.2% TORITON  $\times$  100, 50 mM DTT and 0.2% ampholyte. Homogenates were cleaned up using the ProteoExtract Protein Precipitation Kit (Calbiochem, Darmstadt, Germany) and total protein concentration was determined as per the BCA procedure (Pierce, Rockford, IL, USA). The proteins (10  $\mu$ g) were heated to 95 °C for 30 s with SDS, cooled to room temperature, and then separated using electrophoresis on a 5–20% polyacrylamide gradient gel (ATTO Corporation, Tokyo, Japan). The proteins in the gel were transferred to PVDF membranes (Immuno-Blot, Amersham Pharmacia Biotech, Piscataway, NJ, 30 min, 15 V). Then, the membranes were blocked with 5% nonfat dry milk (#9999, Cell Signaling) in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h. The membranes were washed with TBS-T and then incubated overnight at 4 °C with TBS-T containing one of the following primary antibodies: anti-MCT2 (sc-166925, 1:500), anti-MCT1 (sc-50325, 1:500), anti-MCT4 (sc-50329,

1:500, Santa Cruz Biotechnology) and anti- $\beta$ -actin (A5441, 1:50000, Sigma-Aldrich Co). After incubation, the membranes were again washed with TBS-T, and then incubated with TBS-T containing one of the following secondary antibodies: horseradish peroxidase (HRP)-conjugated goat anti-mouse was used for MCT2 (1:5000) and  $\beta$ -actin (1:50000, sc-2005, Santa Cruz Biotechnology) and HRP-conjugated goat anti-rabbit for MCT1 and MCT4 (1:5000, sc-2030, Santa Cruz Biotechnology). Immunocomplexes were made visible using chemiluminescence analysis with the Chemilumi One Super Kit (Nakalai Tesque, Kyoto, Japan). The immunoblotting images were digitally scanned and then quantified using image-analysis software (GE Healthcare, Bio-Science). The band intensities of the proteins were normalized against the band intensity of  $\beta$ -actin.

### Statistical analysis

Data are expressed as mean  $\pm$  standard error (SEM) and were analyzed using Prism 7 (MDF Co., Ltd, Tokyo, Japan). Before running the analysis for group comparisons, we strictly checked the normality of raw data distribution using histograms. When the data were normally distributed, parametric tests (unpaired *t* test) were used for statistical analyses (for glycogen levels in the hypothalamus, MCT2 in all brain regions, MCT1 in the hippocampus, hypothalamus, cerebral cortex and brainstem, and MCT4 in the hippocampus, hypothalamus and cerebral cortex). On the other hand, if we could not confirm that data was distributed normally, non-parametric tests (Mann–Whitney test) were used for statistical analyses (for glycogen levels in the hippocampus, cerebral cortex, cerebellum and brainstem, MCT1 in the cerebellum, and MCT4 in the cerebellum and brainstem). Correlations were calculated using Pearson's (parametric) correlation analysis. Statistical significance was set at  $p < 0.05$ .

## Results

Physiological and biochemical parameters in experimental rats are listed in Table 1. OLETF rats exhibited higher body weight ( $p < 0.05$ ) with an increased ratio of fat to body weight and hyperphagia ( $p < 0.001$ ) compared to the genetic control LETO rats. In addition, OLETF rats exhibited T2DM symptoms, such as hyperglycemic state ( $p < 0.01$ ) and hyper-HbA<sub>1C</sub> levels ( $p < 0.001$ ), compared to LETO rats.

### Glycogen levels in T2DM brain regions

OLETF rats exhibited significantly higher glycogen levels in the hypothalamus and cerebral cortex (about 23 and

**Table 1** Baseline of physiological and biochemical parameters

	LETO	OLETF
Body weight (g)	548.4 ± 8.52	613.1 ± 20.4*
Food intake (g/day)	19.6 ± 0.17	28.4 ± 0.73***
Fat/BW (mg/g)	23.5 ± 1.30	68.6 ± 3.96***
Blood glucose (mM)	6.01 ± 0.10	10.5 ± 1.50**
HbA <sub>1C</sub> (%)	4.75 ± 0.06	6.49 ± 0.34***

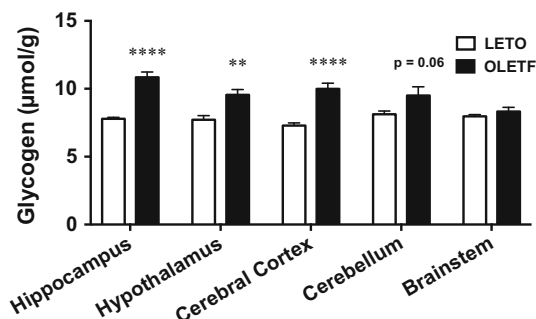
Data are expressed as mean ± SEM, *n* = 10 rats for each group

\* *p* < 0.05, \*\* *p* < 0.01, \*\*\* *p* < 0.001 vs. LETO rats

37%, respectively) compared to the LETO control rats (Fig. 1; *p* < 0.01, *p* < 0.0001, respectively). Significant association was noted between levels of glycogen and circulatory HbA<sub>1C</sub> in these brain regions (ESM Fig. S1b and S1c; hypothalamus: *r* = 0.68, *p* < 0.001, cortex: *r* = 0.88, *p* < 0.0001), as well as in the hippocampus (Fig. 1; *p* < 0.0001, ESM Fig. S1a; *r* = 0.84, *p* < 0.0001). In contrast, the other two regions of the brain, the cerebellum and brainstem did not differ significantly in glycogen levels (Fig. 1; *p* = 0.06, *p* > 0.05, respectively) between the OLETF and LETO rats. Although there was no significant difference in glycogen levels in the cerebellum and brain stem in T2DM and non-T2DM rats, there was a clear association between levels of glycogen and circulatory A<sub>1C</sub> in these important brain regions (ESM Fig. S1d and S1e; cerebellum: *r* = 0.76, *p* < 0.0001, brainstem: *r* = 0.53, *p* < 0.05).

### MCT-series protein levels in T2DM brain regions

MCT-series protein levels are expressed based on LETO rats as 100%. MCT2 protein levels in the hypothalamus and the hippocampus of OLETF rats was low (Fig. 2b; both *p* < 0.05), and there were no significant changes in MCT2 protein levels in the other brain regions (cerebral



**Fig. 1** Glycogen levels in the hippocampus, hypothalamus, cortex, cerebellum, and brainstem of control LETO and T2DM OLETF rats. Data are expressed as mean ± SEM. *n* = 10 rats for each group. \*\**p* < 0.01, \*\*\*\**p* < 0.0001 vs. LETO rats

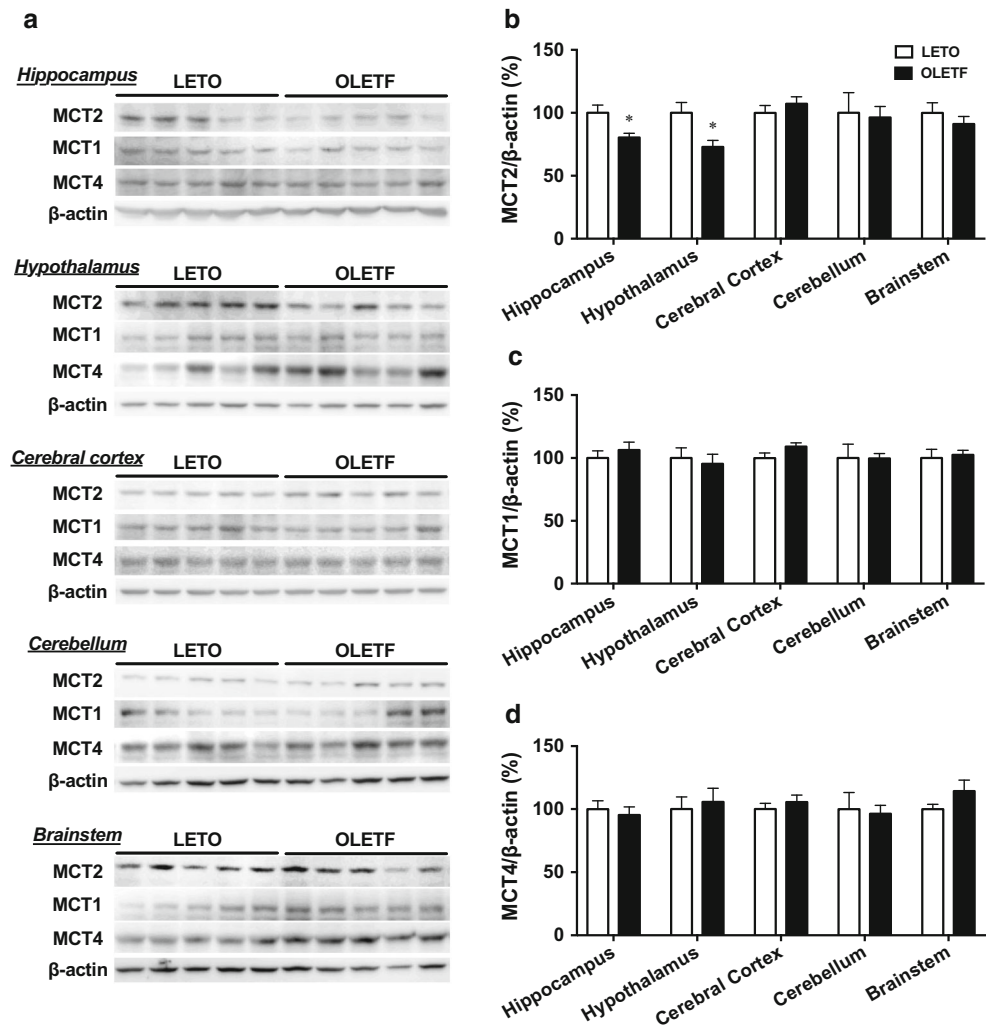
cortex, cerebellum, and brainstem) compared to those of the control LETO rats (Fig. 2b; *p* > 0.05). In addition, MCT2 protein levels had a significant negative correlation with circulatory HbA<sub>1C</sub> levels in the hypothalamus (ESM Fig. S2b; *r* = −0.47, *p* < 0.05), as well as in the hippocampus (ESM Fig. S2a; *r* = −0.50, *p* < 0.05), but not in the other brain regions (ESM Fig. S2c, S2d and S2e; cortex: *r* = 0.13, *p* > 0.05, cerebellum: *r* = 0.01, *p* > 0.05, brainstem: *r* = −0.13, *p* > 0.05). MCT2 protein levels in the hypothalamus had a significant negative correlation with food intakes (ESM Fig. S3; *r* = −0.56, *p* < 0.01). Of note, the MCT1 and four protein levels in the brain were not significantly altered by T2DM rats compared to those in LETO rats (Fig. 2c, d). Furthermore, MCT2 protein levels had a significant negative correlation with glycogen levels in the hippocampus (Fig. 3a; *r* = −0.53, *p* < 0.05) but not in the other brain regions (Fig. 3b–e; hypothalamus: *r* = −0.38, *p* = 0.09, cortex: *r* = 0.08, *p* > 0.05, cerebellum: *r* = −0.03, *p* > 0.05, brainstem: *r* = 0.13, *p* > 0.05).

### Discussion

In the present study, we found region-specific alterations in two crucial components of glycometabolism associated with the ANLS system in the brain, namely the MCT-series protein levels and the local glycogen levels, in a T2DM rat model. Among the several brain regions (hippocampus, hypothalamus, cerebral cortex, cerebellum, and brainstem) investigated in the present study, significant hyper-glycogen levels and downregulated MCT2 protein levels were found in the hippocampus and hypothalamus in T2DM rats compared to those of the control rats, and cerebral cortex glycogen levels were significantly higher in T2DM rats compared to control rats. Additionally, there was a significant negative correlation between glycogen levels and MCT2 protein levels only in the hippocampus.

The physiological, biological, and clinical parameters of OLETF rats used in the present study are well established and were consistent with previous studies [8, 17] (details in Table 1). These T2DM rats had significantly higher glycogen levels not only in the hippocampus but also in the hypothalamus and cerebral cortex compared to those of control rats (Fig. 1). In addition, the glycogen levels in these regions had a significant positive correlation with circulatory glycemic state (ESM Fig. S1a, S1b, and S1c). This pattern of T2DM-induced hyper-glycogen levels, as well as their association with a circulatory glycemic state, was also observed in the hippocampus of T2DM rats in our recent study [8]. Although we currently have no mechanistic explanation to account for the observed changes in T2DM, such as increased glycogen levels, the possibility of

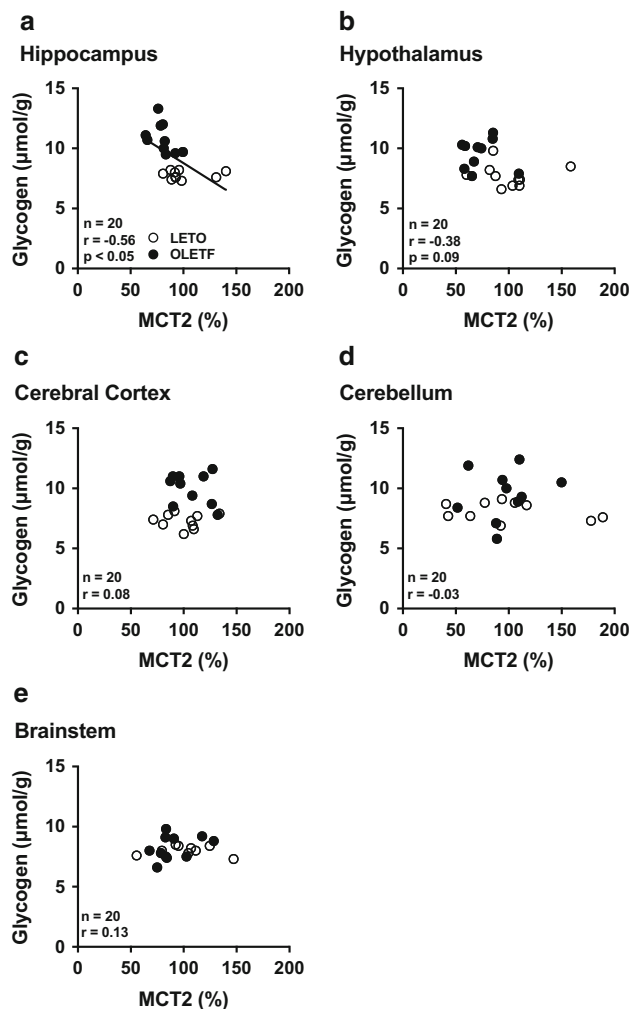
**Fig. 2** **a** Representative immunoblotting images of MCT2, MCT1 and MCT4. Immunoblot analysis for MCT2 (**b**), MCT1 (**c**) and MCT4 (**d**) protein levels of control LETO and T2DM OLETF rats in the hippocampus, hypothalamus, cortex, cerebellum and brainstem, respectively. Protein levels were normalized against  $\beta$ -actin as an internal standard. Data are expressed based on LETO rats as 100%,  $n = 10$  rats for each group. \* $p < 0.05$  vs. LETO rats



metabolic adaptation cannot be completely ruled out. Indeed, previous reports have shown that the heart in T2DM subjects also exhibits hyper-glycogen levels [10, 22], which is likely a metabolic adaptation. Thus, based on these studies, it is reasonable to assume that the hyper-glycogen levels associated with T2DM reported here may be a metabolic adaptation to compensate for the decreased utilization of lactate and glucose in the brain [8]. Although increased glycogen levels in the cerebellum and brainstem were not induced in T2DM rats, the present study revealed that glycogen levels in these brain regions have a positive correlation with HbA<sub>1C</sub> levels (Fig. 1, ESM Fig. S1d and S1e). A time-course study to track molecular and biochemical changes beginning with pre-diabetes and continuing through to severe diabetes and the associated end-stage complications is essential in order to explore and chronicle the sequence of changes in glycogen levels in various brain regions in T2DM subjects.

As a crucial part of the ANLS system, in the present study, we also investigated MCT-series protein levels in

the brain. Compared to those of the genetic control rats, MCT1 and MCT4 (which release lactate from astrocytes into extracellular fluid) protein levels in T2DM rats were not altered in any brain regions investigated in the current study (Fig. 2c, d), the protein levels of MCT2, which plays an important role in the uptake of lactate by neurons [5], were significantly decreased in the T2DM hypothalamus as well as hippocampus, but not in the cerebral cortex, cerebellum, or brainstem (Fig. 2b). Based on previous studies, decreased MCT2 protein levels result in impairment of brain function [3, 8, 9]. The hypothalamus regulates feeding behavior [11], and we found in the present study that there is a significant negative correlation between MCT2 protein levels and food intake (ESM Fig. S3). These data imply that diminished transport of lactate to hypothalamic neurons through downregulated MCT2 protein levels is a possible contributor to hyperphagia in T2DM subjects. This current finding again signifies that MCT2 protein levels are important in maintaining brain function in not only the hippocampus, as demonstrated in



**Fig. 3** Correlation between MCT2 protein levels and glycogen levels in the hippocampus, hypothalamus, cortex, cerebellum and brainstem (a–e), respectively. The line in the scatter diagram indicates significant correlation

our recent study [8], but also the hypothalamus. In addition, MCT2 protein levels had a significant negative correlation with circulatory HbA<sub>1C</sub> levels in the hypothalamus and hippocampus, but not in the other three brain regions investigated here (ESM Fig. S2). Based on these data, it can be stated that not only are local glycogen levels in the T2DM brain region-specific, but also that MCT2 is expressed distinctly, based on brain region. Further, MCT2 protein levels had a significant negative correlation with glycogen levels only in the hippocampus, not in the other brain regions, in the current study (Fig. 3). T2DM-induced glycogen increase in brain may be a metabolic adaptation to compensate for the decreased utilization of lactate via MCT2 [8], and the hippocampus may be the first-line most vulnerable region to be affected in regards to ANLS-related glycometabolism induced by T2DM. However, this statement is still premature based on the evidence from current

study warranting in-depth investigation of the cause–effect relationship in between glycogen and MCT2 levels in T2DM hippocampus.

The current study has several other limitations. We investigated glycometabolism in only five broad regions of the T2DM brain; we did not study other brain regions with sub-regions with different functions. This limitation should be addressed promptly in future studies. In addition, more mechanism-based studies are needed to directly link the present observations to ANLS-related glycometabolism in the T2DM brain. More essentially, intervention studies should also be planned to explore ways to overcome the alterations of ANLS-related glycometabolism in the T2DM brain.

In summary, here we found that T2DM rats exhibit decreased MCT2 protein levels and increased glycogen, associated with HbA<sub>1C</sub> levels, in both the hippocampus and hypothalamus, suggesting the possible dysregulation of glycometabolism in both these brain loci with T2DM. This observed change, possibly linked to ANLS-related glycometabolism, in the hypothalamus (glycogen increase and MCT2 decrease) with T2DM is similar to the findings for the hippocampus found in our earlier study [8]. Further, since MCT2 protein levels have a significant negative correlation with glycogen levels only in the hippocampus, it may be more vulnerable than the other regions of the brain to T2DM in the context of ANLS-related glycometabolism alterations. The current findings lay a foundation and provide insights for exploring the pathogenesis of brain dysfunction in T2DM subjects in regards to the ANLS system.

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#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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