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Cognitive decline in type 2 diabetic *db*/*db* mice may be associated with brain region-specific metabolic disorders

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ABSTRACT

Type 2 diabetes has been associated with cognitive decline, but its metabolic mechanism remains unclear. In the present study, we attempted to investigate brain region-specific metabolic changes in *db*/*db* mice with cognitive decline and explore the potential metabolic mechanism linking type 2 diabetes and cognitive decline. We analyzed the metabolic changes in seven brain regions of two types of mice (wild-type mice and *db*/*db* mice with cognitive decline) using a ^{1}H NMR-based metabolomic approach. Then, a mixed-model analysis was used to evaluate the effects of mice type, brain region, and their interaction on metabolic changes. Compared with the wild-type mice, the *db*/*db* mice with cognitive decline had significant increases in lactate, glutamine (Gln) and taurine as well as significant decreases in alanine, aspartate, choline, succinate, γ-Aminobutyric acid (GABA), glutamate (Glu), glycine, N-acetylaspartate, inosine monophosphate, adenosine monophosphate, adenosine diphosphate, and nicotinamide adenine dinucleotide. Brain region-specific metabolic differences were also observed between these two mouse types. In addition, we found significant interaction effects of mice type and brain region on creatine/phosphocreatine, lactate, aspartate, GABA, N-acetylaspartate and taurine. Based on metabolic pathway analysis, the present study suggests that cognitive decline in *db*/*db* mice might be linked to a series of brain region-specific metabolic changes, involving an increase in anaerobic glycolysis, a decrease in tricarboxylic acid (TCA) and Gln-Glu/GABA cycles as well as a disturbance in lactate-alanine shuttle and membrane metabolism.

1. Introduction

Type 2 diabetes (T2D) is caused by insulin resistance or impaired insulin secretion and thereby results in hyperglycemia. T2D is a progressive disease which causes a series of complications. Cognitive decline is one of T2D-associated complications and seriously affect the quality of life of patients [1]. Several mechanisms were proposed between T2D and cognitive decline. Cerebrovascular mechanism has been suggested as the main reason from epidemiologic, imaging and autopsy studies [2]. Manschot et al. [3] and Cui et al. [4] revealed that T2D-related cognitive decline may be attributed to gray and white matter atrophy. Moreover, hippocampal atrophy was also found to impair cognitive function [5]. Hyperglycemia-induced neuronal apoptosis and dysfunction may also be one of potential mechanisms of T2D-related cognitive decline [6]. In addition, hyperinsulinaemia as a common characteristic of T2D patients was associated with cognitive decline [7]. However, there are still more questions than answers to fully understand the link between T2D and cognitive decline [8]. Recently, we found that cognitive decline in T2D may be induced by an unbalanced metabolism between astrocyte and neuron as well as an increase in gluconeogenesis [9]. Yet, these findings were obtained on the whole brain. Investigation of metabolic changes in different brain regions will advance understanding of brain metabolic mechanism linking T2D and cognitive decline.

Metabolomics as a relatively new omics technique aims to analyze a comprehensive set of metabolites in biological samples and examine their changes under a particular condition such as disease occurrence or drug intervention. It has been used as a promising tool in research field of brain metabolism [10]. Ivanisevic et al. [11] have used an untargeted metabolomics approach to profile metabolic phenotypes of eight brain regions in normal mice and thereby to give context to study brain metabolism during physiological and pathological events. Lalandea et al. [12] reported that metabolic alterations were first occurred in the hippocampus and rhinal cortex and then extended to

Abbreviations: ADP, adenosine diphosphate; Ala, alanine; Asp, aspartate; AMP, adenosine monophosphate; Cho, choline; Cre/PCre, creatine/phosphocreatine; FWT, fresh weight tissue; GABA, γ-aminobutyric acid; Gln, glutamine; Glu, glutamate; Gly, glycine; IMP, inosine monophosphate; Ino, inosine; Lac, lactate; Myo, myo-inositol; NAA, N-acetylaspartate; NAD+, nicotinamide adenine dinucleotide; Suc, succinate; T2D, type 2 diabetes; Tau, taurine; wt, wild-type.

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cerebellum and midbrain during Alzheimer's disease (AD) development. Moreover, they also found that a reduction in glutamate and N-acetylaspartate levels before 6 months and an increase in taurine and creatine levels by 6 months were main metabolic characteristics in the brain of AD mice [12]. In addition, using a metabolomics approach, metabolic changes were also examined in different brain regions from rat models of chronic unpredictable mild stress [13] and neonatal Borna disease [14]. However, brain region-specific metabolic changes in diabetic mice with cognitive decline have not reported yet. In the field of diabetic research, *db*/*db* mouse has been commonly used as a preclinical rodent model. It should be noted that, however, *db*/*db* mouse is a leptin receptor-deficient diabetic model and leptin was involved in the development of brain in mouse embryos [15]. Therefore, cognitive decline happened in 7-week-old *db*/*db* mice [16]. Yet, Stranahan et al. [17] and Dinel et al. [18] reported that leptin receptor deficiency may be not the main cause of cognitive decline in *db*/*db* mice. The aims of the present study were: (1) to analyze metabolic changes in different brain regions of *db*/*db* mice with cognitive decline and (2) to explore the potential metabolic mechanism in T2D-related cognitive decline.

2. Materials and methods

2.1. Animals

Twelve-week-old diabetic (*db*/*db*, BKS.Cg-m$^{+/+}$Leprdb/J, body weight = 43.20 ± 4.35, n = 10) and wild-type (*wt*, C57BLKS/J, body weight = 25.15 ± 2.32, n = 10) mice were purchased from the Mode Animal Research Center of Nanjing University, China. All mice were male and of the same genetic background (C57BLKS). All mice were housed in specific pathogen-free colony under standard condition (room temperature 22 °C with a 12:12-h light-dark cycle) at the Laboratory Animal Center of Wenzhou Medical University (Wenzhou, China). Mice were given free access to standard rat chow and tap water throughout the experimental period. This study was performed according to the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University (IRB number: wydw2015-0083).

2.2. Morris water maze (MWM) test

After 5 weeks, the MWM test was conducted to evaluate learning and memory performance according to a previously reported method [19]. Briefly, the test was performed in a circular pool with a diameter of 110 cm and a height of 30 cm, filled with opaque water at 26 ± 1 °C. The escape platform with a diameter of 7 cm was submerged 1 cm below the surface of the water. During 4 days of training (continuous and 4 trials per day), mice were guided to reach the escape platform by the operator, if they could not find it within 60 s. After training, trained mice were placed in the same start location and subjected to a 90 s probe trial without the escape platform. The behavior was tracked and recorded by an overhead video camera and a computer system equipped with 'Viewer 2' software (Biobserve GmbH, Bonn, Germany) to calculate the swimming path length to reach the original platform location as well as the number of crossings over the original platform location.

2.3. Sample preparation

Mice were sacrificed by decapitation and then brains were isolated immediately. The brain was firstly dissected into cerebellum, hippocampus, striatum, and cerebral cortex. Then according to anatomical boundaries cerebral cortex was further divided into parietal lobe, occipital lobe, frontal lobe, and temporal lobe. All tissues were frozen in liquid nitrogen immediately and stored at −80 °C until analysis. The frozen tissue was weighed into an Eppendorf tube and extracted using our previous published method [9]. Briefly, 4 ml/g of cold methanol and 0.85 ml/g of cold distilled water were added into the sample tube. After homogenizing using a handheld homogenizer, 2 ml/g of cold chloroform and 2 ml/g of cold distilled water were added into the mixture. Then, the mixture was vortex mixed, kept on ice for 15 min, and centrifuged for 15 min at 10,000 *g* at 4 °C. Finally, the supernatant was transferred into a new Eppendorf tube, lyophilized for about 24 h, and stored at −80 °C until use. The lyophilized sample was reconstituted in the Eppendorf tube with 0.6 ml of D_2O (99.5%) containing 0.05% of sodium trimethylsilyl propionate-d_4 (TSP) and then transferred to a 5 mm NMR tube for metabolomic analysis.

2.4. NMR-based metabolomic analysis

^{1}H NMR spectra were recorded using a Bruker AVANCE III 600 MHz NMR spectrometer equipped with a 5-mm TXI probe (Bruker BioSpin, Rheinstetten, Germany) at 25 °C. A standard single-pulse sequence with water signal pre-saturation (zgpr) was used in the present study. The typical acquisition parameters were set as follows: scans, 256; data points, 64 K; spectral width, 12,000 Hz; relaxation delay, 6 s; acquisition time: 2.65 s per scan.

NMR spectra were preprocessed using Topspin software (v2.1 pl4, Bruker Biospin, Germany), with reference to the TSP peak (δ 0.0) and phase/baseline corrected manually. Subsequently, NMR peaks were assigned based on reported data [20,21]. For metabolite quantification, the peak areas were automatically integrated using Topspin software and carefully checked to exclude the overlaid peaks. The concentrations of metabolites were calculated in accordance with their peak areas by reference to the internal TSP concentration and expressed as μmol/g fresh weight tissue (FWT) (see supplementary material).

Metabolic pathways were produced manually using Adobe Photoshop CS6 (Adobe Inc., San Jose CA) according to the KEGG database (www.genome.jp/kegg/).

2.5. Statistical analysis

Principal component analysis (PCA) was used to obtain an overview of the metabolic pattern changes among different brain regions in *wt* and *db*/*db* mice based on quantified metabolites using MetaboAnalyst 3.0 [22]. Metabolite concentrations were Log-transformed and Auto-scaled prior to PCA. The difference in behavioral data between *wt* and *db*/*db* mice was analyzed by Student's *t*-test with Bonferroni correction in SAS 9.2 (SAS Institute Inc., Cary, NC). In addition, a linear mixed-model analysis of variance (ANOVA) was conducted using MIXED procedure in SAS 9.2. This model included mice type, brain region, and their interaction as fixed effects, while individuals and the intercept of model were set as random effects. The restricted maximum likelihood (REML) was performed to estimate variance components in the mixed-model [23]. The optimal model was identified by Akaike information criterion [24]. Data are presented as least-square (LS) means and standard errors (SE) by LS-means procedure, and pairwise-tests for multiple comparisons were estimated using Student's *t*-test with Bonferroni correction. In the present study, the difference was considered statistically significant when Bonferroni-adjusted P value < 0.05.

3. Results

3.1. Cognitive decline in db/db mice

Fig. 1A illustrates the swimming path of *wt* and *db*/*db* mice to reach the original platform location in the probe test of the MWM test. Compared with age-matched *wt* mice, *db*/*db* mice showed significantly longer swim path length to cross the original platform location (Fig. 1B). In addition, the number of crossings over the original platform location was significantly reduced in *db*/*db* mice than *wt* mice, as shown in

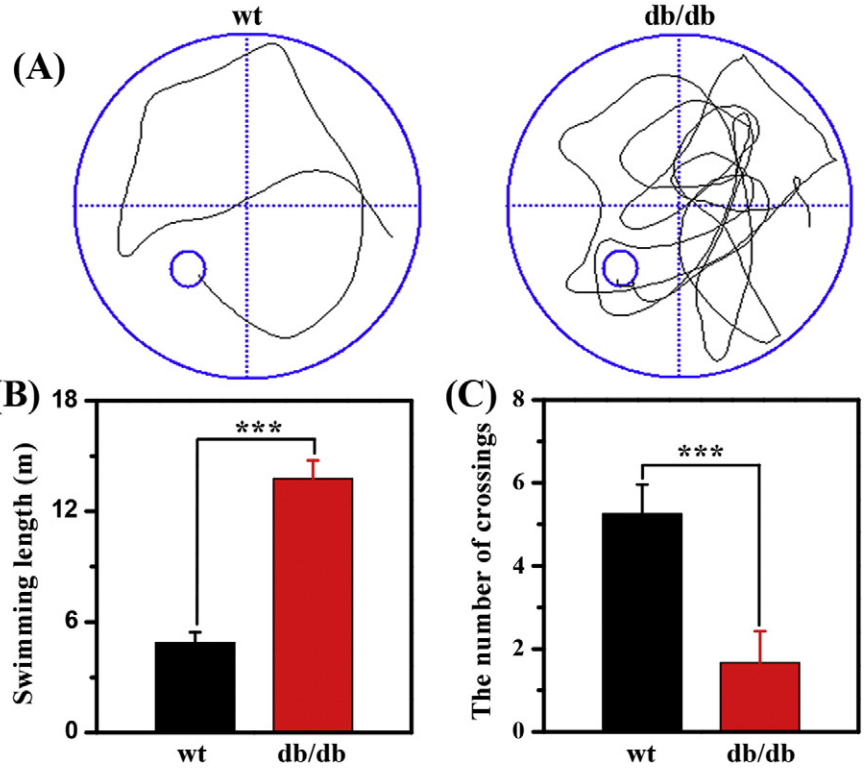

Fig. 1. Learning and memory performance of *wt* and *db*/*db* mice evaluated using a 90 s probe trial of the Morris water-maze test: (A) the swimming path to reach the original platform location; (B) swimming length to reach the original platform location; (C) the number of crossings over the original platform location. The small circle in Fig. 1A represents the original platform location, but the escape platform has been removed in the probe test. Significant level: $^{***}P < 0.001$.

Fig. 1C. Taken together, the MWM test suggests that learning and memory performance was impaired in *db*/*db* mice relative to *wt* mice.

3.2. Metabolic profiles in brain extract of db/db mice with cognitive decline

Fig. 2 shows a typical ^{1}H NMR metabolic profile of brain extract (hippocampus) in *db*/*db* mice with cognitive decline. All brain regions demonstrate similar metabolic profiles (data not shown). A total of 18 metabolites were identified involving energy metabolism (Lac, lactate; Ala, alanine; Cre/PCre, creatine/phosphocreatine; Suc, succinate), neurotransmitters (Glu, glutamate; Gln, glutamine; GABA, γ-Aminobutyric acid; Asp, aspartate; Gly, glycine), membrane metabolism (Cho, choline; NAA, N-acetylaspartate), osmoregulation (Tau, taurine; Myo, myo-inositol), and nucleotides (AMP, adenosine monophosphate; ADP, adenosine diphosphate; IMP, inosine monophosphate; NAD+, nicotinamide adenine dinucleotide; Ino, inosine).

3.3. The change of brain metabolic patterns in db/db mice with cognitive decline

The overview of metabolic pattern changes among different brain regions between *wt* mice and *db*/*db* mice with cognitive decline was illustrated in Fig. 3. Results show that metabolic patterns in cerebellum, hippocampus and striatum were largely separated from other brain regions in both of *wt* mice (Fig. 3A) and *db*/*db* mice with cognitive decline (Fig. 3B). However, it is worth noting that metabolic patterns in four regions of the cerebral cortex were slightly differentiated in *db*/*db* mice with cognitive decline relative to *wt* mice.

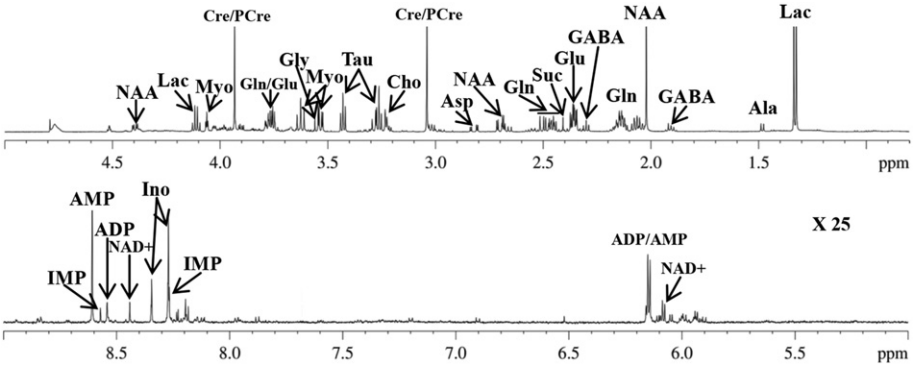

Fig. 2. Typical 600 MHz ^{1}H NMR spectra of brain tissue (hippocampus) extract in *db*/*db* mice. Assignments: Ala, alanine; Asp, aspartate; Cho, choline; Cre/PCre, creatine/phosphocreatine; GABA, γ-Aminobutyric acid; Gln, glutamine; Glu, glutamate; Gly, glycine; Lac, lactate; Myo, myo-inositol; NAA, N-acetylaspartate; Suc, succinate; Tau, taurine; AMP, adenosine monophosphate; ADP, adenosine diphosphate; IMP, inosine monophosphate; NAD+, nicotinamide adenine dinucleotide; and Ino, inosine.

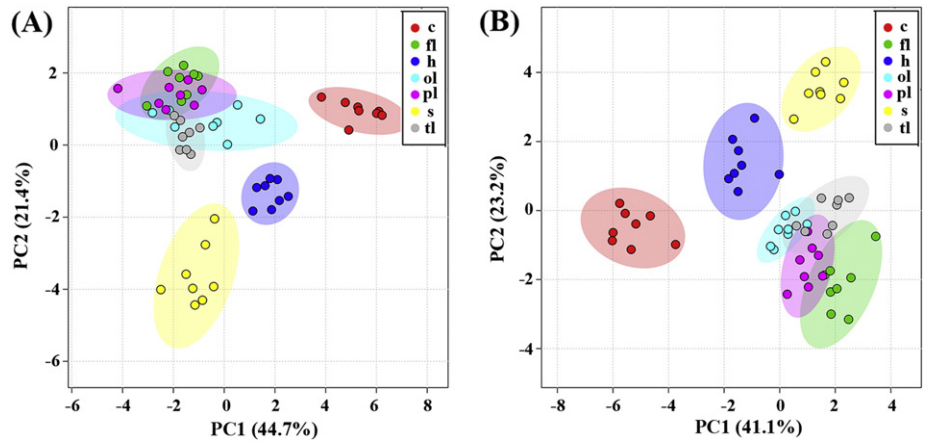

Fig. 3. Overview of metabolic pattern changes among different brain regions in (A) *wt* mice and (B) *db/db* mice with cognitive decline analyzed using principal component analysis: pl, parietal lobe; ol, occipital lobe; fl, frontal lobe; tl, temporal lobe; h, hippocampus; s, striatum; and c, cerebellum.

3.4. Brain region-specific metabolic changes in db/db mice with cognitive decline

In this study, we used a mixed-model analysis to assess the effects of mice type, brain region, and their interaction on metabolic changes, and the detailed results were listed in Tables S1–S4. Fig. 4 illustrates the overview of the whole brain metabolic changes between *wt* mice and *db/db* mice with cognitive decline.

Table S1 shows energy metabolite changes in different brain regions between *wt* mice and *db/db* mice with cognitive decline. Compared with

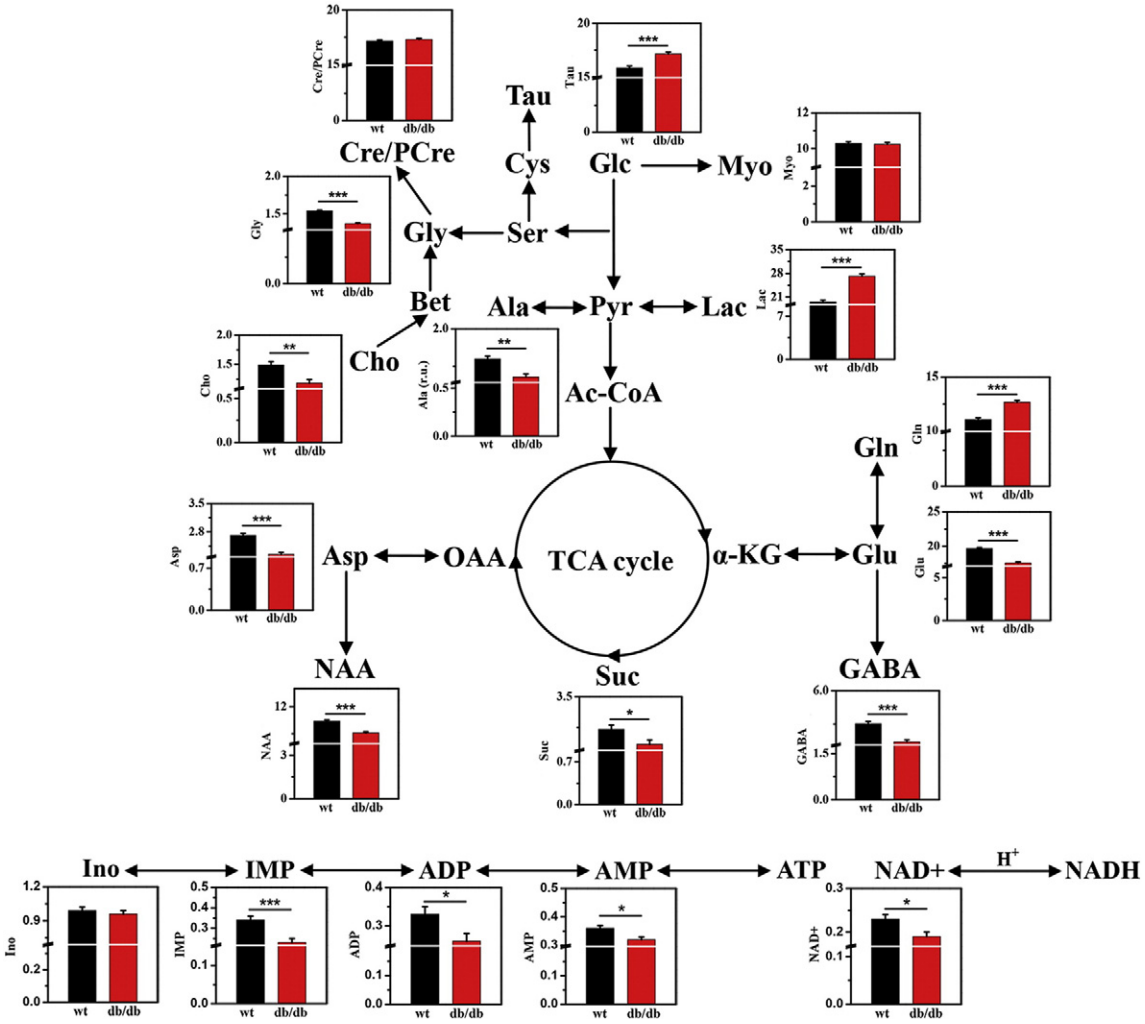

Fig. 4. Metabolic changes in the whole brain of *wt* mice and *db/db* mice with cognitive decline: Ala, alanine; Asp, aspartate; Cho, choline; Cre/PCre, creatine/phosphocreatine; GABA, γ-Aminobutyric acid; Gln, glutamine; Glu, glutamate; Gly, glycine; Lac, lactate; Myo, myo-inositol; NAA, N-acetylaspartate; Suc, succinate; Tau, taurine; AMP, adenosine monophosphate; ADP, adenosine diphosphate; IMP, inosine monophosphate; NAD +, nicotinamide adenine dinucleotide; and Ino, inosine. Data are obtained from mixed-model analysis and presented as LS-means ± SE (μmol/g FWT). Significant level: $^{*}P < 0.5$; $^{**}P < 0.01$; $^{***}P < 0.001$.

wt mice, a significant increase in Lac and a significant decrease in Ala and Suc were found in *db*/*db* mice with cognitive decline (Fig. 4). Yet, there was no significant difference in Cre/PCre between them. Brain region-specific differences were statistically significant in Ala, Cre/PCre and Suc ($P < 0.0001$), but not in Lac ($P = 0.2$). The concentration of Ala was significantly higher in hippocampus than in striatum and cerebellum, while no significant difference from parietal lobe, occipital lobe, frontal lobe, and temporal lobe. The cerebellum had a highest concentration of Cre/PCre, followed by hippocampus, striatum, occipital lobe, parietal lobe, temporal lobe, and frontal lobe. Table S1 also shows that the cerebellum and hippocampus showed a significantly higher concentration of Suc as compared with other brain regions. Moreover, there were significant interaction effects of mice type and brain region on Cre/PCre ($P = 0.006$) and Lac ($P < 0.0001$) and their changes were illustrated in Figs. 5A and B, respectively. We found that only in parietal lobe *db*/*db* mice with cognitive decline had a significant higher concentration of Cre/PCre than *wt* mice. However, a significant higher concentration of Lac was obtained in all brain regions of *db*/*db* mice with cognitive decline (Fig. 5B).

Variations in neurotransmitter metabolism in different brain regions between *wt* mice and *db*/*db* mice with cognitive decline were listed in Table S2. A higher concentration of Gln was observed in *db*/*db* mice with cognitive decline relative to *wt* mice, while the concentrations of Asp, GABA, Glu, and Gly were significantly decreased (Fig. 4, $P < 0.05$). There were significant brain region effects on all neurotransmitters analyzed in the present study (Table S2, $P < 0.0001$). The hippocampus and striatum had a significantly lower concentration of Asp compared with other brain regions. The highest concentration of GABA was detected in hippocampus, followed by striatum, cerebellum and cerebral cortex. The hippocampus showed a significantly higher concentration of Glu than parietal lobe, occipital lobe and striatum (Table S2). For Gln concentration, the highest and lowest concentrations were observed in cerebellum and parietal/frontal lobes, respectively. In addition, Gly concentration was significantly lower in cerebral cortex than other brain regions. It is worth noting that significant interaction effects of mice type and brain region on Asp ($P < 0.0001$) and GABA ($P = 0.02$) were found in the present study. We found that Asp concentration was significantly reduced in all brain regions of *db*/*db* mice with cognitive decline relative to *wt* mice (Fig. 5C). Moreover, in *wt* mice, the highest concentration of Asp was obtained in cerebellum, but in frontal lobe in *db*/*db* mice with cognitive decline. Compared with *wt* mice, *db*/*db* mice with cognitive decline had a significant decrease in GABA concentration in hippocampus and parietal lobe, while no significant differences were found in other brain regions between them, as shown in Fig. 5D. In addition, the hippocampus in *wt* mice possessed a significantly higher GABA concentration than striatum, but there was no significant difference between these two brain regions in *db*/*db* mice with cognitive decline.

Table S3 shows changes in membrane metabolism and osmoregulation in different brain regions between *wt* mice and *db*/*db* mice with cognitive decline. Membrane metabolism-related metabolites, Cho and NAA, were significantly decreased in *db*/*db* mice with cognitive decline, while an opposite result was observed in Tau concentration (Fig. 4). Significant brain region-specific differences were found in NAA, Myo and Tau ($P < 0.0001$), but not in Cho ($P = 0.07$). The hippocampus had a significantly lower concentration of NAA than

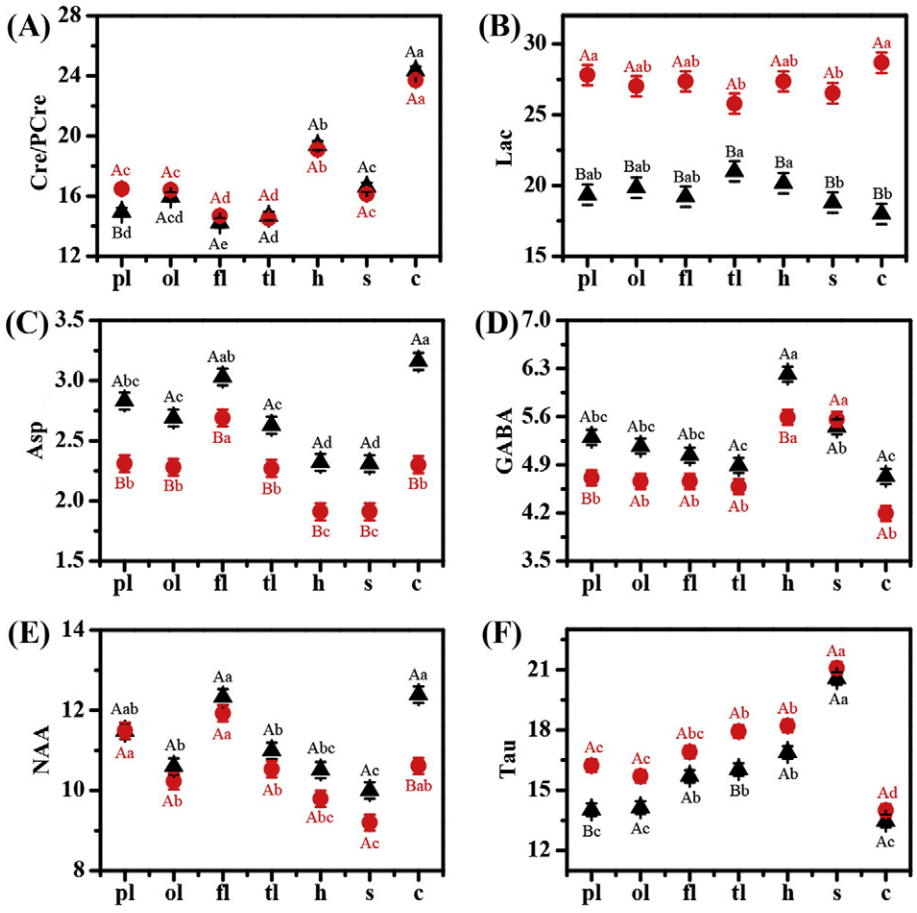

Fig. 5. Changes in metabolites of different brain regions in (▲) *wt* mice and (●) *db*/*db* mice with cognitive decline: (A) creatine/phosphocreatine; (B) lactate; (C) aspartate; (D) γ-Aminobutyric acid; (E) N-acetylaspartate; and (F) taurine. Brain regions: pl, parietal lobe; ol, occipital lobe; fl, frontal lobe; tl, temporal lobe; h, hippocampus; s, striatum; c, cerebellum. Different uppercase and lowercase letters indicate significant differences between *wt* and *db*/*db* mice and among different brain regions, respectively (Bonferroni-adjusted *P* value < 0.05). Data are obtained from mixed-model analysis and presented as LS-means ± SE (μmol/g FWT).

parietal lobe, frontal lobe, and cerebellum. However, a higher Myo concentration was obtained in hippocampus and cerebellum relative to other regions (Table S3). For Tau concentration, the striatum showed the highest concentration, followed by hippocampus, temporal lobe, frontal lobe, parietal lobe, occipital lobe and cerebellum. Table S3 also shows that there were significant interaction effects of mice type and brain region on NAA ($P = 0.001$) and Tau ($P = 0.02$). It can be seen from Fig. 5E that NAA concentration was significantly decreased in *db*/*db* mice with cognitive decline in cerebellum, but not in other brain regions. Moreover, compared with *wt* mice, *db*/*db* mice with cognitive decline showed a significantly increased concentration of Tau only in parietal lobe (Fig. 5F).

In addition, significantly lower concentrations of AMP, ADP, IMP and NAD+ were found in *db*/*db* mice with cognitive decline as compared with *wt* mice, while no significant difference in concentration of Ino (Table S4). The effect of brain region was statistically significantly different on all nucleotides in this study ($P < 0.0001$). We found that the concentrations of AMP, IMP, NAD+ and Ino were significantly lower in hippocampus and cerebellum than other brain regions (Table S4). For ADP, the highest concentration was observed in striatum. Moreover, there was no significant interaction effect of mice type and brain region on all nucleotides in the present study ($P > 0.05$).

4. Discussion

T2D as a progressive metabolic disease has gained considerable attention for its adverse effect on the central nervous system. In the present study, we report that learning and memory performance was impaired in 17-week-old *db*/*db* mice relative to age-matched *wt* mice. Therefore, we examined metabolic changes in seven brain regions of *db*/*db* mice with cognitive decline to explore the potential metabolic mechanism linking T2D and cognitive decline. Our results suggest that this link may be implicated in changes of energy metabolism, neurotransmitter metabolism, membrane metabolism and osmoregulation in different brain regions.

4.1. Brain energy metabolism in db/db mice with cognitive decline

The brain requires high energy for maintaining its normal function. Although the brain accounts for only 2% of the total body mass, approximately 25% of glucose is dedicated to brain energy metabolism [25]. Glucose can be converted to pyruvate and then pyruvate is oxidized to CO_2 and H_2O via tricarboxylic acid (TCA) cycle or transformed into Lac by anaerobic glycolysis. In this study, compared with *wt* mice, a significantly higher concentration of Lac may indicate that anaerobic glycolysis was increased in the brain of *db*/*db* mice with cognitive decline, which was further confirmed by decreased concentrations of Suc as a TCA intermediate and Ala as an amino acid with a close link to glycolysis and the TCA cycle. Furthermore, we also found that the energy biomolecules including IMP, AMP, ADP and NAD+ were significantly reduced in the brain of *db*/*db* mice with cognitive decline relative to *wt* mice. Sickmann et al. [26] reported that the TCA cycle was down-regulated more than glycolysis in the brain of T2D rats. Although Lac can be further utilized as an energy substrate in the brain [27], an increased Lac level has been associated with cognitive decline in human [28,29] and animal models [30]. In addition, a lactate-alanine shuttle would be responsible for nitrogen exchange in mammalian brain [31,32]. Compared with *wt* mice, a decrease in Ala and an increase in Lac were observed in this study, indicating a disturbance of lactate-alanine shuttle in the brain of *db*/*db* mice with cognitive decline. However, in our previous study, we found an increased transfer from Lac to Ala in neurons of *db*/*db* mice with cognitive decline using ^{13}C NMR spectroscopy [9]. A significant interaction effect of mice type and brain region on Lac was obtained by mixed-model analysis and Lac concentration was significantly increased in all brain regions of *db*/*db* mice with cognitive decline relative to *wt* mice. The interaction effect was also found on Cre/PCre, which plays an important role in brain energy homeostasis [33]. We found that Cre/PCre concentration was significantly increased in parietal lobe of *db*/*db* mice with cognitive decline as compared with *wt* mice, but not in other brain regions examined in this study. Moreover, Ala, Cre/PCre and Suc were brain region-specific, for instance, the hippocampus and cerebellum had a higher concentration of Suc than other brain regions. Thus, cognitive decline in *db*/*db* mice may be linked to an increase in anaerobic glycolysis, a decrease in the TCA cycle and a disorder in lactate-alanine shuttle. Moreover, a disturbance in brain region-specific energy metabolism may also contribute to cognitive decline in *db*/*db* mice.

4.2. Brain neurotransmitter metabolism in db/db mice with cognitive decline

Neurotransmitter metabolism is also vital to keep normal brain function. The Gln-Glu/GABA cycle between astrocytes and neurons plays a critical role in regulation of neurotransmitter homeostasis [34]. In this cycle, Gln as an amino acid can directly synthesize the excitatory neurotransmitter, Glu, by ammonia metabolism, and then indirectly generate the inhibitory neurotransmitter, GABA. In the present study, a significant increase in Gln concentration and a significant decrease in Glu and GABA concentrations were observed in the brain of *db*/*db* mice with cognitive decline, indicating an inhibition of the Gln-Glu/GABA cycle. Neurotransmitter metabolism is closely linked to learning and memory performance in the water maze. For example, the impairment of spatial learning and memory was observed after systemic administration of Glu [35] and GABA [36] receptor antagonists in rats. Moreover, there were brain region-specific differences in the concentrations of Gln, Glu and GABA. The hippocampus had relatively higher concentrations of Glu and GABA, while higher Gln concentration was observed in cerebellum, followed by hippocampus. It is worth noting that a significant effect of mice type and brain region interaction was obtained on GABA concentration. We found that GABA concentration was significantly reduced only in parietal lobe and hippocampus of *db*/*db* mice with cognitive decline. Sickmann et al. [26] reported that the Gln-Glu/GABA cycle was less affected in cortex and cerebellum than in hippocampus due to T2D.

Asp as another excitatory neurotransmitter is directly derived by transamination from a TCA cycle intermediate, oxaloacetate. We found that the concentration of Asp was significantly decreased in all brain regions of *db*/*db* mice with cognitive decline compared with *wt* mice. This is in agreement with a reduction in the TCA cycle observed in *db*/*db* mice with cognitive decline. In addition, Asp concentration was brain region-specific, and the hippocampus showed a lower concentration of Asp than other brain regions. Like GABA, Gly as an inhibitory neurotransmitter has an essential role in inhibitory and excitatory synapses in the central nervous system [37,38]. Compared with *wt* mice, the present study shows a significantly decreased concentration of Gly in the brain of *db*/*db* mice with cognitive decline. Xin et al. [39] reported that Gly was highly concentrated in brain stem. In the present study, however, we found a higher concentration of Gly in hippocampus, striatum and cerebellum than in cerebral cortex. Therefore, an inhibition of the Gln-Glu/GABA cycle, perturbed Asp and Gly concentrations as well as brain region-specific neurotransmitter metabolic disorder might be implicated in cognitive decline in *db*/*db* mice.

4.3. Membrane metabolism and osmoregulation in db/db mice with cognitive decline

Cho is essential for phospholipid synthesis of cell membranes [40]. In the present study, a reduction in Cho concentration was found in the brain of *db*/*db* mice with cognitive decline, indicating that phospholipid synthesis was decreased and in turn the structural integrity of cell membranes cannot be maintained. Cho deficiency during fetal development has been reported to affect learning and memory performance due to

impaired brain development [41]. In addition, Nitsch et al. [42] and Pettegrew et al. [43] reported that the phospholipid abnormality in brain may be a potential pathogenic mechanism underlying Alzheimer disease. Cho deficiency can also cause neuronal cell death [44]. Thus, a decreased Cho concentration may be one of reasons for cognitive decline in *db*/*db* mice. Since NAA is mainly synthesized and stored in neurons, it has been considered as a neuronal marker [45]. In this study, thus, a decrease in NAA concentration was observed in hippocampus, striatum and cerebellum of *db*/*db* mice with cognitive decline, while the difference was significant only in cerebellum.

Osmoregulation is also important for the maintenance of cell structure and function. Myo and Tau have been considered as markers for astrocytic activity and play a vital role in brain osmoregulation [46,47]. In the present study, a significantly increased level of Tau in *db*/*db* mice with cognitive decline as compared with *wt* mice may suggest a proliferation of astrocyte cells under diabetic conditions [48]. However, no significant difference was recorded in Myo concentration. There was a significant interaction effect of mice type and brain region on Tau concentration in this study. We found a decrease in Tau concentration in cerebral cortex and hippocampus of *db*/*db* mice with cognitive decline, while the significant difference was only observed in parietal lobe. Moreover, both Myo and Tau showed brain region-specific differences. For instance, a higher concentration of Myo was detected in hippocampus and cerebellum, while for Tau striatum had a relatively higher concentration, followed by hippocampus. Hence, taken together, our results suggest that brain region-specific disturbances in membrane metabolism and osmoregulation may also be related to cognitive decline in *db*/*db* mice.

In summary, we expectedly found that learning and memory performance was impaired in *db*/*db* mice. Therefore, we analyzed metabolic changes in seven brain regions of *db*/*db* mice with cognitive decline to explore the potential metabolic mechanism underlying this phenomenon. Our results suggest that cognitive decline in *db*/*db* mice may be implicated in a series of metabolic changes, including an increase in anaerobic glycolysis, a decrease in the TCA cycle, a disturbance in lactate-alanine shuttle, an inhibition of the Gln-Glu/GABA cycle, and a reduction in membrane metabolism. Moreover, brain region-specific differences were presented in these metabolic changes. There are several limitations to the present study: (1) the causal association between metabolic changes and cognitive dysfunction needs to be further established; (2) since *db*/*db* mouse is a leptin receptor-deficient diabetic model, the effect of leptin on cognitive decline cannot be completely excluded; (3) metabolites cannot be comprehensively detected in a specific metabolic pathway (e.g. the TCA cycle) by NMR, so the use of multi-analytical techniques is recommended to achieve a more detailed metabolic pathway; (4) the results proposed here were based on the metabolite level, and we believe that the result at gene and protein levels will advance understanding of the mechanisms of cognitive decline.

Author contributions

HCG, ZHY and HZ contributed to experimental design. YQZ, LCZ, MJC, GHB, YSH and WYH contributed to animal experiment and NMR analysis. HZ and HCG contributed to data analysis, result interpretation and writing. All authors have read, revised and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2016.11.003.

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