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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