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Type 1 diabetes induces cognitive dysfunction in rats associated with alterations of the gut microbiome and metabolomes in serum and hippocampus

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ABSTRACT

Cognitive decline is a common symptom at advanced stage of type 1 diabetes (T1D), but its potential pathogenesis remains unclear. In this study, therefore, we investigated changes in the gut microbiome and metabolome in serum and hippocampus between advanced-stage T1D (AST1D) rats with cognitive decline and age-matched controls (AMC), and explored the possible mechanism of the gut-microbiota-metabolite axis in T1D-induced cognitive dysfunction. The results demonstrated that AST1D rats possessed peculiar metabolic phenotypes in serum and hippocampus relative to AMC rats, as characterized by decreases in tricarboxylic acid (TCA) cycle and amino acid and choline metabolism as well as disturbances in glutamate/GABA-glutamine cycle and astrocyte-neuron metabolism. We also found that AST1D rats had higher relative abundances of *Prevotella_9*, *Bacteroides* and *Lachnospiraceae_NK4A136_group* as well as lower relative abundances of *Clostridium_sensu_stricto_1*, *Romboutsia* and *Turicibacter* than AMC rats. Microbiota-host metabolic correlation analysis suggests that metabolic alterations in serum and hippocampus may be modulated by the gut microbiota, especially *Clostridium_sensu_stricto_1*, *Romboutsia* and *Turicibacter*. Therefore, our study implies that the modification of host metabolism by targeting the gut microbiota may be a novel avenue for prevention and treatment of diabetic encephalopathy in the future.

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disorder characterized by hyperglycemia due to destruction of pancreatic β-cells [1], and its incidence is rapidly increasing globally [2]. In 2017, over one million children and adolescents suffered from T1D [3]. Of note, T1D has been reported to be associated with cognitive impairment [4–6]. Several potential mechanisms have been proposed, for example, hyperglycemia may cause diabetic neuropathy through increased levels of reactive oxygen species and advanced glycation end-products, excessive release of cytokines, activation of polyol pathway and inflammation [7,8]. Additionally, T1D has a higher risk of vascular disease, resulting in cognitive decline [9]. In our previous study, we found that diabetic cognitive dysfunction may be also attributed to unbalanced metabolic cooperation between neurons and astrocytes [10] as well as brain region-specific metabolic disorders [11,12]. However, the pathogenesis of T1D-induced cognitive decline is still far from being fully understood.

In recent years, accumulating evidences suggest that the gut microbiota plays an important role in cognitive function via the gut-brain axis [13–15]. Sampson et al. [16] reported that the gut microbial changes promoted neuroinflammation and resulted in motor dysfunction in a mouse model of Parkinson's disease (PD). Intestinal amyloid deposition altered the gut microbiome and reduced short-chain fatty acids (SCFAs) levels, exacerbating cognitive deficits in mice of Alzheimer's disease (AD) [17]. Moreover, from a large multicenter study, MahmoudianDehkordi et al. [18] observed an association between altered bile acids, AD-related genetic variants and cognitive changes, suggesting a potential link between the gut flora and AD. Excepting PD and AD, the gut microbiota has also been reported to be responsible for other neurologic diseases, such as depression [19], seizure [20], schizophrenia [21], and Huntington disease [22]. In addition, the onset and development of T1D have also been associated with the gut dysbiosis [23–25], but little information is available on the relationship between the gut microbiota and T1D-induced cognitive impairment.

Therefore, in the present study, we examined the structure and composition of the gut microbiota in colonic contents of advanced-stage T1D rats with cognitive decline and age-matched controls through 16S rRNA gene sequencing. Additionally, metabolic profiles of serum and

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hippocampus were measured using an NMR-based metabolomics analysis. Spearman correlation analysis was employed to evaluate the relationship between microbes and metabolites. The purposes of this study were to identify changes in the gut microbiome and metabolomes of serum and hippocampus associated with T1D-induced cognitive decline, and to investigate the microbiota-host metabolic interaction.

2. Materials and methods

2.1. Animals

Fourteen male Sprague-Dawley rats (age = 6 weeks; body weight = 180 ± 20 g) were purchased from the SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All rats were housed in a specific pathogen-free (SPF) colony with free access to standard rat chow and tap water at the Laboratory Animal Center of Wenzhou Medical University (Wenzhou, China). The feeding condition was fully controlled as follows: temperature: 22 ± 1 °C; humidity: 50–60%; light: 12 h/12 h light/dark cycle and lights on at 8:00. This study was conducted 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.

2.2. Streptozotocin-induced diabetic rat model

After one week of acclimation, all rats were weighted and randomly assigned into the normal control (n = 7) and diabetic (n = 7) groups. To develop animal models of type 1 diabetes (T1D), rats received a single intraperitoneal injection with streptozotocin (STZ, Sigma-Aldrich) solution of 65 mg/kg of body weight after a 12-h fasting. The STZ solution was prepared in citrate buffer (0.1 M, pH = 4.5). Therefore, rats in the control group were treated with the same volume of sodium citrate. After 3 days of STZ injection, blood glucose level was determined from a tail nick using a handheld glucometer (One Touch Ultra, Lifescan), and the T1D rat was defined when its blood glucose level exceeded 16.70 mmol/l.

2.3. Morris water maze test

After 10 weeks of STZ treatment, the Morris water maze (MWM) test was employed to assess learning and memory ability of rats in accordance with a previously reported method [12]. In brief, the MWM test was performed in a circular pool (diameter = 110 cm; height = 30 cm) filled with opaque water at 26 ± 1 °C. The escape platform (diameter = 7 cm) was submerged 1 cm below the surface of the water. During 4 days of continuous training (4 trials/day), rats were guided to reach the escape platform by an operator, if they cannot find it within 60 s. Subsequently, trained rats 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). In the current study, we calculated the percentages of total swimming length and time in the original platform area (Q-III) and the number of crossings over the original platform location as indictors of cognitive function in rats.

2.4. Sample collection and preparation

In this study, rats were given 5 days of the MWM test followed by 1 day of rest. Blood sample was collected and centrifuged at 3000*g* at 4 °C for 15 min to separate serum, and then kept at -80 °C until analysis. Serum sample (200 μl) was thawed and diluted with 250 μl of phosphate buffer (0.2 mM Na_2HPO_4/NaH_2PO_4, pH = 7.4) and 50 μl of D_2O. The diluted serum was vortexed for 10 s, centrifuged at 12,000*g* at 4 °C for 15 min, and then 500 μl of supernatant was transferred into a 5 mm NMR tube for metabolomics analysis.

Rats were sacrificed by decapitation under general anaesthesia, and hippocampus was rapidly isolated, frozen in liquid nitrogen immediately and stored at -80 °C until analysis. The frozen tissue was weighed into a centrifuge tube and extracted by our previously used method [12]. Firstly, 4 ml/g of ice-cold methanol and 0.85 ml/g of ice-cold distilled water were added into the sample tube, and homogenized by a handheld homogenizer (Fluko F8, Essen, German). After homogenizing, 2 ml/g of ice-cold chloroform and 2 ml/g of ice-cold distilled water were added. The mixture was then vortexed for 10 s, stood on ice for 15 min, and centrifuged for 15 min at 10,000*g* at 4 °C. Lastly, the supernatant was transferred into a new tube, lyophilized for about 24 h, and stored at -80 °C until use. The lyophilized hippocampal extract was reconstituted with 0.6 ml of D_2O (99.5%) containing 0.05% of sodium trimethlysilyl propionate-d_4 (TSP) and then transferred to an NMR tube for metabolomics analysis.

2.5. NMR-based metabolomics analysis

^{1}H NMR spectra of serum and hippocampal samples were recorded by using a Bruker AVANCE III 600 MHz NMR spectrometer with a 5-mm TXI probe (Bruker BioSpin, Rheinstetten, Germany) at 37 °C. For serum analysis, to minimize the line-broadening effects of macromolecules such as lipids or proteins, a "CPMG" pulse sequence with a fixed receiver-gain value was used to acquire NMR spectra. The main parameters included: data points, 256 K; relaxation delay, 4 s; spectral width, 12,335.5 Hz; acquisition time, 2.66 s/scan. Besides, a standard single-pulse sequence with water signal pre-saturation ("ZGPR") was employed for hippocampal extract and the typical parameters were set as follows: data points, 256 K; relaxation delay, 6 s; spectral width, 12,000 Hz; acquisition time, 2.66 s/scan.

All NMR spectra were manually corrected for phase and baseline using Topspin 3.0 software (Bruker BioSpin, Rheinstetten, Germany). The spectra of hippocampal extract were referenced to TSP peak at 0.00 ppm, while the spectra of serum were referenced to the anomeric signal of α-glucose at 5.23 ppm. Metabolites in the NMR spectrum were assigned by using Chenomx NMR suite 7.0 (Chenomx Inc., Edmonton, Canada) and the Human Metabolome Database [26]. To further confirm tentative identifications, two-dimensional $^{13}C—^1H$ heteronuclear single quantum coherence (HSQC) experiment was employed to analyze the representative sample. Typical ^{1}H NMR spectra obtained from serum and hippocampus were illustrated in Fig. 1A and B, respectively. To quantify these metabolite levels, their peak areas were manually integrated using Topspin 3.0. For serum, the level of metabolite was indicated using its peak area. For hippocampus, the level of metabolite was calculated on the basis of its peak area by reference to the TSP peak area.

2.6. Gut microbiota analysis

Colonic contents were collected in rats after 10 weeks of STZ treatment under SPF condition and kept at -80 °C until use. The microbial DNA was extracted using the stool DNA isolation kit according to manufacturer's instructions (TIANGen, China). The V4 region of the 16S rRNA gene was amplified with the Barcoded primer pair 515F/806R (515F: 5′-GTG CCA GCM GCC GCG GTA A-3′; 806R: 5′-GGA CTA CHV GGG TWT CTA AT-3′). Subsequently, PCR amplicons were sequenced on an Illumina HiSeq2500 PE250 platform (Illumina, San Diego, USA) at Novogene (Novogene, Beijing, China). The sequencing data were analyzed using QIIME software (v1.7.0) [27]. Operational taxonomic units (OTUs) were clustered at a similarity level of 97% by UPARSE software (v7.0.1001) [28]. Taxonomy assignment was performed using the Mothur method and SILVA SSUrRNA database [29,30]. Alpha- and beta-diversity of the gut microbiota were calculated by QIIME software (v1.7.0) and R software (v2.15.3). In addition, linear discriminant analysis effect size (LEfSe) analysis was used to identify important microbial communities at different levels using

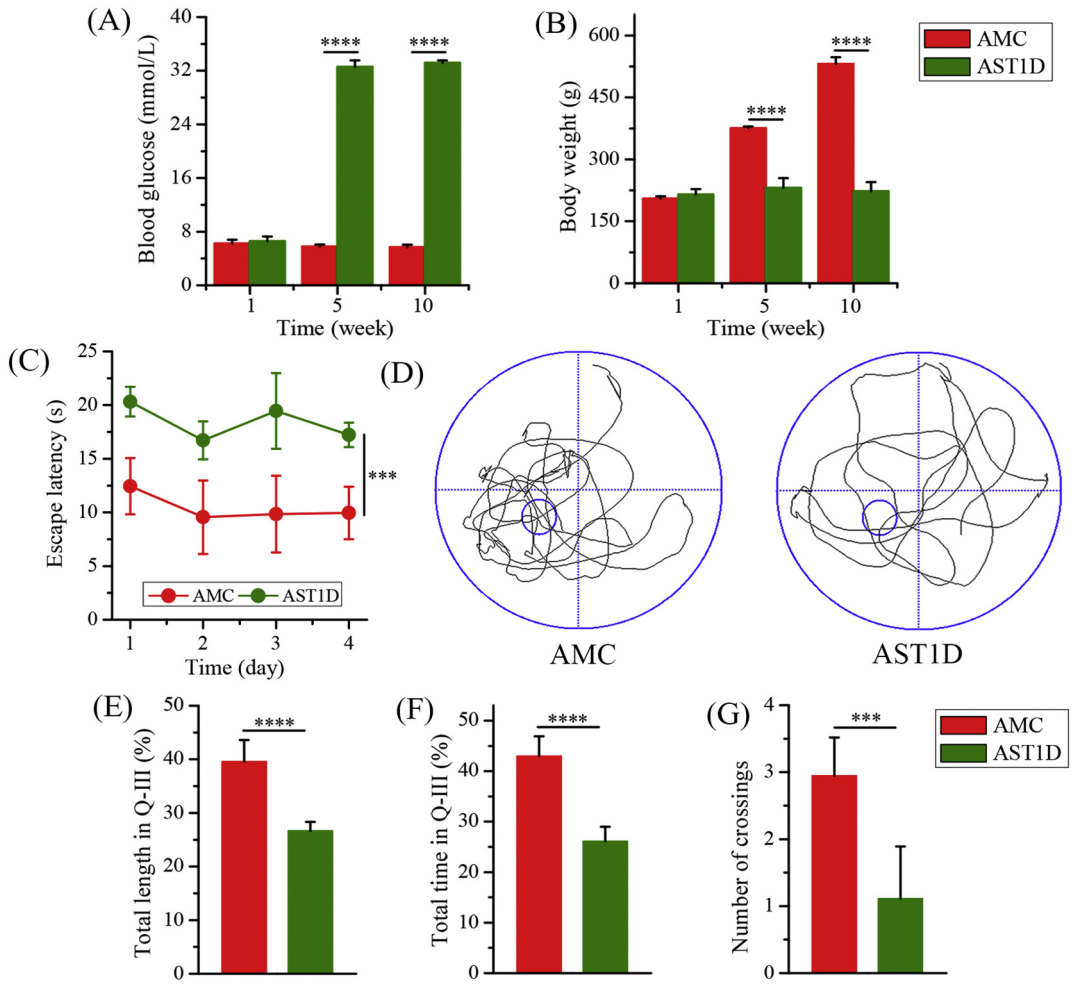


Fig. 1. Cognitive dysfunction in streptozotocin-induced diabetic rats. Changes in (A) blood glucose level and (B) body weight of rats after streptozotocin treatment. (C) Escape latency in diabetic rats at 10 weeks after STZ treatment (AST1D, advanced-stage type 1 diabetes) and age-matched control (AMC) rats during 4 days of continuous training. The escape latency data were analyzed using a repeated-measurement ANOVA. Significant level: ***$p < 0.001$. (D) Swimming path in AST1D and AMC rats during the test period. (E) The percentage of total swimming length in the original platform quadrant (Q-III). (F) The percentage of total swimming time in Q-III. (G) Number of crossings over the original platform location. The difference in behavioral data between AST1D and AMC rats was evaluated by two-tailed unpaired Student's *t*-test with Bonferroni correction. Significant level: ***$p < 0.001$; ****$p < 0.001$.

LEfSe software [31].

2.7. Data analysis and statistics

In the present study, microbial and metabolic analyses were conducted by masking animal labels. All data were log-transformed and auto-scaled prior to multivariate and univariate analyses. Principal component analysis (PCA) was used to examine an overview change in microbial or metabolic pattern between AST1D and AMC rats using MetaboAnalyst 3.0 [32]. Differences in microbial and metabolic data between AST1D and AMC rats were analyzed by Student's *t*-test with Bonferroni correction in SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Spearman rank correlation and the corresponding p value between microbes and metabolites were analyzed using the MATLAB function ("CORR", R2012a, The MathWorks, Inc., Natick, MA, USA). The correlation network was drawn by using Cytoscape software (v3.6.0) [33], and its topological parameters were analyzed using NetworkAnalyzer plug-in in Cytoscape. Metabolic pathway analysis was performed on the basis of KEGG database [34].

3. Results

3.1. Cognitive dysfunction occurs in advanced-stage T1D rats

Fig. 1A shows that blood glucose level was significantly higher in rats at 5 and 10 weeks after STZ injection relative to age-matched controls (AMC). Meanwhile, we also found a significant reduction in body weight of STZ-induced type 1 diabetic (T1D) rats (Fig. 1B). In the present study, the Morris water maze (MWM) test was employed to evaluate learning and memory performance of advanced-stage T1D (AST1D) rats (10 weeks after STZ injection). As can be seen from Fig. 1C, the escape latency in AST1D rats was significantly higher than that in ACM rats, suggesting an impaired learning ability in AST1D rats. Next, the escape platform in the MWM test was removed to assess memory performance in rats, and the swimming trajectory of rats was illustrated in Fig. 1D. We observed that AST1D rats had significantly lower percentages of total swimming length (Fig. 1E) and time (Fig. 1F) in the original platform area as well as the number of crossings over the original platform location (Fig. 1G) than AMC rats. Taken together, the MWM test revealed that an impaired learning and memory ability occurred in AST1D rats.

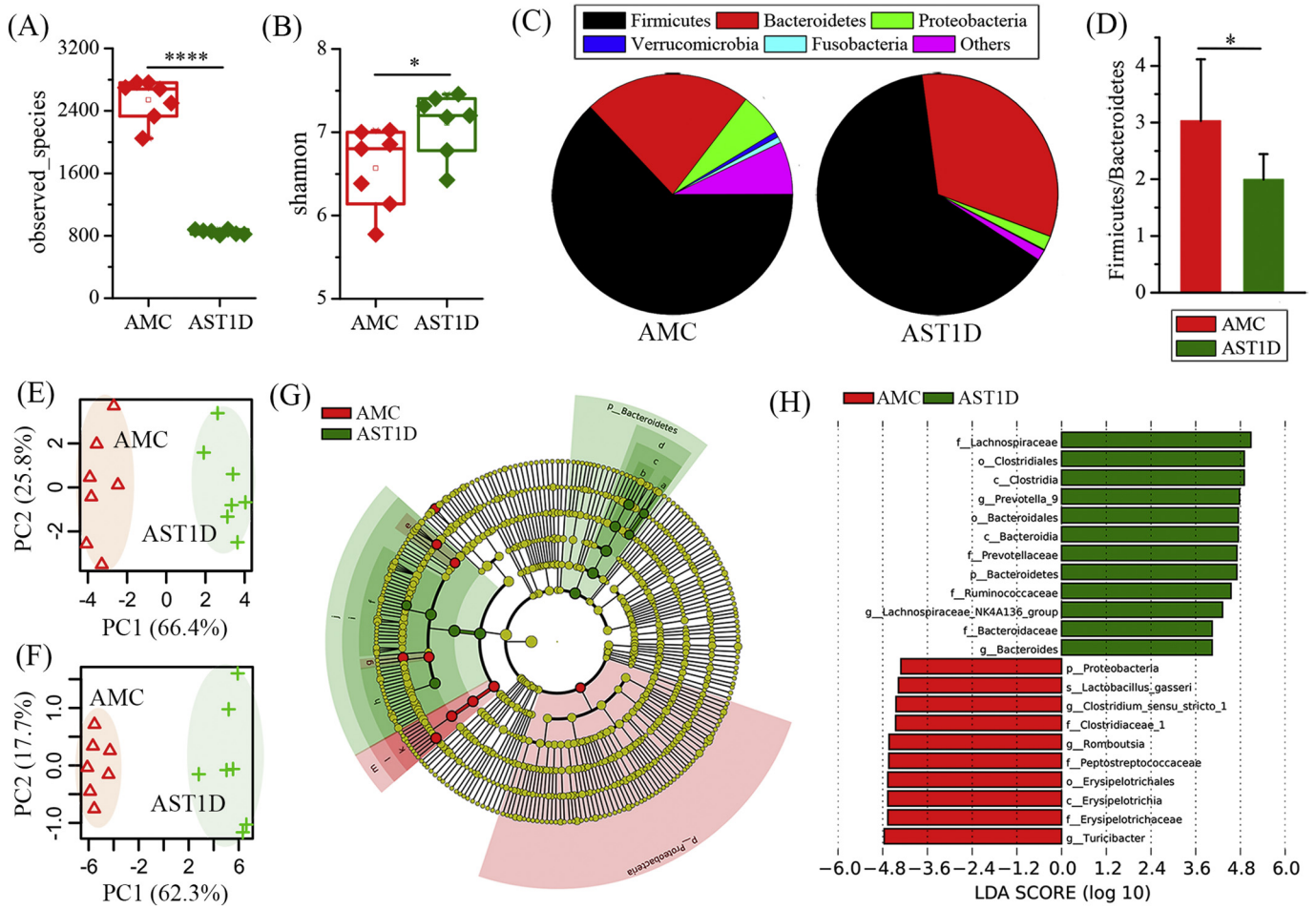


Fig. 2. Gut dysbiosis in diabetic rats with cognitive decline. The observed species (A) and Shannon index (B) of the gut microbiota in advanced-stage type 1 diabetic (AST1D) and age-matched control (AMC) rats. (C) Gut microbiota composition at the phylum level and (D) *Firmicutes/Bacteroidetes* ratio in AST1D and AMC rats. PCA classification based on the gut microbiota at the phylum (E) and genus (F) levels. (G) Linear discriminant analysis effect size (LEfSe) analysis. The red and green shadings indicate bacterial taxa that were significantly higher in AMC and AST1D rats, respectively; the yellow circles indicate bacterial taxa that were not significantly altered between AST1D and AMC rats. (H) Important bacterial taxa were selected using LDA score cutoff of 4.0 (log_{10}). The difference between AST1D and AMC rats was evaluated by two-tailed unpaired Student's *t*-test with Bonferroni correction. Significant level: *$p < 0.05$; ****$p < 0.001$.

3.2. Disturbance of the gut microbiota in advanced-stage T1D rats

In this study, the gut microbiome of colonic contents was analyzed using 16S rRNA gene sequencing and its alpha diversity was evaluated between AST1D and AMC rats. The results show that AST1D rats had a significant decrease in the observed species of the gut microbiota (Fig. 2A) and a significant increase in Shannon index (Fig. 2B) compared with AMC rats. In Fig. 2C, we found that the microbial structure of colonic contents in AST1D rats was noticeably differed from that in AMC rats, as indicated by reduced *Proteobacteria* and increased *Bacteroidetes*. Additionally, a significantly lower *Firmicutes/Bacteroidetes* ratio was observed in AST1D rats relative to AMC rats (Fig. 2D).

PCA results based on the gut microbiome exhibited a clear separation between AST1D and AMC rats at both the phylum (Fig. 2E) and genus (Fig. 2F) levels. Subsequently, linear discriminant analysis effect size (LEfSe) was employed to identify differential microbial communities between AST1D and AMC rats (Fig. 2G and H). Fig. 3 demonstrates changes in the gut microbiota at the genus level identified from LEfSe analysis. The relative abundances of *Prevotella_9* (Fig. 3A, $p = 0.005$), *Lachnospiraceae_NK4A136_group* (Fig. 3B, $p = 0.002$) and *Bacteroides* (Fig. 3C, $p = 0.004$) were significantly increased in AST1D rats relative to AMC rats. Moreover, AST1D rats had lower relative abundances of *Clostridium_sensu_stricto_1* (Fig. 3D, $p < 0.0001$), *Romboutsia* (Fig. 3E, $p < 0.0001$) and *Turicibacter* (Fig. 3F, $p < 0.0001$) when compared with AMC rats.

3.3. Changes in metabolic phenotypes of serum and hippocampus in advanced-stage T1D rats

Typical ^{1}H NMR spectra of serum and hippocampus in healthy control rats are shown in Fig. 4A and B, respectively. Herein, a series of metabolites were identified, involving energy metabolism (citrate, creatine, glucose, fumarate, succinate, AMP, lactate and pyruvate), lipid metabolism (acetate, choline and LDL/VLDL), amino acid metabolism (aspartate, glycine, alanine, isoleucine, leucine, valine, phenylalanine and tyrosine), ketone body metabolism (acetone, acetoacetate and 3-hydroxybutyrate), neurotransmitter metabolism (glutamate, glutamine and γ-aminobutyric acid) as well as astrocyte-neuron metabolism (myo-inositol, *N*-acetylaspartate and taurine).

To visualize the difference in metabolic patterns between AST1D and AMC rats, a principal component analysis (PCA) was carried out, and we found that AST1D rats displayed a peculiar metabolic phenotype relative to AMC rats in both serum (Fig. 4C) and hippocampus (Fig. 4D). The detailed metabolic changes were illustrated in Fig. 4E for serum and Fig. 4F for hippocampus. Besides, serum and hippocampal metabolites that significantly altered between AST1D and ACM rats were highlighted in metabolic pathways as shown in Fig. 4G and H, respectively. Amino acid metabolism in serum was significantly down-

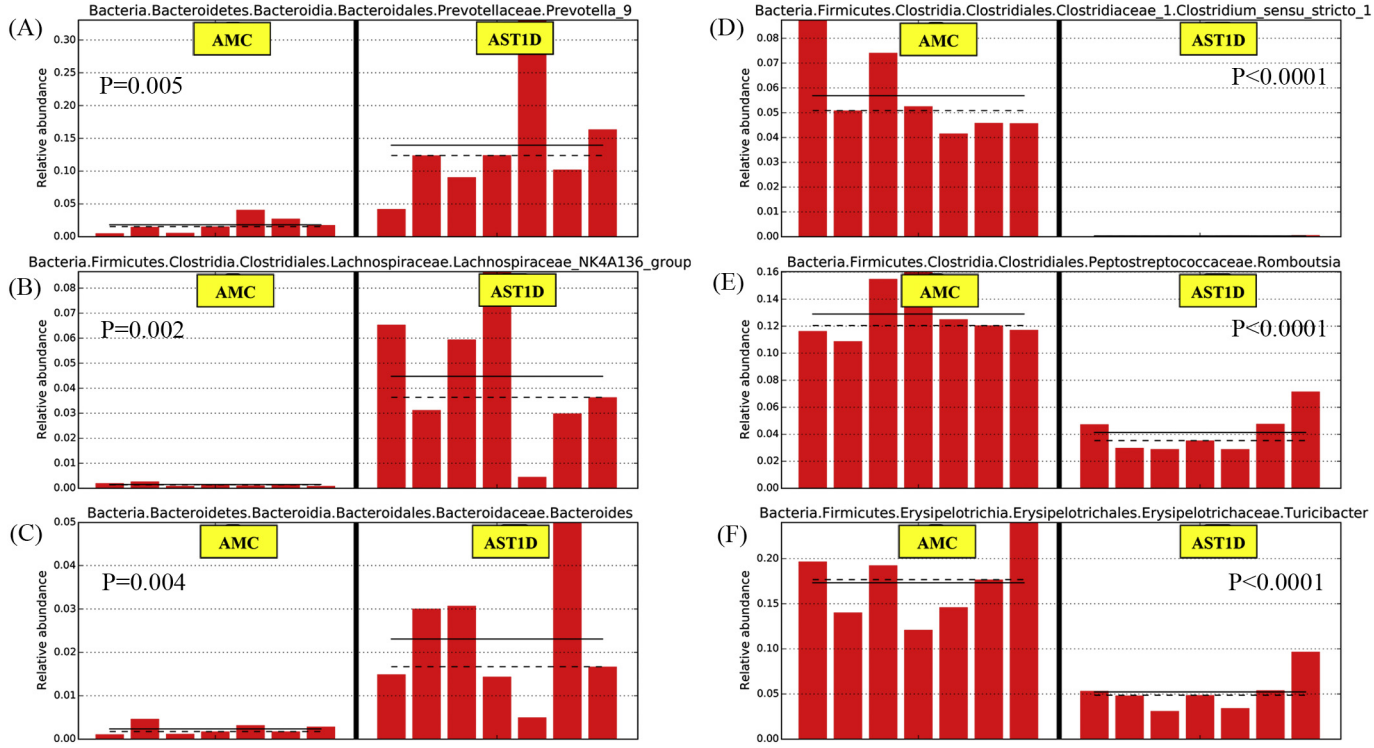


Fig. 3. Changes in the gut microbiota at the genus level selected from LEfSe analysis. (A) *Prevotella_9*; (B) *Lachnospiraceae_NK4A136_group*; (C) *Bacteroides*; (D) *Clostridium_sensu_stricto_1*; (E) *Romboutsia*; (F) *Turicibacter*. AST1D, advanced-stage type 1 diabetic rats; AMC, age-matched control rats. The difference between AST1D and AMC rats was evaluated by two-tailed unpaired Student's *t*-test with Bonferroni correction.

regulated in AST1D rats relative to AMC rats (Fig. 4G), as indicated by decreased levels of alanine, phenylalanine and glutamine (Fig. 4E). The levels of citrate, pyruvate, creatine and lactate in serum were also significantly lower in AST1D rats than those in AMC rats, implying a reduction of energy metabolism (Fig. 4G). Moreover, AST1D rats had a lower choline level and higher glucose and acetoacetate levels in serum than AMC rats (Fig. 4E and G). When compared with AMC rats, we found that AST1D rats had significantly lower levels of leucine, isoleucine, valine, alanine, glycine, glutamine, GABA, NAA, fumarate, succinate, creatine and choline as well as higher levels of glutamate, lactate, taurine and myo-inositol in hippocampus (Fig. 4F). Fig. 4H shows that these metabolic changes were mainly implicated in energy metabolism, neurotransmitter metabolism, choline metabolism and astrocyte-neuron metabolism.

3.4. Microbiota-host metabolic interaction

To explore the relationship between microbes and metabolites, a correlation matrix was assessed using Spearman's correlation analysis based on 6 bacterial genera and 22 metabolites that significant altered between AST1D and AMC rats, and presented as a heatmap shown in Fig. 5A. Then, the strong correlations with an absolute r value > 0.80 ($p < 0.05$) were further illustrated as a correlation network in Fig. 5B, and its main topological parameters included: number of nodes, 25; average number of neighbors, 4.00; characteristic path length, 1.91; shortest paths, 506; network density, 0.17; network heterogeneity, 1.18. The results show that *Turicibacter*, *Romboutsia* and *Clostridium_sensu_stricto_1* had stronger correlations with metabolic changes in serum and hippocampus than other bacterial genera. These three bacterial genera were positively associated with amino acid metabolism (alanine, phenylalanine and glutamine) and energy metabolism (citrate, pyruvate, creatine and lactate) in serum (Fig. 5B). *Turicibacter* had a positive relationship with glycine, GABA, NAA, creatine and choline in hippocampus. Similarly positive correlations were also obtained between *Romboutsia* and hippocampal metabolites, including alanine, glycine, leucine, valine, GABA, NAA, creatine, fumarate and choline (Fig. 5B). Moreover, as can be seen from Fig. 5B, *Clostridium_sensu_stricto_1* exhibited a positive relationship with alanine, glycine, leucine, glutamine, GABA, NAA, creatine, fumarate and choline in hippocampus. However, these three bacterial genera were found to be negatively correlated with hippocampal lactate. Myo-inositol in hippocampus was negatively related to *Romboutsia* and *Clostridium_sensu_stricto_1*. Moreover, a negative relationship between *Lachnospiraceae_NK4A136_group* and hippocampal choline was also observed (Fig. 5B). Collectively, our results suggest that T1D-induced microbial disturbances altered host metabolism and caused cognitive decline (Fig. 6).

4. Discussion

Cognitive dysfunction is one of the most common complications at an advanced stage of diabetes [35,36]. In the current study, we also found that the learning and memory ability was impaired in AST1D rats, which is in agreement with our previous findings [11,12]. Interestingly, AST1D rats had a peculiar gut microbial pattern relative to AMC rats. For example, the observed species of the gut microbiota was dramatically reduced in AST1D rats, which has been also reported by Patterson et al. [37]. Another feature identified herein is that AST1D rats had a higher proportion of *Bacteroidetes* and a lower *Proteobacteria* than AMC rats. Besides, the *Firmicutes* to *Bacteroidetes* ratio, as an indicator of body weight [38,39], was notably decreased in AST1D rats, indicating diabetes-induced weight loss. In addition, we found that the relative abundances of *Prevotella_9*, *Lachnospiraceae_NK4A136_group* and *Bacteroides* were significantly increased in AST1D rats as compared with AMC rats, while an opposite result was detected for *Clostridium_sensu_stricto_1*, *Romboutsia* and *Turicibacter*. However, whether there exists a causal relationship between these microbial communities and T1D-induced cognitive decline still need to be further explored.

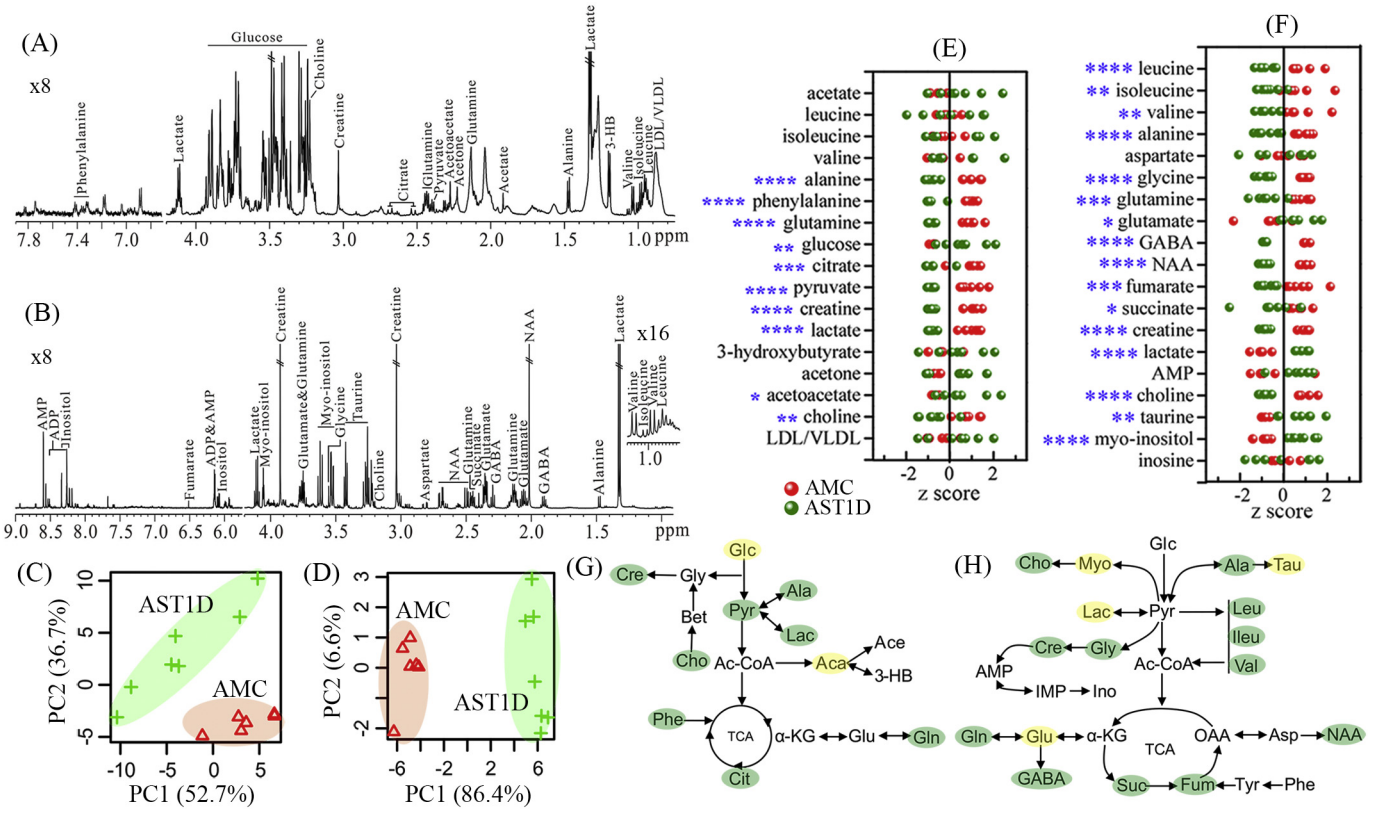
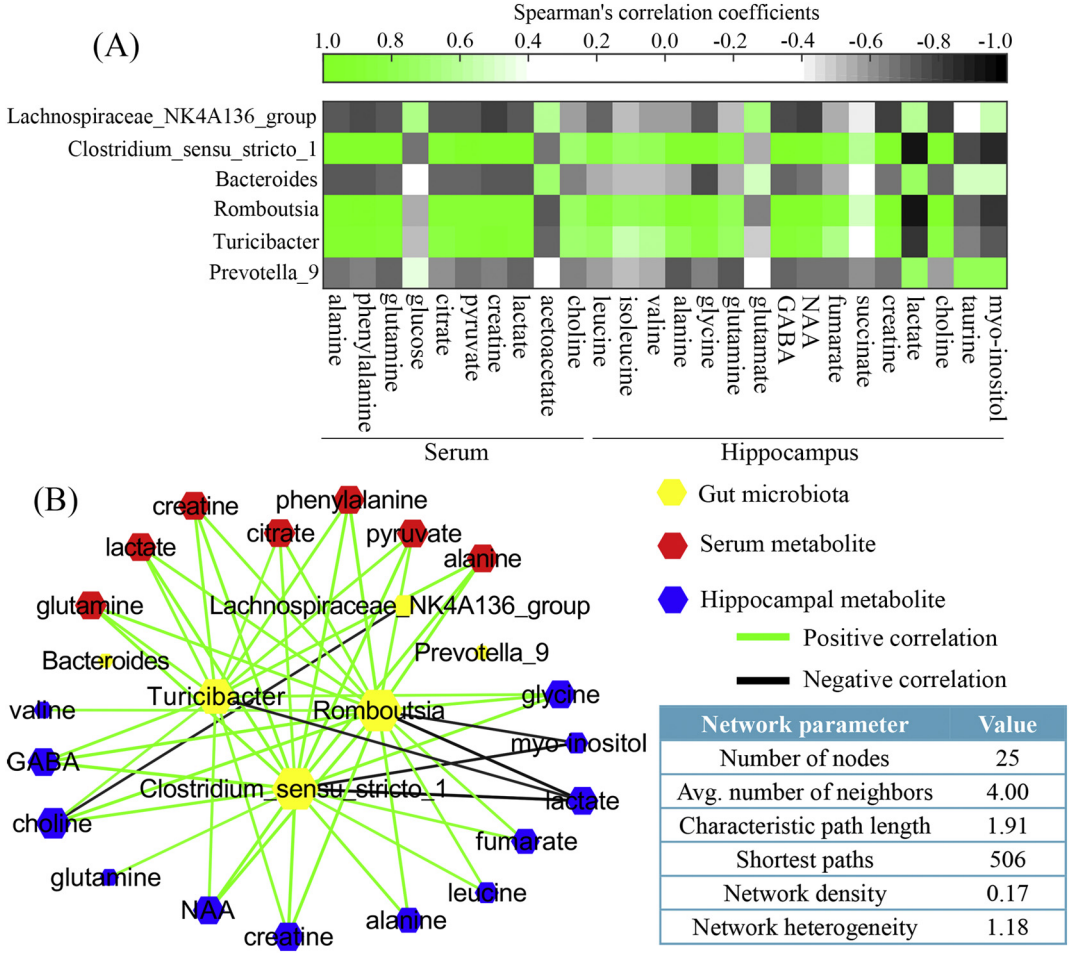


Fig. 4. NMR-based metabolomics analysis. Typical 600 MHz ^{1}H NMR spectra in (A) serum and (B) hippocampus of healthy control rats. Principal component analysis based on the metabolome of (C) serum and (D) hippocampus in advanced-stage type 1 diabetic (AST1D) and age-matched control (AMC) rats. Metabolic changes in (E) serum and (F) hippocampus, and the difference between AST1D and AMC rats was evaluated by two-tailed unpaired Student's *t*-test with Bonferroni correction. Metabolic pathway changes in (G) serum and (H) hippocampus of AST1D rats. The green or yellow shadings represent the significantly decreased or increased level of metabolite in AST1D rats relative to AMC rats. Metabolite: Cit, citrate; Cre, creatine; Glc, glucose; Fum, fumarate; Suc, succinate; Lac, lactate; Pyr, pyruvate; Cho, choline; Gly, glycine; Ala, alanine; IIeu, isoleucine; Leu, leucine; Val, valine; Phe, phenylalanine; Aca, acetoacetate; Glu, glutamate; Gln, glutamine; GABA, γ-aminobutyric acid; Myo, myo-inositol; NAA, *N*-acetylaspartate; Tau, taurine.

Meanwhile, we found that metabolic phenotypes in serum and hippocampus were considerably altered between AST1D and AMC rats. Metabolomics analysis identified several altered metabolic pathways, mainly involving energy metabolism, amino acid metabolism, neurotransmitter metabolism, choline metabolism as well as astrocyte-neuron metabolism.

The vital role of energy metabolism is unquestionable for maintaining normal life activities in mammals. In this study, however, we found that energy metabolism in serum and hippocampus was notably reduced in AST1D rats than that in AMC rats, as indicated by significantly decreased TCA cycle intermediates, citrate, succinate and fumarate. Excepting TCA cycle, creatine or creatine phosphate is also implicated in energy metabolism, particularly for organs/tissues with high energy demand, such as brain and muscle [40]. In this study, we also observed a significantly lower creatine level in both serum and hippocampus of AST1D rats relative to AMC rats. It has been well known that energy metabolism plays a key role in cognitive function [41,42]. Therefore, we speculate that reduced energy metabolism could be responsible for diabetes-induced cognitive impairment, which is consistent with our previous study [12]. Interestingly, we herein identified that the levels of citrate, fumarate and creatine were positively correlated with the shifts of *Clostridium_sensu_stricto_1*, *Romboutsia* and *Turicibacter*, suggesting that the gut flora may regulate host energy metabolism [43]. Glucose can be metabolized to pyruvate and then pyruvate is oxidized to CO_2 and H_2O through TCA cycle or transformed into lactate by anaerobic glycolysis. In serum, we expectedly found a down-regulation of glucose metabolism in AST1D rats, as characterized by lower levels of pyruvate, citrate and lactate and higher glucose level. However, of note, a higher lactate level in hippocampus of AST1D rats may imply an enhanced anaerobic glycolysis. Lactate has also been regarded as a neuronal energy substance [44], but a long-term increase in cerebral lactate level could result in cognitive decline in both human [45] and animal models [46]. Additionally, our results demonstrated that hippocampal lactate level was negatively associated with *Clostridium_sensu_stricto_1*, *Romboutsia* and *Turicibacter*. This finding indicates a potential role of the gut microbiota on hippocampal lactate level, which is also evidenced by a germ-free animal study, where Swann et al. [47] found that germ-free mice had a higher lactate level in hippocampus than specific pathogen-free mice.

Amino acid metabolism was also found to be notably reduced in AST1D rats when compared with AMC rats, such as alanine and phenylalanine in serum as well as alanine, glycine, leucine, isoleucine and valine in hippocampus. This finding may also be one possible explanation for diabetes-induced cognitive decline, since amino acids have essential roles in neurotransmitter synthesis, protein biosynthesis and energy replenishment [48,49]. In our previous study, we have reported that cognitive dysfunction in diabetic animal models could be associated with brain region-specific metabolic disorders of amino acids [11,12]. As far as we know, there is very little information available regarding the role of amino acid metabolism in diabetic encephalopathy, but amino acids never be ignored in the research field of neurological diseases. For example, an increased alanine level in plasma was identified as a therapeutic marker for schizophrenia [50], and alanine supplementation can ameliorate cognitive function in schizophrenia [51], hypoglycaemia [52] and stress exposure [53]. BCAAs, including leucine, isoleucine and valine, have been reported to improve



Network parameter	Value
Number of nodes	25
Avg. number of neighbors	4.00
Characteristic path length	1.91
Shortest paths	506
Network density	0.17
Network heterogeneity	1.18

Fig. 5. Microbiota-host metabolic correlation analysis. (A) Heatmap showing Spearman's correlation coefficients between the gut microbiota and metabolites in serum and hippocampus. (B) Correlation network between the gut microbiota and metabolites in serum and hippocampus ($|r| > 0.8$, $p < 0.05$) and its main topological parameters.

injury-induced cognitive decline [54,55]. In addition, glycine is an inhibitory neurotransmitter and plays an important role in inhibitory and excitatory synapses of the central nervous system [56]. Kodama et al. [57] have suggested a potential therapeutic method for chronic pain with memory impairment by inhibiting glycine transporter 1. Glycine transporters could also be novel therapeutic targets in schizophrenia [58] and epilepsy [59]. Another attractive finding in this study is that changes in amino acids in serum and hippocampus had a close association with the gut microbiota, mainly including *Clostridium_sensu_stricto_1*, *Romboutsia* and *Turicibacter*. In fact, the gut microbiota modulating amino acid metabolism has been reported by Mardinoglu et al. [60] and Sridharan et al. [61]. Moreover, Kawase et al. [62] found that amino acid metabolism in brain may also be affected by the gut microbiota. Thus, we speculate that the modification of amino acid metabolism by targeting the gut microbiota is a possible strategy for treatment of diabetes-induced cognitive decline.

Neurotransmitter metabolism has a key role for maintaining normal cognitive function, especially glutamate/GABA-glutamine (Glu/GABA-Gln) cycle [63]. In this cycle, glutamine can be directly transformed to the excitatory neurotransmitter glutamate and then indirectly to the inhibitory neurotransmitter GABA [63]; glutamate and GABA have been identified as the top two neurotransmitters associated with cognitive impairment [64]. Our results demonstrated that AST1D rats had a higher glutamate and lower GABA in hippocampus than AMC rats. Relative to AMC rats, we also found a significant decrease of glutamine in both serum and hippocampus of AST1D rats. In our previous study, disturbance of Glu/GABA-Gln cycle has been observed in type 2 diabetic *db/db* mice [10,12] and STZ-induced diabetic rats [11]. Therefore, diabetes-induced cognitive decline could be attributed to disrupted Glu/GABA-Gln cycle in brain. Additionally, *Clostridium_sensu_stricto_1*, *Romboutsia* and *Turicibacter* were identified to be strongly positively correlated with changes in serum glutamine and hippocampal GABA, suggesting that the gut flora may modulate Glu/GABA-Gln cycle and thereby affect cognitive function. This hypothesis still needs to be elucidated, but a recent study performed by Zheng et al. [21] may give promising indirect evidence, where they reported that the gut microbiota from schizophrenic patients altered Glu/GABA-Gln cycle in hippocampus and caused schizophrenia-relevant behaviors in mice.

Choline is associated with cognitive dysfunction in neurodegenerative diseases owing to its role in acetylcholine synthesis and cholinergic transmission [65]. Tabassum et al. [66] reported that chronic choline administration improved cognitive function in healthy adults through reduced oxidative stress and enhanced cholinergic transmission. Moreover, choline is an essential component in phospholipid synthesis for cellular membranes [67]. Brain phospholipid dysregulation was proved as a potential pathogenic mechanism of Alzheimer's disease [68,69]. Here, we observed a significant decrease of choline in both serum and hippocampus of AST1D rats compared with AMC rats, which is in agreement with our previous findings in type 2 diabetic *db/db* mice [12] and STZ-induced diabetic rats [11]. We speculate that diabetes-induced cognitive decline could also be attributed to brain choline deficiency. More interestingly, a reduced choline level in hippocampus was significantly associated with decreased abundances of

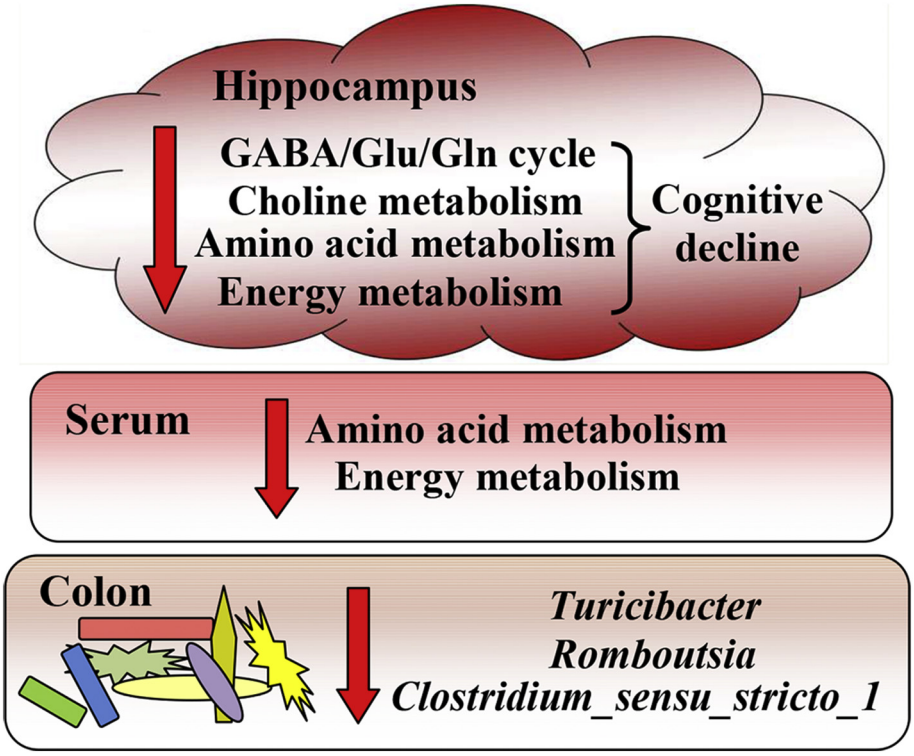


Fig. 6. The potential mechanism of diabetes-induced cognitive dysfunction in rats.

Clostridium_sensu_stricto_1, *Romboutsia* and *Turicibacter*, which may indicate that hippocampal choline level can be affected by the gut microbiota. Swann et al. [47] also found a lower choline level in hippocampus of the germ-free rats compared to the conventional rats. In addition, the level of choline in anterior cingulate cortex may be linked with *Clostridiales*, *Prevotella* and *Lactobacillus ruminis* in patients at ultra-high risk for psychosis [70]. Collectively, these findings imply a possible association between the gut microbiota and brain choline level, and this relationship may need to be paid more attention in diabetes-induced cognitive decline.

N-acetylaspartate (NAA), a neuron-specific brain metabolite, has been employed as a neuronal marker [71,72]. In this study, a significantly lower level of NAA was detected in hippocampus of AST1D rats when compared with AMC rats. This result is consistent with our previous studies [11,12], and indicates that hippocampal neuronal loss may be implicated in diabetes-induced cognitive decline [73,74]. In addition, myo-inositol and taurine have been regarded as markers of astrocytes and play a key role in osmoregulation in brain [74–78]. Our results demonstrate that the levels of these two metabolites in hippocampus were significantly higher in AST1D rats, which may suggest that diabetes resulted in the proliferation of hippocampal astrocytes [79]. Another interesting finding is that these metabolites as markers of neuron and astrocyte may be affected by the gut microbiota. Swann et al. [69] reported that the germ-free rats had a lower NAA level in hippocampus than the conventional rats, suggesting a possible relationship between the gut microbiota and brain NAA level. Janik et al. [80] found that administration of *Lactobacillus rhamnosus* increased total NAA level in brain of mice. In addition, brain NAA has also been associated with *Ruminococcus* in the young pig [81]. In the current study, however, we identified *Clostridium_sensu_stricto_1*, *Romboutsia* and *Turicibacter* may also be potential mediators of hippocampal NAA change. Additionally, hippocampal myo-inositol showed a negative association with *Clostridium_sensu_stricto_1* and *Romboutsia.* O'Hagan et al. [82] reported that *Lactobacillus* and *Bifidobacterium* supplements in middle-aged rats increased the level of myo-inositol in frontal cortex, but not in hippocampus. Together, these findings indicate the potential role of the gut microbiota in regulating astrocyte-neuron metabolism. The gut microbiota has been evidenced as an integral contributor for development of the central nervous system [83]. Therefore, we suggest that exploring the causal relationship between the gut microbiota and astrocyte-neuron metabolism will facilitate a better understanding of the gut-brain signaling mechanism in diabetic encephalopathy.

5. Conclusions

In summary, our results reveal that the learning and memory ability was impaired in AST1D rats, accompanied by disturbances of the gut microbiome and metabolome in serum and hippocampus. We identified several metabolic pathways that significantly altered between AST1D and AMC rats, such as energy metabolism, amino acid metabolism, neurotransmitter metabolism, choline metabolism and astrocyte-neuron metabolism. Moreover, microbiota-host metabolic correlation analysis suggests that these metabolic changes may be modulated by the gut microbiota, especially *Clostridium_sensu_stricto_1*, *Romboutsia* and *Turicibacter*. To elucidate the role of the gut-microbiota-metabolite axis in diabetes-induced cognitive decline, future work should include studies confirmed the causal association between microbes and metabolites. This study will give a possible direction for prevention and treatment of diabetic encephalopathy through modification of the gut microbiota and host metabolism.

Abbreviations

AST1D	advanced-stage type 1 diabetes
AMC	age-matched control
BCAAs	branched-chain amino acids
Glu	glutamate
Gln	glutamine
GABA	γ-aminobutyric acid
NAA	*N*-acetylaspartate
T1D	type 1 diabetes

Transparency document

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

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Author contributions

HZ and HCG contributed to the experimental design. HJ, JN and QYJ contributed to animal experiments. QYJ, HJ, JN and CL contributed to the sample collection and NMR metabolomic analysis. HZ and HCG contributed to the data analysis, result interpretation and writing. All authors have read, revised and approved the final manuscript.

Declaration of competing interest

The authors have no conflict of interest to report.

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