The Alteration of Proteomic Profiles in Hippocampus of Type 2 Diabetic Mice Associated With Cognitive Impairment

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ABSTRACT: Clinical and experimental studies have demonstrated that type 2 diabetes mellitus (T2DM) affects the brain structure and function, in particular the hippocampus, leading to cognitive impairments. However, the molecular mechanisms underlying cognitive deficits induced by T2DM are not fully understood. In this study, we aimed to investigate the effects of T2DM on behavior, the proteome profile in the hippocampus, and the potential molecular pathways involved in the development of cognitive dysfunction in T2DM mice. We found that the diabetic mice exhibited cognitive impairment in the novel object location recognition test and the novel object recognition test. The proteomic analysis revealed that various molecular pathways were involved in this context. These included the upregulation of proteins in the protein synthesis and folding pathway (EIF5A, RSP24, and PPIB), endocytosis and cellular trafficking (VPS24, SNX12, and ARP2/3), cannabinoid receptor interacting (CRIP1), ubiquitination (SKP1), and oxidative stress response (NUDT3). Downregulated proteins were related to mitochondria function (ANT1), neuronal development (ELP1), protein glycosylation (RPN2), and endocytosis (VPS4). Our study shows that T2DM mice exhibit neurocognitive impairment, which is linked to the dysregulation of hippocampal proteins involved in various molecular pathways. These findings contribute to a better understanding of the pathophysiology of T2DM-related cognitive impairment and may identify molecular targets for drug development to treat T2DM-associated cognitive impairment conditions.

KEYWORDS: diabetes mellitus, hippocampus, mouse model, cognitive deficit, proteomics

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Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder that affects millions of people worldwide, and its prevalence is increasing due to the epidemic of obesity.[1] T2DM is characterized by insulin resistance, hyperglycemia, and chronic inflammation, which are associated with various complications, including cardiovascular disease, renal disease, and retinopathy.[2] T2DM also increases the risk of developing mild cognitive impairment (MCI) and enhances the conversion from MCI to dementia.[3] Cognitive impairments affect the quality of life of patients who suffer from T2DM, and these conditions impose a heavy financial burden on families because of the need for home care.[4] Thus, better understanding of the neuropathological substrates of cognitive impairment induced by diabetes mellitus is urgent.

Mounting evidence suggests that T2DM can also affect brain function and structure, particularly in the hippocampus, which plays a crucial role in learning and memory.[5-7] There is a growing body of evidence linking brain insulin resistance, inflammation, hypercortisolism and amyloid accumulation to cognitive impairments and the pathology of Alzheimer disease (AD) in experimental models of diabetes.[8] Mitochondrial dysfunction and neurodegeneration, blood-brain barrier injury, Ca^{2+} signaling disruption, and abnormal neuronal structure and function have also been proposed to contribute to DM-induced cognitive impairments.[9,10] However, the exact molecular mechanisms underlying cognitive impairments induced by T2DM remain unclear.

Proteomics is a powerful tool for the comprehensive analysis of protein expression and modification of complex biological systems and has been used to investigate the molecular basis of neurodegenerative diseases.[11] Despite the recognition of hippocampal involvement in T2DM-related cognitive dysfunction, the molecular mechanisms underlying this condition are not yet fully elucidated. To address this gap, researchers have employed proteomic analysis approaches to investigate protein expression profiles in the hippocampus of animal models. However, few studies are available in this context.

Previous studies demonstrated that proteins involved in energy metabolism, oxidative stress response, protein transport, and inflammatory pathways were altered in the hippocampus of a streptozotocin/high fat diet-induced diabetic animal model, correlating with observed cognitive deficits.[12,13] Similarly, significant alterations in the levels of proteins implicated in the

arrangement of myelin sheath and morphology of axons have been reported in the hippocampus of diabetic Zucker fatty rats.[14] Although these studies provide valuable insights, the proteomic data obtained from animal models are quite different in terms of protein alterations and molecular pathway involvement. In addition, these studies have predominantly used the 2D gel electrophoresis (2DE) approach. This method has limitations, such as the pH range and potential loss of some proteins during separation, which may result in an incomplete proteomic profile. Therefore, employing an additional proteomic approach, such as Gel-Liquid Chromatography (GeLC), is necessary to overcome these limitations and provide a more comprehensive understanding of the molecular mechanism underlying T2DM-related cognitive dysfunction.

Therefore, the main objective of this study was to identify the changes in the hippocampus proteome of T2DM mice compared with controls. We aimed to use a GeLC shotgun proteomic approach to comprehensively analyze the hippocampus proteome and identify potential molecular pathways involved in the development of cognitive dysfunction in T2DM. We additionally applied the novel object location recognition test (NOLT) and novel object recognition test (NORT) to assess and confirm the effect of T2DM on cognitive function. The results obtained from this study contribute to a better understanding of the molecular basis of T2DM-related cognitive impairment in mouse models and may facilitate the development of new treatments of T2DM-associated cognitive dysfunction.

Materials and Methods

Animals and treatment

Eight-week-old male C57BL/6N mice were obtained from Nomura Siam International, Thailand. The animals were housed in a climate-controlled environment with alternating 12 h light–dark cycles, 4 to 5 animals per cage. After 1 week of acclimatization, animals were randomly assigned to receive either a control diet ($n = 12$) offering a total of 3.04 kcal/g (containing 4.5% crude fat, 24% crude protein) (the National Animal Center, Salaya Campus, Mahidol University, Bangkok, Thailand) or a high-fat diet (HFD; $n = 12$) supplemented with 25% sucrose (containing 14.4% crude fat, 24.3% crude protein) (Quick fat; CLEA, Japan) offering a total of 4.11 kcal/g for 4 weeks.[15] The diabetic animal model was generated by 2 intraperitoneal injections of streptozotocin (STZ), dissolved in cold citrate buffer (0.01 M, pH 4.5) (Sigma-Aldrich, Saint Louis, USA) at the dose of 100 mg/kg with the injection volume of 10 mL/kg, while the normal control (ND) group received the same volume injection of citrate buffer (vehicle).[16] The second injection was performed 48 h after the first injection. One week after the first injection of STZ, the fasting blood glucose (FBG) test was performed to confirm the diabetic model. The diabetic group (DM group) mice which met the criterion (FBG > 200 mg/dL) were included in the study.[16,17] Two mice from the DM group did not meet the criterion and were excluded for data analysis. In total, 12 normal control mice (ND) and 10 diabetic mice (DM) were included in the behavioral tests and metabolic profile analysis. The sample size was calculated by using the G*Power program, configuring *t*-test (2-tailed) with the effect size of 1.41[15] a significance level (α) of 0.05 for type I error and a test of power (1-β) of 0.8. The resulting total sample size calculated was 9 animals per group. However, the attrition rate of 25% has been taken into account. Therefore, the total number of sample size was equal to 24 animals which were assigned into the normal control (ND) group ($n = 12$) and the diabetic group (DM) ($n = 12$). The behavioral tests were performed 4 weeks after the diabetes status confirmation. The experimenters were blinded to the treatment group. After the behavioral test, mice were euthanized, and hippocampi were dissected and frozen at -20°C for proteomic analysis. This study adheres to the ARRIVE guidelines for the reporting of animal experiments. All experiments were conducted in accordance with the Animal Care and Use Committee of Burapha University (IACUC Number 006/2564).

Novel object location recognition test

The protocol carried out was an adapted version used by previous studies.[15,18] Briefly, the test was performed in the white plastic open-field box (40 × 40 × 25 cm). The test consisted of 2 phases: (1) the training phase; mice were allowed to freely explore the box containing 2 identical objects (glass bottle) for 10 minutes and (2) the retention phase was performed 3 hours after the training phase. Animals were allowed to re-explore the objects for 5 minutes where one object remained in the same position (nondisplaced object), but the location of the other object was changed (displaced object). The minimal time that animals had to explore the objects during both the training and test phases to be included in the analysis was 10 seconds. The percentage of recognition index was represented as the time spent exploring the displaced object divided by the total time spent exploring the displaced object and the nondisplaced object X 100. The counterbalancing was used to minimize the order of measurement effect.

Novel object recognition test

The NORT was conducted as described previously.[19,20] Briefly, the test consisted of 3 phases: habituation phase, training phase, and test phase. During the habituation phase, mice were exposed to an open-field box in the absence of objects for 5 minutes. Mice were then returned to their home cage. On the following day (training phase), mice were placed in the same open field with 2 identical objects and were allowed to freely explore the environment and objects for 10 minutes. Twenty-four hours later (test phase), mice were placed back in the open field and, at this time, allowed to explore a novel object and a familiar one for 10 minutes. During the experiment, the open-field box and objects were cleaned using 70% alcohol to eliminate the olfactory cue. Time spent exploring each object was

collected in the test phase in which the mouse's nose was within 2 cm of the object. The minimal time that animals had to explore the objects during both the training and test phases to be included in the analysis was 10 seconds. The percentage of time spent exploring the novel object versus the total object exploration time was presented.

Measurement of body weight and blood glucose level

Body weight was monitored weekly across the experiment. The blood glucose levels were evaluated at 1 week after STZ injection and 4 weeks after STZ injection. After a 6-hour fast, blood samples were collected from a small incision at the end of each mouse's tail.[13] A drop of blood was placed onto a glucose strip and blood glucose was measured with a glucose meter according to the user instructions guideline (ACCU-CHEK Guide, Roche, Thailand). The counterbalancing was also used to minimize the order of measurement effect.

Plasma insulin measurement and insulin resistance determination

Plasma samples from each group of mice were collected and stored at -20°C until they were thawed for the assay. Plasma insulin was detected using ELISA kits (MyBioSource, San Diego, CA, USA) according to the manufacturer's instructions. The optical density (OD) value of the sample at 450 nm was collected by a microplate reader (Thermo Fisher Scientific, Waltham, USA). The sample concentration was calculated according to a corresponding standard curve.

The homeostatic model assessment for insulin resistance (HOMA-IR) was calculated as previously described.[21]

Proteomic analysis

Sample preparation. The hippocampal proteome of T2DM mice was compared with the controls using the GeLC shotgun proteomic approach. Mice from the diabetic group and control group were selected based on 2 criteria including FBG and the NOLT values, which reflect hippocampus function. Four DM with the highest FBG levels and poorest cognitive function in NOLT were selected while 4 normal control mice (ND) with highest cognitive performance in NOLT were selected for hippocampal proteome analysis. Hippocampal proteins were extracted according to our previous work.[22] A total of 10 µg protein/individual was separated on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel using 100 V for 15 minutes to obtain protein migration of approximately 0.5 cm in a separating gel and stained with Coomassie blue for gel band visualization.

In-gel digestion and MS/MS analysis

Gel bands that appeared in the separating gel were cut into approximately 1 × 1 mm, washed and destained. Proteins fixed in the gel were reduced with dithiothreitol and alkylated with iodoacetamide. In-gel digestion was performed with trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega, Madison, WI, USA) at a final trypsin concentration of 20 ng/µL in 50 mM aqueous ammonium bicarbonate and 5 mM $CaCl_2$. Digestion proceeded for 8 hours at 37°C. Afterward, peptides were extracted with 5 changes of 50 µL of 5% trifluoro acetic acid in 50% aqueous acetonitrile supported by ultrasonication for 10 minutes per change. Extracted peptides were dried down in a vacuum concentrator (Eppendorf, Hamburg, Germany). Dried peptides were resuspended in 100 µL 0.1% trifluoroacetic acid (TFA) for Q Exactive Orbitrap LC-MS/MS analysis and 5 µL were injected in technical duplicates. All procedures for Q Exactive Orbitrap LC-MS/MS analysis were performed according to Gutiérrez et al.[23]

The GeLC shotgun proteomic approach offers significant advantages in enhancing protein separation and resolution by separating proteins in a gel prior to digestion and analysis. This prefractionation step reduces sample complexity and helps remove impurities and contaminants, leading to more accurate and detailed protein identification and quantification.[24] In addition, this method allows the identification of a broader range of proteins, including those with extreme isoelectric points (pI) or molecular weights that may not be well-resolved by 2DE proteomic approach, resulting in a more comprehensive proteome profile.

Database search and pathway analysis

Database search was performed using the Proteome Discoverer Software 2.4.0.305 (Thermo Fisher Scientific) with the following settings: Protein Database: Uniprot mouse (tx1009) (www.uniprot.org) and the database of common contaminants (cRAP, http://ftp.thegpm.org/fasta/cRAP/crap.fasta), Enzyme Name: Trypsin (Full), Max. Missed Cleavage Sites: 2, Precursor Mass Tolerance: 10 ppm, Fragment Mass Tolerance: 0.02 Da, Dynamic Modification: Oxidation/+15.995 Da (M), Dynamic Modification: Deamidated/+0.984 Da (N, Q), N-Terminal Modification: Gln->pyro-Glu/-17.027 Da (Q), N-Terminal Modification: Acetyl/+42.011 Da (N-Terminus), N-Terminal Modification: Met-loss/-131.040 Da (M), N-Terminal Modification: Met-loss+Acetyl/-89.030 Da (M), Static Modification: Carbamidomethyl/+57.021 Da (C).

Bioinformatic and pathway analysis

In the proteomic experiment, intensity-based, label-free quantification protein abundance raw values were generated in Proteome Discoverer software. Raw data were filtered for proteins containing no or at least 4 values per group. After aggregation by the mean, further statistical analysis by *t*-test comparison was performed with the R programming language using the DEP package including normalization and imputation of missing values by zero. Proteins were considered

differentially expressed when protein abundance differed by a factor of 2-fold change (upregulation when $\log_2$-fold change $\geqslant$ 1 or downregulation when $\log_2$-fold change $\leqslant$ 1), and an adjusted P value $<.05$.

For pathway analysis, differential proteins were annotated using Gene Ontology (GO) information from Uniprot. Protein-protein interactions (PPI) among these differential proteins were predicted using the STRING database. Gene names were searched against *Mus musculus* with minimum required interaction score of high confidence (0.700). Metabolic pathway enrichment analysis was conducted using the Kyoto Encyclopedia of Genes and Genomes (KEGG).

Statistical analysis

Data obtained from NOLT, NORT, the body weight and blood glucose, plasma insulin level, and HOMA-IR are presented as mean ± standard error of mean (SEM). The independent t-test was used to compare the means of parameters addressed between the 2 groups. Two-way analysis of variance (ANOVA) with repeated measures was used to analyze the body weight and the FBG at different time points by the GraphPad version 5. Shapiro-Wilk test was used to test the normality for data distribution and Levene test was used to test the homogeneity of variance. P value $<.05$ was considered as significant.

Results

The effect of diabetes mellitus on the body weight, FBG plasma insulin levels, and insulin resistance index

Two-way ANOVA with repeated measure for the body weight revealed a significance of time effect, $F_{(4,80)}=3.395$, $P=.013$, group effect, $F_{(1,80)}=5.621$, $P=.028$, and interaction between time and group effect, $F_{(4,80)}=94.820$, $P<.001$. A Bonferroni post hoc test revealed that after 4 weeks of HFD feeding when the injection of streptozotocin (STZ) was applied (W0), the diabetic group (DM) had a body weight significantly heavier than that of the normal control group (ND), $t(20)=4.317$, $P<.001$. However, 4 weeks after STZ injection, the body weight of the DM group was significantly lower than that of the ND group, $t(20)=7.121$, $P<.001$ (Figure 1A). This result indicated that diabetes status induces body weight loss in this model.

We further investigated the effect of diabetic induction by a combination of HFD feeding and STZ injections on the FBG levels. Two-way ANOVA with repeated measure for the FBG revealed a significant time effect, $F_{(1,20)}=8.291$, $P=.009$, group effect, $F_{(1,20)}=1003$, $P<.001$, and interaction between time and group effect, $F_{(1,20)}=12.290$, $P=.002$. A Bonferroni post hoc test revealed that diabetic mice exhibited an increase in the FBG levels at both 1 week after STZ injection (W1), $t(20)=13.34$, $P<.001$, and 4 weeks after STZ injection (W4), $t(20)=19.35$, $P<.001$ (Figure 1B). This result indicates that a combination of HFD feeding and STZ injections induces hyperglycemia in this model.

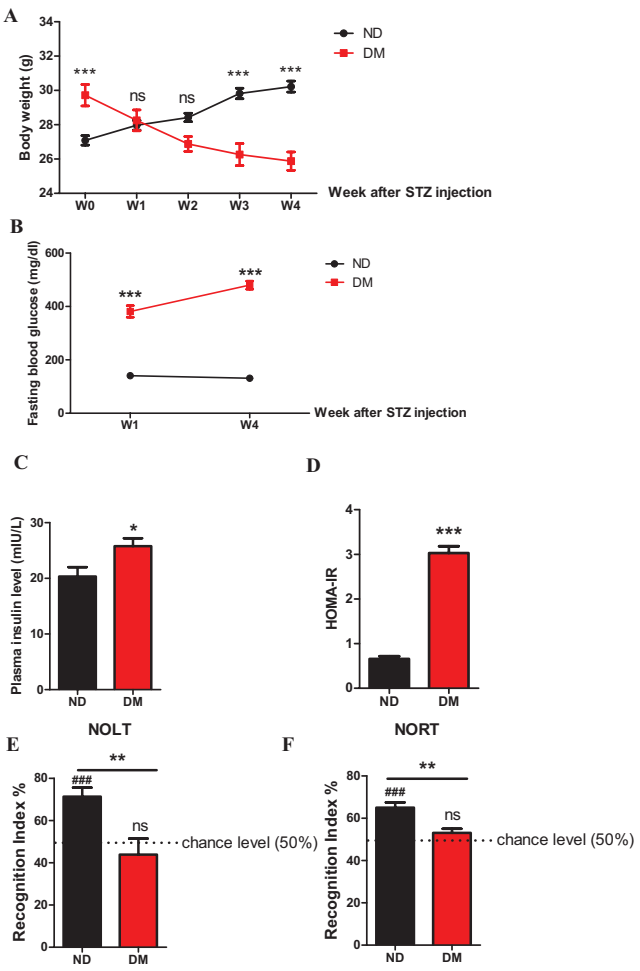

Figure 1. The effects of diabetes mellitus on metabolic parameters and cognitive functions. Diabetes status induces body weight loss (A), increase in fasting blood glucose level (B), increase in plasma insulin level (C), augment HOMA-IR (D) and impaired cognitive function in novel object location recognition test (NOLT) (E) and affects cognitive function in novel object recognition test (NORT) (F). Normal control mice (ND) ($n=12$), diabetic mice (DM) ($n=9$-10). ***$P<.001$, **$P<.01$, *$P<.05$ when compared with the normal control group (ND), ###$P<.001$ when compared with the chance level (50%) and ns = nonsignificant.

We next investigated the effect of diabetic induction on plasma insulin level. The independent t-test revealed an elevation in plasma insulin levels of diabetic mice (DM) compared with the normal control group (ND), $t(20)=2.418$, $P=.025$ (Figure 1C). We further evaluated the insulin resistance in these mice and found that the diabetic group (DM) exhibited insulin resistance as indicated by the HOMA-IR value higher than 2.5. Moreover, the diabetic mice (DM) presented a significant increase in the HOMA-IR when compared with the normal control mice (ND), $t(20)=15.38$, $P<.001$ (Figure 1D). This result suggests that combination of HFD feeding and STZ injections induces hyperinsulinemia and insulin resistance in this model.

The effect of diabetes mellitus on cognitive functions

The cognitive functions of these mice were evaluated in the NOLT and the NORT. In the NOLT, 1 sample *t*-test for the percentage of recognition index during test phase revealed that the normal control mice (ND) presented a good performance in object location memory retention, $t(11) = 5.053$, $P < .001$. In contrast, the diabetic mice (DM) did not show a preference for the object in the novel location, $t(9) = 0.806$, $P = .441$. Moreover, the percentage of recognition index of the diabetic mice (DM) was significantly lower than that of the normal control group (ND), $t(20) = 3.290$, $P = .004$ (Figure 1E). The total exploration time of the objects during the training phase and the test phase was not different between group, $t(20) = 0.513$, $P = .614$ and $t(20) = 0.268$, $P = .792$ (Supplementary material, Figure S1A and S1B).

We next evaluated the cognitive functions of these mice in the NORT. One sample *t*-test for the percentage of recognition index during the test phase revealed that the normal control mice (ND) presented a good performance in object recognition memory, $t(11) = 6.095$, $P < .001$, whereas the diabetic mice (DM) did not show preference for the novel object, $t(8) = 1.583$, $P = .152$. The percentage of recognition index in NORT significantly differed between 2 groups, $t(19) = 3.602$, $P = .002$ (Figure 1F). The total exploration time of the objects during the training phase and the test phase in NORT was not statistically different between groups, $t(19) = 1.036$, $P = .313$, and $t(19) = 0.972$, $P = .343$, respectively (Supplementary material, Figure S1C and S1D). This indicates that the diabetic mice exhibit deficits in both object location memory and object recognition memory.

We further investigated the correlation between the FBG and the cognitive performance in NOLT and NORT tests. We found a negative correlation between FBG and the cognitive performance in NOLT ($r = -0.53$, $P = .014$) and a negative correlation between FBG and the cognitive performance in NORT ($r = -0.53$, $P = .014$) (Supplementary material, Figure S2A and S2B).

Proteomic results

In this study, approximately 2430 hippocampal proteins were identified by Q Exactive Orbitrap LC-MS/MS. By differential analysis, 13 proteins were differentially expressed (adj. *P* value $< .05$; ± 2-fold change). Of these, 9 proteins were upregulated while 4 proteins were downregulated in the hippocampus of diabetic mice as compared with the controls (Figure 2). A list of differential proteins and detailed analysis of protein functions are shown in Table 1. Upregulated proteins are composed of a variety of functions, including protein synthesis and folding (EIF5A, RSP24, and PPIB), endocytosis and retromer complex (VPS24, SNX12, and ARP2/3), cannabinoid receptor interacting (CRIP1), ubiquitination (SKP1) and oxidative stress response (NUDT3), while downregulated proteins are composed of proteins relating to mitochondria function (ANT1), neuronal development (ELP1), protein glycosylation (RPN2), and endocytosis (VPS4).

Moreover, PPI enrichment results revealed that most differential proteins are involved in the ribosome pathway and endocytosis pathway (Figure 3). KEGG pathway analysis also showed that many proteins involved in various steps of endocytosis are identified including clathrin-coated vesicle (ARP2/3), early endosome (VSP29, SNX12, and ARP2/3), and late endosome (VPS4) (Figure 4).

Discussion

The effect of diabetes mellitus on the body weight, FBG plasma insulin levels, and insulin sensitivity

We found that the diabetic mice exhibited significant weight loss and hyperglycemia throughout the experiment. Our results were consistent with previous studies that demonstrated streptozotocin (STZ) treatment was sufficient to induce diabetes mellitus, characterized by significant weight loss and hyperglycemia.[16,25] Moreover, in our model, the diabetic mice induced by the combination of an HFD and STZ injection showed a significant increase in plasma insulin levels, which was similar to previous experiments.[26] Overall, these findings indicate that the combination of an HFD and STZ injection induces weight loss, hyperglycemia, hyperinsulinemia, and insulin resistance in mice.

The effect of diabetes mellitus on cognitive functions

In this study, we demonstrated that diabetic mice exhibited cognitive deficits in object location recognition memory (NOLT) and in novel object recognition memory (NORT). These 2 types of memory are dependent on the hippocampus function.[27] Our finding is in accordance with previous studies showing that diabetes mellitus affected cognitive functions both in clinical studies[28,29] and in animal models of diabetes.[15,16,30] This alteration in cognitive function was associated with changes in structure and function of the hippocampus, not only in humans,[31,32] but also in animal experiments of the diabetes model.[33,34] However, the molecular mechanisms underlying diabetes-induced cognitive impairments are not yet fully understood. Therefore, the GeLC shotgun proteomic approach was employed to investigate the proteome response in this context.

Proteomics

In the current work, we demonstrated that T2DM affected cognitive functions evaluated by NOLT and NORT, which are hippocampal-dependent tasks. This opens the window for us to explore proteomic alteration of T2DM mice as compared with the controls to obtain a comprehensive view of the

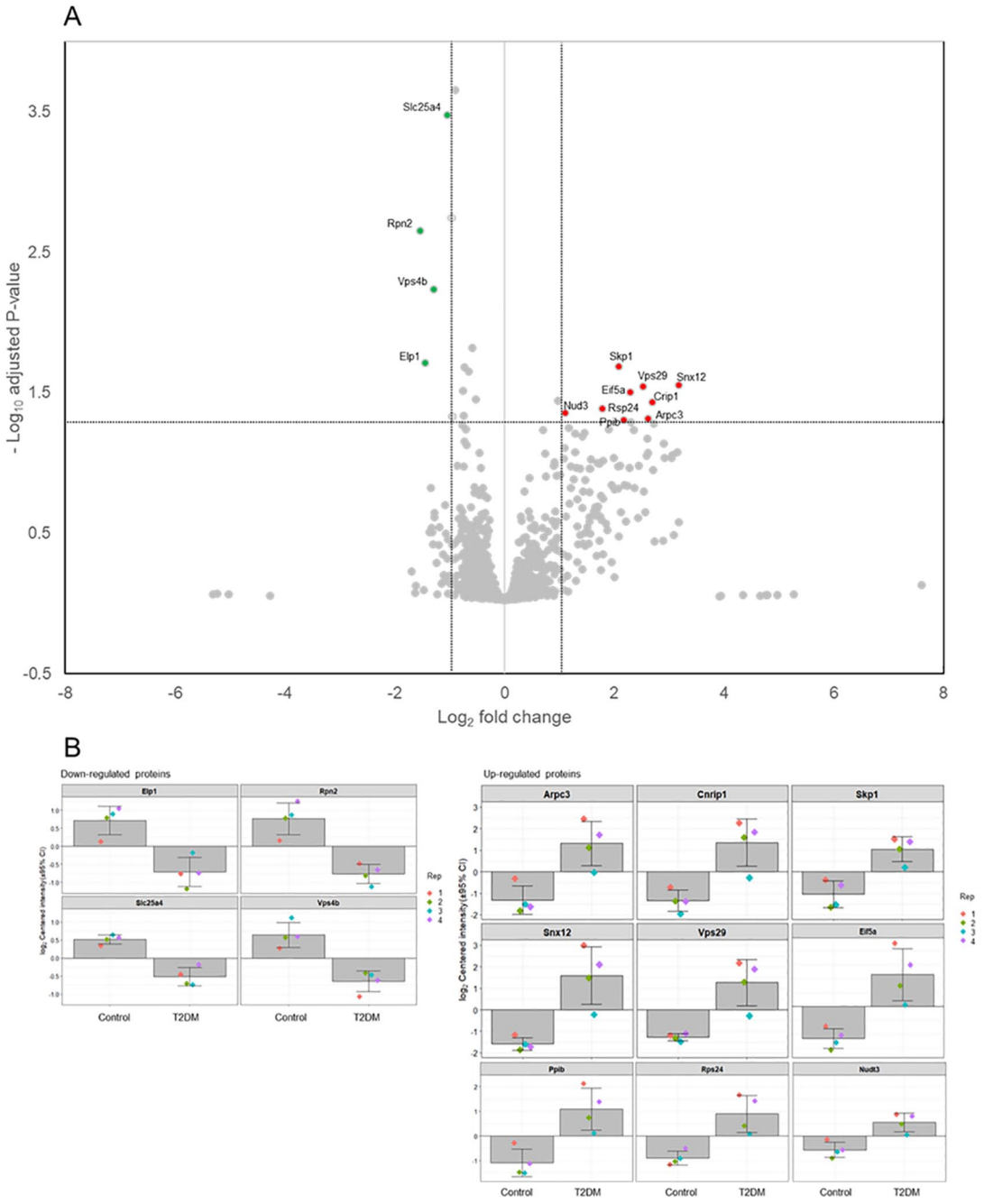

Figure 2. Protein expression profile of T2DM mice as compared with the control. (A) Volcano plot of differential proteins ($P > .05$). The horizontal axis represents Log_2 fold-change, the vertical axis represents $-Log_{10}$ adjusted *P* value, and values closer to the center represents smaller differences. Upregulated and downregulated differential proteins are shown in red and green, respectively. (B) Expression levels of downregulated and upregulated proteins which were obtained from proteomic analysis.

molecular mechanisms in the hippocampus underlying the cognitive deficits of T2DM.

Based on the proteomic analysis, 13 differential proteins in the hippocampus are implicated in neurocognitive function in the context of T2DM. Seven of these proteins were previously reported to play an important role in neurocognitive function. These include Eukaryotic translation initiation factor 5A, Peptidyl-prolyl cis-trans isomerase B, Actin-related protein 2/3 complex subunit 3, CB1 cannabinoid receptor-interacting protein 1, S-phase kinase-associated protein 1, ADP/ATP translocase 1, and Elongator complex protein 1. However, the specific mechanisms of these proteins have never been explained in regard to cognitive impairment in T2DM context. The 5 novel proteins that are potentially associated with T2DM-induced cognitive dysfunction include 40S ribosomal protein S24, Vacuolar protein sorting-associated protein 29, Sorting nexin-12, Diphosphoinositol polyphosphate phosphohydrolase 1, Dolichyl-diphosphooligosaccharide-protein

Table 1. List of differential proteins (adjusted *P* value < .05; ±2-fold change) in hippocampus of type 2 diabetic mice with cognitive impairment as compared with the control.

PROTEIN IDS	PROTEIN NAME	PROTEIN FUNCTION	GENE NAME	PEPTIDES	UNIQUE PEPTIDES	LOG2 FOLD CHANGE	ADJ. *P* VALUE
Upregulation							
Protein synthesis and folding							
A0A0A0MQM0	Eukaryotic translation initiation factor 5A (Fragment)	Translation elongation factor activity	EIF5A	7	5	2.30	.0317
Q3TIF8	40S ribosomal protein S24	Small ribosomal subunit rRNA binding	RPS24	2	2	1.79	.0413
P24369	Peptidyl-prolyl cis-trans isomerase B	Assist protein folding	PPIB	7	7	2.18	.0498
Endocytosis and cellular trafficking							
Q9QZ88	Vacuolar protein sorting-associated protein 29	Retromer complex binding	VPS29	7	7	2.54	.0288
Q3TGS7	Sorting nexin-12	Intracellular trafficking	SNX12	5	5	3.18	.0282
Q9JM76	Actin-related protein 2/3 complex subunit 3	Formation of branched actin networks in the cytoplasm	ARP2/3	4	4	2.63	.0485
Cannabinoid receptor interacting							
Q5M8N0	CB1 cannabinoid receptor-interacting protein 1	Suppresses cannabinoid receptor CNR1-mediated tonic inhibition of voltage-gated calcium channels	CNRIP1	6	6	2.70	.0372
Ubiquitination							
Q9WTX5	S-phase kinase-associated protein 1	Protein ubiquitination	SKP1	7	7	2.09	.0207
Oxidative stress							
Q9JI46	Diphosphoinositol polyphosphate phosphohydrolase 1	Oxidative stress may play a role in signal transduction	NUDT3	4	4	1.11	.0443
Downregulation							
Mitochondria dysfunction							
P48962	ADP/ATP translocase 1	Plays a role in mitochondrial uncoupling by acting as a proton transporter	SLC25A4	16	8	-1.03	.0003
Neuron development							
Q7TT37	Elongator complex protein 1	Migration and branching of projection neurons in the developing cerebral cortex	ELP1, IKBKAP	3	3	-1.43	.0195
Protein glycosylation							
Q3U505	Dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit 2 (Fragment)	Protein glycosylation	RPN2	6	6	-1.53	.0022
Endocytosis							
Q3TN07	Vesicle-fusing ATPase	Involved in late steps of the endosomal multivesicular bodies (MVB) pathway	VPS4	2	2	-1.28	.0058

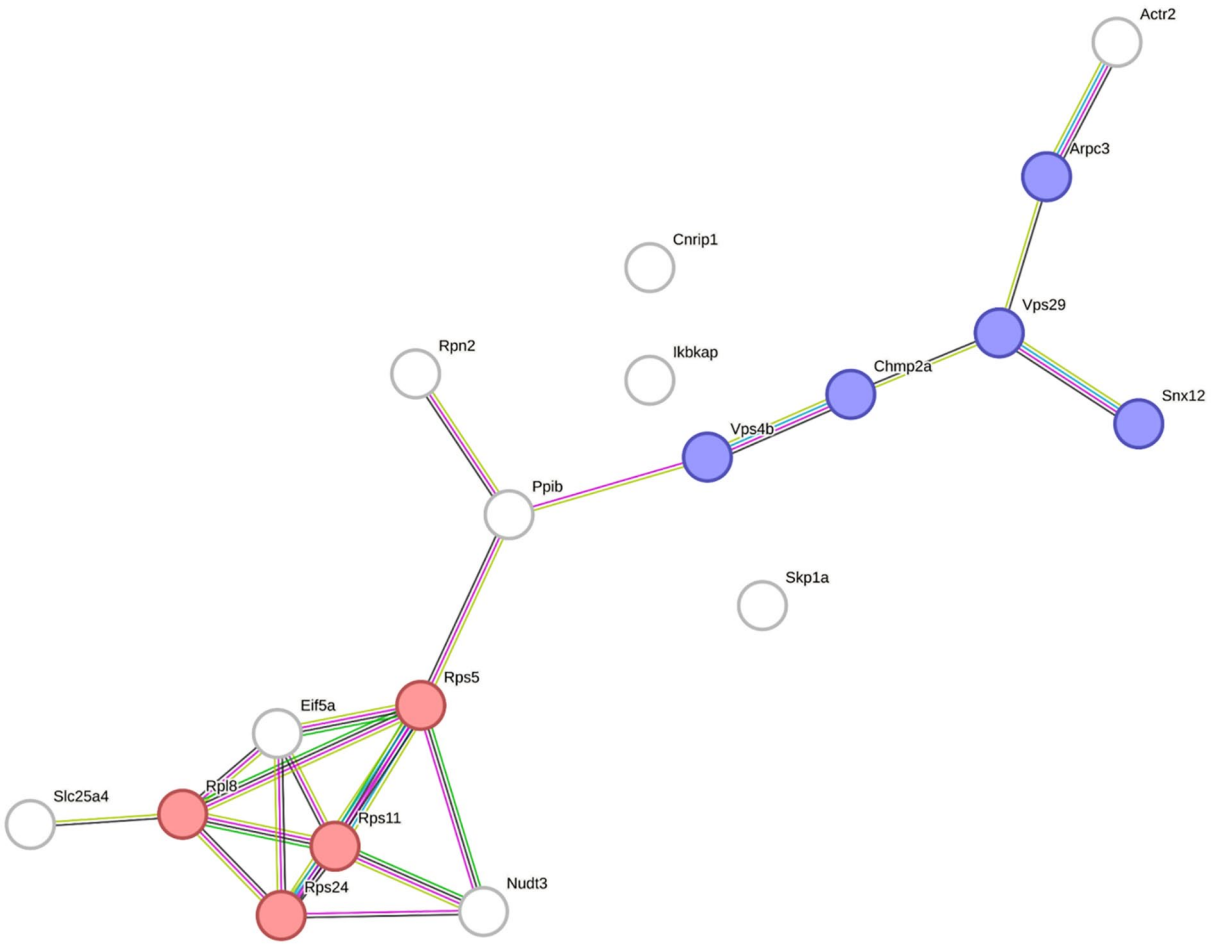

Figure 3. Enrichment PPI of differential proteins represents the majority of proteins related to the ribosome pathway (red circle) and endocytosis pathway (blue circle). (PPI enrichment *P* value = .00137).

glycosyltransferase subunit 2, and Vesicle-fusing ATPase. All of these differential proteins are of particular interest as they will improve current knowledge of T2DM-associated cognitive dysfunction.

The PPI enrichment results clearly demonstrated that the hippocampal proteins involved in the ribosome pathway (protein synthesis), and endocytosis pathway were among the most dysregulated in diabetic mice with cognitive impairment. Alteration of those proteins may theoretically affect the protein synthesis steps and molecular trafficking of cargo proteins within hippocampal neurons. In addition, some of the differential proteins were previously reported to be involved in neurodegenerative diseases.

Protein synthesis and folding

In this study, proteins involved in protein synthesis pathway including 40S ribosomal protein S24, (RSP24) and eukaryotic translation initiation factor 5A (EIF5A) were upregulated in T2DM mice with cognitive impairment. Moreover, a protein involved in the folding pathway, peptidyl-prolyl cis-trans isomerase B (cyclophilin B or PPIB), was also upregulated. These indicate that altered dynamics of protein production and modification affect hippocampal function.

Upregulation of RSP24 and EIF5A also indicates altered protein production and quality control mechanisms of misfolded protein since RSP24 is involved in protein production and EIF5A is involved in protein synthesis and folding. A recent study of EIF5A in the mouse nervous system has shown that EIF5A is crucial for neurodevelopment and cognitive function. Knockout of EIF5A in the mouse nervous system led to impairments in growth, viability, neurodevelopment, and cognitive function in mice.[35] The evidence from the recent study showed that an increase of EIF5A is observed in the T2DM mice. Altogether, it revealed that EIF5A is important to neurocognitive function and an imbalance of EIF5A expression is involved in T2DM neurocognitive impairment.

PPIB encodes a protein CypB, which is involved in the protein folding pathway. Our results showed that PPIB is upregulated in the hippocampus of diabetic mice. The CypB was shown to protect against Endoplasmic reticulum stress and play a role in synaptic transmission through synapsin signaling.[36] In addition, CypB was shown to be protective against amyloid beta (Aβ)-induced neurotoxicity through regulating

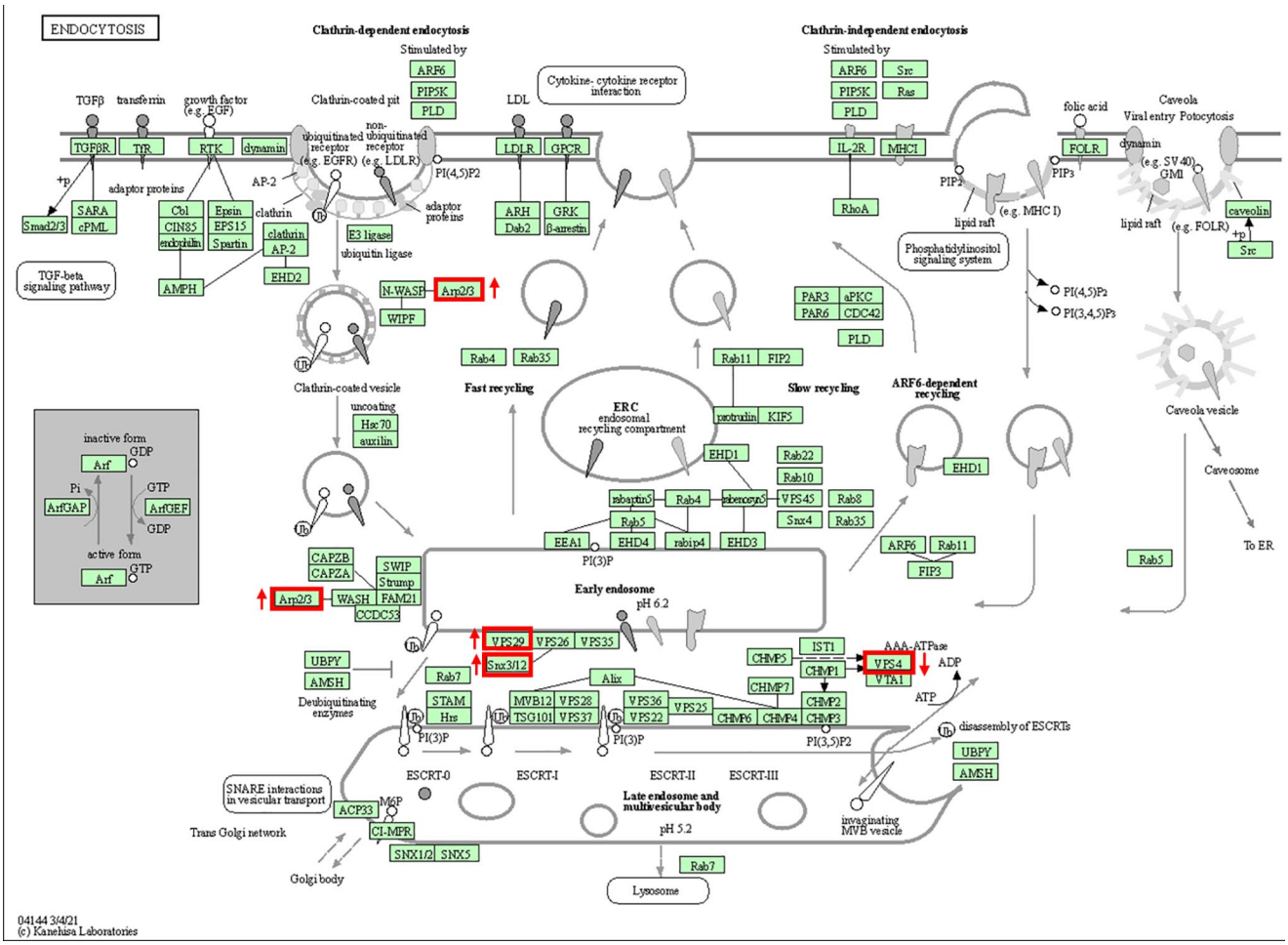

Figure 4. KEGG pathway maps. ARP2/3, VPS29, SNX3/12, and VPS4 were particularly mapped in the endocytosis pathway (*Mus musculus*).

ROS levels and activation of kinases phosphoinositide 3-kinase and mitogen-activated protein kinase.[37] However, the function of PPIB regarding T2DM context and hippocampal function has not been reported. More research is needed to elucidate the precise the role of the hippocampal PPIB in cognitive function in this diabetic model.

Endocytosis and cellular trafficking

Our findings highlight that proteins associated with endocytosis and the retromer complex pathway were among the most prominent groups in the context of diabetes-induced cognitive impairment in mice hippocampus (Figure 4). The observed imbalance in these proteins such as Actin-related protein 2/3 complex subunit 3 (ARP2/3), Vacuolar protein sorting-associated protein 29 (VPS29), Sorting nexin 12 (SNX12), and Vesicle-fusing ATPase (VPS4), indicates a disturbance of endocytosis, sorting and trafficking pathways. Specifically, the increase of ARP2/3, a key factor in dendritic spine plasticity in the hippocampus of diabetic mice suggests induction of the endocytosis pathway.[38,39] This increase signifies a potential augmentation in the dynamics of endocytosis within the neural context. Moreover, the increase of VPS29, a component of the retromer complex and SNX12, a component of sorting nexin, indicate a potential cascade of events. This includes the continuous fusion of endocytosis vesicles with lysosomes and the directed trafficking of cargo to specific cellular destinations.[40] These observed alterations suggest a complex interaction of molecular events and show that endocytosis and intracellular trafficking are involved in diabetic mice hippocampus dysfunction.

VPS4 is a member of the ATPases, which play an important role in the lysosomal degradation pathway and intracellular protein trafficking. A recent study showed that VPS4B is mainly located in the cytoplasm of neurons and may play an important role in the adult rat hippocampus after middle cerebral artery occlusion (MCAO).[41] However, the role of VSP4 related to cognitive function induced by diabetes mellitus has never been reported. Our study introduces VSP4 as a novel player in the context of diabetes-induced cognitive dysfunction, warranting exploration of its specific involvement in this context.

Cannabinoid receptor interacting protein

In this study, we found that the protein expression of cannabinoid receptor interacting protein 1 (CRIP1) was upregulated in the hippocampus of diabetic mice. This is the first study to

reveal alteration of CRIP1 expression in the hippocampus of diabetic mice with memory deficits.

CRIP1 is an important CB1 cannabinoid receptor-associated protein and it was shown that CRIP1 negatively modulates CB1 receptor function.[42] In the hippocampus, CB1 receptor and CRIP1 co-expressed in both glutamatergic and GABAergic neurons.[43] However, the largest expression of CB1 receptor resides in GABAergic interneurons.[44] Previous experiments demonstrated that episodic-like memory processes and synaptic plasticity are controlled by hippocampal CB1 receptor.[45,46] In addition, it has been shown that CRIP1 modulated CB1 receptor signaling in the hippocampus.[43] Downregulation of CRIP1 protein expression in the hippocampus induced by subchronic pyridoxin treatment was associated with an enhancement of object recognition memory.[47] Furthermore, knockdown of CRIP1 expression in the medial prefrontal cortex rescued cognitive flexibility in long-term estrogen-deprived female mice.[48] Taken together, these findings indicate that upregulation of hippocampal CRIP1 expression may participate in diabetes-induced cognitive deficits.

Ubiquitination

S-phase kinase-associated protein 1 (SKP1) is a core component of an E3 ubiquitin ligase (SCF complex) that is involved in protein ubiquitination. The ubiquitination of proteins is an important part of several biological processes including the regulation of protein homeostasis, autophagy, mitochondrial function, endoplasmic reticulum homeostasis, DNA repair, and cell cycle regulation.[49] Recent studies reported the involvement of SCF complex in neurodegenerative disease[50] via modulation of the expression of tau and Aβ, 2 proteins which are involved in the pathogenesis of AD,[51] alteration of mitochondria function,[52] and induction of neuroinflammation.[53] This finding suggests that diabetes mellitus may enhance the ubiquitination of hippocampal proteins, which in turn, may lead to the impairment of memory.

Mitochondrial dysfunction

A detailed analysis of protein function and disease relevance revealed that ADP/ATP translocase 1 (ANT1) was downregulated in diabetic mice. ANT1, a protein in AD pathway (KEEG ID mmu05010) and Parkinson disease pathway (KEGG ID mmu05012), is involved in the exchange of cytosolic adenosine diphosphate (ADP) and mitochondrial adenosine triphosphate (ATP). ADP/ATP exchange by ANT1 is vital for the maintenance of ATP synthase activity, thus its deficiency may contribute to the mitochondrial pathogenesis.[54] It was proposed that diabetes mellitus is associated with mitochondrial dysfunction and mitochondrial dysfunctions have been identified as a key factor in cognitive impairment in diabetes mellitus.[55,56] A previous study reported the downregulation of ANT1 expression in the mouse brain, including the midbrain, the striatum, the brainstem, and the hippocampus, of a Parkinson disease model.[49] However, the direct implications of the hippocampal ANT1 in cognitive deficits induced by diabetes mellitus are unknown. Further study is needed to unravel the causal link between the cognitive impairments and the hippocampal ANT1 in the context of diabetes mellitus.

Oxidative stress response

In this study, we found that the protein expression of diphosphoinositol polyphosphate phosphohydrolase I (NUDT3) was upregulated in the hippocampus of diabetic mice. It was reported that Nud3, a subfamily of diphosphoinositol-pyrophosphate-polyphosphatases (DIPPs), is involved in the degradation of inositol pyrophosphates. Specifically, it has been demonstrated that NUDT3 plays a vital role in limiting DNA damage and maintaining cell survival upon the oxidative stress in the presence of Zinc (Zn^{2+}).[57] It is well-known that hyperglycemia and diabetes mellitus promotes oxidative stress which is linked to cognitive deficits observed in both human and animal studies.[58,59] It seems that the upregulation of hippocampal NUDT3 in diabetic mice is the protective mechanism of neurons to prevent cellular damage caused by the DM-induced oxidative stress.

Neuronal development

Our results also suggest that the development of hippocampal neurons might be disrupted via the downregulation of elongator complex protein 1 (ELP1). Loss of ELP1 function has been linked to neurodevelopment disruption.[60] This may result in structural change of the hippocampus. ELP1 was identified as a noncatalytic scaffolding protein within the heterohexameric transcriptional elongator complex.[61] Transcriptional factor function of ELP1 was proposed to be involved in alpha-tubulin acetylation which affects the regulation of cortical neuron migration and differentiation.[62] Recently, it has been further shown that ELP1 protein is localized in the cytoplasm of cells, where it is essential for retrograde nerve growth factor (NGF) signaling and neuron target tissue innervation and survival.[63]

Protein glycosylation

Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 2 (RPN2) is an integral membrane protein found only in the rough ER.[64] RPN2 is involved in protein glycosylation by catalyzing the transfer of an oligosaccharide from a lipid donor to a nascent protein during protein synthesis. This modification is important for protein folding, stability, and function. However, the importance of RPN2 in the hippocampus of T2DM mice regarding cognitive function is unknown. Since RPN2 plays an important role in protein glycosylation and quality control, regulation of RPN2 may be critical for the proper function of glycoprotein production in

hippocampal neurons, with downregulation leading to misfolding and malfunctioning of glycoproteins in ER lumen. The alterations of the protein synthesis, folding and modification pathway suggest that they may play an important role in the pathophysiology of cognitive impairment in diabetic mice.

Proteomic results in the context of T2DM-associated cognitive impairment have been previously reported as mentioned earlier.[12-14] In this study, we found 9 upregulated proteins which are involved in protein synthesis and folding (EIF5A, RSP24, and PPIB), endocytosis and trafficking (VPS24, SNX12, and ARP2/3), cannabinoid receptor interacting (CRIP1), ubiquitination (SKP1) and oxidative stress response (NUDT3), and 4 downregulated proteins which are involved in mitochondria function (ANT1), neuronal development (ELP1), protein glycosylation (RPN2), and endocytosis (VPS4). Downregulation of proteins with known function implicated in the arrangement of myelin sheath and morphology of axons (MBP), the structure of astrocytes (GFAP), and response to oxidative stress and inflammation (Apo-AI) have been reported in the hippocampus of spontaneous diabetic Zucker fatty rats when compared with the control littermates.[14] Also, significant alterations in hippocampal protein expressions involved in cytoskeletal regulation, oxidoreductase activity, protein deubiquitination, energy metabolism, GTPase activation, heme binding, hydrolase activity, iron storage, neurotransmitter release, protease inhibitor, and transcription factor have been observed in STZ/HFD induced-diabetic mouse model.[12] Changes in protein expression with the functional role in the response to oxidative stress, energy metabolism and neuronal transport, which were associated with cognitive decline, have been found in the hippocampus of STZ/HFD induced-diabetic rat model.[13] To the best of our knowledge, our study is the first to show that T2DM-associated cognitive dysfunction is linked to alterations in protein synthesis, endocytosis and cellular trafficking pathways in the hippocampus.

The differential proteins found in the hippocampus of T2DM mice in our study differ from those found in previous works. Several factors could explain this discrepancy in results obtained including the differences in technique used to analyze the proteomic profile, animal model of T2DM, and the onset age and duration of diabetes between studies.

In this study, we employed the GeLC shotgun proteomic approach, whereas the previous study used 2DE-based proteomic approaches. Each technique may have different limitations, which can lead to different results. The GeLC shotgun proteomic approach is effective in obtaining a broad overview of proteome alteration, but offers semiquantitative data. Therefore, gel-free label-based quantitative proteomic approaches such as iTRAQ should be considered for future studies to confirm these findings.

Regarding the diabetic animal model, the diabetic model in our study was induced by the combination of long-term HFD feeding and 2 intraperitoneal injections of STZ at the dose of 100 mg/kg in mice, while the diabetic mouse model in the study of Matsuura et al[12] was induced by intraperitoneal injections of STZ at the dose of 50 mg/kg for 5 consecutive days. Shi et al[13] used the diabetic rat model by feeding rats with HFD for 4 weeks followed by a single injection of STZ (30 mg/kg, i.p.). Nam et al[14] investigated the hippocampal proteome profile in the diabetic Zucker fatty (ZDF) rats that develop advanced insulin resistance and hyperglycemia due to a genetic mutation of the leptin receptor gene. These diabetes model differences between studies may affect the type and timing of protein expression differences.

In this study for the proteomic analysis, only 4 mice per group were used for the analysis and this could be considered a limitation of sample size. Further studies with a larger sample size are needed to confirm the hippocampal proteome results of T2DM mice.

It is noted that all 5 novel proteins identified in this study including 40S ribosomal protein S24, Vacuolar protein sorting-associated protein 29, Sorting nexin-12, Diphosphoinositol polyphosphate phosphohydrolase 1, Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 2, and Vesicle-fusing ATPase, have not been previously reported in the context of T2DM-associated cognitive dysfunction. These proteins are of particular interest as potential drug targets for the treatment of T2DM-associated cognitive dysfunction. However, further studies are required to validate their roles, as well as to investigate the causal relationships between these proteins and T2DM-associated cognitive dysfunction.

Conclusion

Overall, this study provides a new insight into the molecular mechanisms underlying the impact of T2DM on hippocampal function and cognitive impairment. Utilizing the GeLC shotgun proteomic approach, we have discovered additional molecular pathways that have not been previously reported in this context. Our findings suggest that the alteration in hippocampus proteome of T2DM mice may contribute to cognitive dysfunction and provide potential therapeutic targets for T2DM-associated neurodegenerative disorders. These findings enhance the understanding of the pathophysiology of T2DM-related cognitive impairment and identify molecular targets for drug development to treat T2DM-associated cognitive impairment conditions.

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Ethics

This study adheres to the ARRIVE guidelines for the reporting of animal experiments. The welfare of the animals used in this research was a priority, and all efforts were made to minimize suffering. Detailed protocols are provided to ensure reproducibility and adherence to ethical standards. All experiments were conducted in accordance with the Animal Care and Use Committee of Burapha University (IACUC Number 006/2564).

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Supplemental material

Supplemental material for this article is available online.

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