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Probing the risk markers of mild cognitive impairment and Alzheimer's disease in alloxan-induced diabetic rats

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Alzheimer's disease (AD) exemplifies a form of diabetes mellitus that selectively affects the brain. In this study we evaluated serum markers and genes linked to AD and investigated the possible link between AD and diabetes. We measured the serum levels of insulin, lactate, citrulline, argininosuccinate, homocitrulline, and AD-associated lipids (2,4-dihydroxybutanoic acid and sphinganine-1-phosphate). We used semi-quantitative reverse transcription polymerase chain reaction to determine the relative gene expression of *Rest*, *Bace1*, and *Rtn3* in the brain. Western blotting was used to determine the relative protein expression of REST, BACE1, and RTN3 in the brain. Serum from diabetic rats showed significantly higher levels of citrulline, homocitrulline, lactate, 2,4-dihydroxybutanoic acid, and sphinganine-1-phosphate, but lower levels of argininosuccinate and insulin compared with serum from control rats. At the gene level, *Bace1* and *Rest* were significantly increased, whereas *Rtn3* was considerably reduced in diabetic compared with control rat brains. At the protein level, BACE1 and RTN3 were significantly increased whereas REST was significantly reduced in diabetic compared with control rat brains. These findings indicate that hyperglycemia and insulin resistance in the brain may be the common element that connects urate turnover and alterations in neural regulatory genes.

Keywords: Amyloid, Urate turnover, Lactate, Serum markers, Gene

Researchers have shown that comorbid medical disorders have a significant role in the development of Alzheimer's disease (AD)[1]. One of the most common comorbidities in people with AD is diabetes. Epidemiological studies have shown that people with diabetes are more likely to develop AD than those without the condition[2,3]. The AD etiology has been thoroughly described in individuals with type 1 or type 2 diabetes[4]. People with type 2 diabetes have a 60% greater chance of developing dementia[5], while those with recent-onset type 2 diabetes have a 16% higher risk of developing dementia[6]. Researchers have also reported a link between type 1 diabetes and cognitive problems[7] as well as AD, implicating the role of tau, β-amyloid (Aβ), and insulin resistance in synaptic and cognitive deficits[4]. People with type 1 diabetes have a greater rate of cognitive impairment and synaptic abnormalities[4,7], which provides supporting evidence for the association between type 1 diabetes and AD[8]. Diabetic ketoacidosis type 1 is seen in almost 80% of people with AD[9]. Our earlier work supports hypometabolism and hypoxia as important risk factors in the vasculature that contribute to AD[10]. As diabetes and mild cognitive impairment (MCI)/AD are well connected with each other, identifying the risk factors of MCI/AD in people with diabetes could be useful to overcome neurovascular abnormalities and to implement early intervention.

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Serum uric acid (sU) measurements in ischemic brain following a stroke and in patients diagnosed with vascular dementia have revealed that uric acid is harmful[11]. Other studies, particularly those focusing on people with vascular or mixed dementia[12, 13], have shown that those with a higher sU content (hyperuricemia) are more likely to develop dementia. Diabetic rats have altered uric acid metabolism and purine metabolite levels in plasma and liver tissues, suggesting the significance of cognitive risk indicators in the disease[14,15].

Amino acid catabolism and stimulation of the urea cycle have been linked to insulin resistance and hypometabolism in AD[16]. Additionally, amyloid protein aggregation may have a role in brain energy functions[17]. Citrulline is synthesized from ornithine and carbamoyl phosphate in the urea cycle. Nitric oxide (NO) is synthesized from L-arginine and L-citrulline through the nitric oxide synthase (NOS) pathway[18]. Depletion of ornithine in the urea cycle causes the accumulation of carbamoyl-phosphate; this phenomenon might enhance the synthesis of homocitrulline and lead to hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome[19]. The urea cycle and NOS pathway activation have been increasingly reported in the pathogenesis and progression of neurological disorders such as AD and Huntington's disease (HD) with ammonia toxicity[20,21]. Therefore, an exploration of changes in the metabolites (e.g., citrulline and homocitrulline) of those pathways could be useful to connect diabetes and neurodegenerative AD.

Some of the markers associated with AD risk progression include 2,4-dihydroxybutanoic acid (2,4-DHB) and sphinganine-1-phosphate[22]. In fact, upregulated sphinganine-1-phsophate is one of the markers used to indicate the conversion of MCI to probable AD. Few studies have looked at the significance of such indicators in diabetes. Despite the importance of multiple genes in the pathophysiology of AD in the brain, including RE1-silencing transcription factor (*Rest*), reticulon-3 (*Rtn3*), and beta-secretase 1 (*Bace1*), no research has investigated a possible connection between these genes and diabetes. Moreover, studies on serum AD markers such as citrulline, homocitrulline, lactate, and argininosuccinate are missing in diabetic models. Therefore, we explored several AD risk genes in brain tissue and serum markers of MCI and AD in alloxan-induced diabetic rats.

Materials and Methods

Chemicals and reagents

Standard reagents, including alloxan monohydrate, citrulline, homocitrulline, arginosuccinate lyase, arginate lyase, and arginase, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Proteinase K was procured from Thermo Fisher Scientific (Waltham, MA, USA). The RNeasy and reverse-transcription polymerase chain reaction (RT-PCR) kits were obtained from Qiagen (Hilden, Germany). Other analytical-grade solvents and chemicals were obtained from SD Fine Chemicals (Mumbai, India).

Animal experiments

The procedures involving the animals were carried out in accordance with the recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals. The Basaveshwara Medical College and Hospital IAEC in Chitradurga, India, approved the procedures (BMCH/IAEC/05 Biochem/2015, dated 04.06.2015).

Wild-type male rats were purchased from Biogen (Bangalore, India). They were used when they were 7-9 weeks old and weighed 180-240 g (without any exclusions). The rats were housed in polycarbonate cages under a normal 12h photoperiod at 22±2°C. The rats were divided at random to ensure that each group had a similar number of animals of the same age. The experimental treatments were concealed from the researchers. The rats were fed with Amruth rat feed (Pranav Agro Industries, Pune, India), and they had free access to water.

Diabetes was induced in rats that had been fasted for 18h by intraperitoneally administering 150 mg/kg of alloxan monohydrate as a single dose. The control group (n=6) comprised rats that were administered 1 mL/kg normal saline intraperitoneally. The diabetic rats were given access to 5%-10% glucose in the drinking water to inhibit hypoglycemia due to the alloxan effect. Blood glucose levels were measured 48h after injection by using a point of care glucometer to confirm hyperglycemia. Blood glucose >250 mg/dL confirmed diabetes in the rats.

Twenty-one days after the alloxan monohydrate injection, the rats were anaesthetized via isoflurane inhalation, and blood was collected in vacutainer tubes (with clot activator) by retro-orbital puncture. Then, rats were subjected to cardiac perfusion with saline; brains were removed and stored at –20°C. The blood was centrifuged at 1700 *g* for 15 min at 8°C; the serum was removed and frozen at –80°C for future use.

Estimation of insulin

The serum insulin concentration was estimated by using a commercially available enzyme linked immunosorbent assay (ELISA) kit (Enzo Life Science, Farmingdale, NY, USA) according to the manufacturer's instructions. The absorbance at 450 nm was measured with a microplate reader (Mindray, Shenzhen, China).

Estimation of lactate

The serum lactate concentration was determined by using a kit from Cell Biolabs, Inc. (San Diego, CA, USA) according to the manufacturer's protocol. The absorbance at 570 nm was measured with a microplate reader.

Estimation of citrulline

The serum citrulline concentration was determined by using an ELISA kit from Life Technologies (New Delhi, India); it was performed according to the manufacturer's instructions. The absorbance at 450 nm was measured with a microplate reader.

Estimation of homocitrulline

The serum homocitrulline concentration was estimated by using a kit purchased from Cell Biolabs Inc., performed according to the manufacturer's instructions. The absorbance was measured at 560 nm with a microplate reader.

Estimation of argininosuccinate

The serum samples were mixed with 0.5 mL of 100 mM potassium phosphate buffer (incubated at 37°C), followed by 2.2 mL of 40 mM potassium sulfate and 0.5 mL arginase. All components were mixed well and equilibrated to 37°C with a thermostat spectrophotometer. The initial absorbance at 240 nm for both the test and blank were recorded. Next, argininosuccinate lyase (0.1 mL) was added and mixed rapidly by inversion; the reaction was permitted to continue to completion (around 5 min). The absorbance was measured at 240 nm.

Estimation of sphinganine-1-phosphate

This assay was performed in 96-well plates. Each well received 50 µL of standard, blank, or serum sample, followed immediately by 50 µL of mouse monoclonal anti-sphinganin-1-phsophate (diluted 1:1000, RayBioTech, Peachtree Corners, GA, USA). Following incubation for 1h at 37°C, the plate was washed with 1× wash solution (350 µL per well) for 1-2 min to remove any unbound antibody. Then, 100 µL of working solution containing an anti-mouse IgG antibody conjugated to horseradish peroxidase was added to each well. The plate was incubated at 37°C for 30 min. The plate was washed to eliminate any remaining antibody (90 µL per well). After incubating the plate at 37°C for 10-20 min, a substrate solution was applied to each well. Fifty microliters of stop solution was added to each well to halt the reaction. The absorbance at 450 nm was measured immediately with a microplate reader.

Estimation of 2,4-DHB by gas chromatography-mass spectrometry (GC-MS)

The 2,4-DHB reference standard was acquired from Sigma-Aldrich. The gas chromatograph had a split/splitless injector (model 6890, Agilent Technologies, Santa Clara, CA, USA). An Rtx-5MS (30 m × 0.25 mm × 0.32 mm) fused-silica capillary column was utilized in a GC-MSQP2010 Plus system, and 1 µL of each derivatization solution was injected at a split ratio of 1:25. The injection temperature was 230°C, the interface temperature was 250°C, and the ion source temperature was 200°C. The carrier gas was helium, and the flow rate was 2.5 mL/min. The column temperature was originally maintained at 100°C for 2 min, then increased by 3°C/min to 185°C, held for 2 min at 192°C at 0.5°C/min, 10°C/min to 245°C, and then increased by 3°C/min to 280°C, held for 15 min. The scan range was 45-600 amu, and the settings included an electron ionization source, an ion source temperature of 200°C, an interface temperature of 280°C, 70 eV of electron energy, a multiplier voltage of 1.0 kV, a 5.5-minute solvent delay, and a 2.8°C temperature difference. The MASSLAB method format now includes a retention time and mass spectrum library for automated peak quantification of metabolite derivatives. The identity of 2,4-DHB in the serum samples was confirmed by comparison with a standard peak. Principal component analysis was performed with the SIMCA-P software (Umetrics, Sweden).

Semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR)

The RNeasy kit (Qiagen) was used to extract RNA from brain samples. A one-step RT-PCR kit was used for all the PCR reactions, strictly adhering to the manufacturer's protocol. The RT-PCR samples were shuffled, and the gel was run by someone who was unaware of the sample identities. Each 20 µL reaction contained 2 µL of reverse and forward primer mixture (10 pmol of each primer), 1 µg of RNA, and diethyl pyrocarbonate (DEPC-treated water). Reverse transcription was performed at 50°C for 1h, followed

by enzyme inactivation for 5 min at 94°C. The primer sequences for the genes of interest (as well as the housekeeping gene *β-actin* [*Actb*]) are presented in Table 1. For *Bace1*, the PCR conditions were 32 cycles of 30 seconds at 95°C, 45 seconds at 60°C, and 1 min at 72°C. For *Rtn3*, the PCR conditions were 40 cycles of 30 seconds at 95°C, 15 seconds at 60°C, and 1 min at 60°C, then 95°C for 15 seconds, 60°C for 15 seconds, and 95°C for 15 seconds to acquire a melting curve to confirm the amplification specificity. The *Rest* PCR conditions were 30 cycles of 30 seconds at 94°C, 45 seconds at 60°C, and 1 min at 68°C. The PCR products were observed in 2% agarose gels with ethidium bromide. The densitometry of each band of interest was determined with ImageJ software and normalized to the intensity of the *Actb* band. The primers were selected based on previous publications and gene database software.

Western blotting

Brain tissues were homogenized in lysis buffer containing 1% protease and phosphatase inhibitor (HiMedia Laboratories, Thane, Maharashtra, India) at 4°C. Then, samples were centrifuged at 10,000 *g* at 4°C for 15 min. Supernatants were collected and the protein concentration was determined using a Bradford protein assay kit. Equal amounts (20 µg) of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with 5% nonfat milk for 1h at room temperature (25°C) prior to incubation overnight at 4°C with polyclonal anti-rabbit β-actin (1:1000; Sigma-Aldrich), polyclonal anti-rabbit REST (diluted 1:500; Invitrogen, Carlsbad, CA, USA), polyclonal anti-rabbit BACE1 (1:500; Invitrogen), or polyclonal anti-rabbit RTN3 (diluted 1:500; Invitrogen). The next day, the membranes were washed three times with Tris-buffered saline and incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (1:500) for 1h at room temperature and then washed three times with Tris-buffered saline with 0.5% Tween-20. The membranes were incubated with an enhanced chemiluminescence (ECL) reagent and exposed to X-ray film to visualize protein bands. The densities of specific protein bands were quantified with the Image J software (version 12.0). Western blotting was performed by a technician who was blind to the animal group information and sample details. The technician ended up running the control and diabetic samples on two separate gels on the same day for each target protein (REST, BACE1, and RTN3). The densitometry for each sample was normalized to a loading control (β-actin) and then quantified.

Statistical analysis

Statistical analysis was performed using Sigma plot 14.5 (Systat Software, Inc., USA). The experimental results are shown as the mean and standard error of the mean (SEM). For statistical analysis, two-tailed Student's *t*-test (with the appropriate null hypothesis of equal or unequal variances) was used to compare the groups. A P value < 0.05 was considered significant.

Results

The serum lactate level was approximately three times as high in the diabetic group compared with the control group (Table 2; P = 0.0013). In contrast, the

Table 2 — Serum levels of citrulline, homocitrulline, argininosuccinate, lactate, and insulin in control and diabetic rats

Parameter	Group	Mean ± SEM	*P*-value
Citrulline (µmol/L)	Control	0.43 ± 0.03	
	Diabetic	0.62 ± 0.05	< 0.001
Homocitrulline (mmol/mol lysine)	Control	0.15 ± 0.006	< 0.001
	Diabetic	0.23 ± 0.008	
Argininosuccinate (µmol/L)	Control	0.35 ± 0.06	< 0.001
	Diabetic	0.14 ± 0.043	
Lactate (mmol/L)	Control	1.21 ± 0.03	0.0013
	Diabetic	3.49 ± 0.38	
Insulin (ng/mL)	Control	4.34 ± 0.098	< 0.001
	Diabetic	2.07 ± 0.087	

[SEM, standard error of the mean; n = 6 per group]

Table 1 — Primers used for semi-quantitative reverse transcription polymerase chain reaction

Gene	Forward primer (5′→ 3′)	Reverse primer (5′→ 3′)	Size (bp)
Bace1 (brain)	CGGGAGTGGTATTATGAAGTG	AGGATGGTGATGCGGAAG	320
Actb(for *Bace1* brain)	GGCATCCTGACCCTGAAGTA	GCCGATAGTGATGACCTGACC	201
Rtn3	CTGGCAGCTTTCAGTGTT	AATGGGAAGACTAAGAGCG	565
Actb(for *Rtn3*)	GTCTTCCCCTCCATCGTGGG	TGGCTGGGGTGTTGAAGGTC	198
Rest	TCGAGCTTCAGCACCGCGGACAG TGCCTTG	TCGACAAGGCACTGTCCGCGG TGCTGAAGC	250
Actb(for *Rest*)	TACAATGAGCTGCGTGTGGCT CCCCCCG	AATGGTGATGACCTGGCCGT CAGGC	132

serum insulin level was significantly lower in the diabetic group compared with the control group (Table 2; $P < 0.001$). Moreover, the diabetic group had significantly higher serum levels of the urea cycle metabolites citrulline and homocitrulline (Table 2; $P < 0.001$). The serum level of argininosuccinate, a non-proteinogenic amino acid involved in the urea cycle, was markedly reduced in the diabetic group compared with the control group (Table 2; $P < 0.001$).

We analysed the levels of two lipids, sphinganine-1-phosphate and 2,4-DHB, that are associated with cognitive impairment risk and AD progression. Each marker was elevated more than twofold in the diabetic compared with the control group (Table 3; $P < 0.05$).

We also examined the relative gene expression of *Rest*, *Bace1*, and *Rtn3* in the brain by using semi-quantitative RT-PCR. Beta-cell-function-related genes are suppressed by the transcription factor REST[23]. Rest messenger RNA (mRNA) was almost 7.5 times more abundant in the diabetic brain than in the control brain (Fig. 1; $P = 0.007$). Brain *Bace1* expression was increased 2.5-fold in the diabetic group compared with the control group (Fig. 2; $P = 0.001$). *Rtn3* expression in the diabetic brain was approximately one third of the level in the control brain (Fig. 3; $P = 0.033$).

Finally, we assessed REST, BACE1, and RTN3 protein expression in the brain with western blotting. There was a 2-fold decrease in REST protein in the diabetic group compared with the control group (Fig. 4; $P < 0.001$). On the contrary, there was a >2-fold increase in BACE1 (Fig. 5; $P < 0.001$) and RTN3 (Fig. 6; $P < 0.05$) in the diabetic group compared with the control group.

Table 3 — Serum levels of lipids associated with AD risk in control and diabetic rats

Parameter	Group	Mean ± SEM	*P*-value
2,4-Dihydroxybutanoic acid (μM)	Control	12.6 ± 1.60	0.021
	Diabetic	26.8 ± 4.26	
Sphinganine-1-phosphate (ng/mL)	Control	1.75 ± 0.25	0.005
	Diabetic	3.91 ± 0.49	

[SEM, standard error of the mean; n = 6 per group]

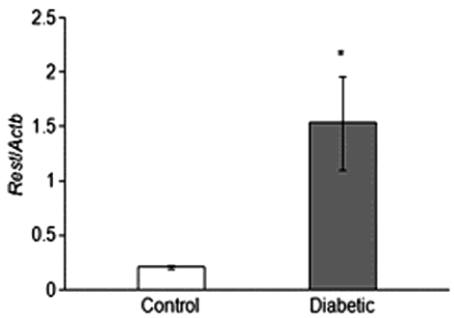

Fig. 1 — Semi-quantitative analysis of *Rest* gene expression (*Rest*/*Actb*). Each bar represents the mean ± standard error of the mean (n = 6 control; n = 5 diabetic). The data were analysed with Student's *t*-test ($P = 0.007$)

Discussion

While there is still debate regarding the link between diabetes mellitus and AD[24], a considerable body of evidence implicates a plausible correlation between AD and diabetes for dementia risk[25-27]. To investigate this possibility, it is necessary to examine AD risk factors and indicators in people with diabetes. In this study, we used a rat model of diabetes to assess MCI/AD markers and genes that have received relatively limited research attention.

Although cerebrospinal fluid sampling is invasive, the levels of AD-related proteins such as Aβ, tau, and phospho-tau in this medium have been suggested as promising biomarkers of pathological processes underlying AD. Magnetic resonance imaging, computed tomography, and 18F-fluorodeoxyglucose or amyloid positron emission tomography are all

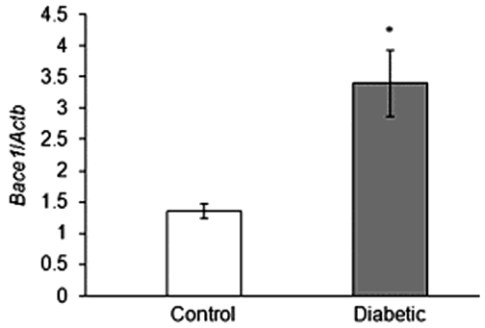

Fig. 2 — Semi-quantitative analysis of *Bace1* gene expression (*Bace1*/*Actb*). Each bar represents the mean ± standard error of the mean (n = 5 for each group). The data were analysed with Student's *t*-test ($P = 0.0011$)

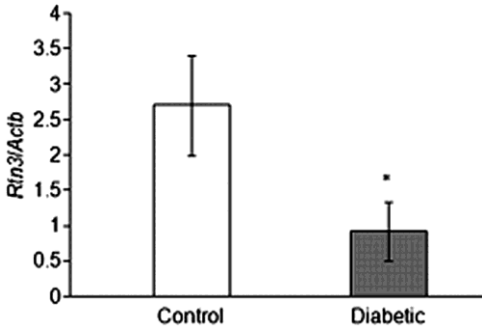

Fig. 3 — Semi-quantitative analysis of *Rtn3* gene expression (*Rtn3*/*Actb*). Each bar represents the mean ± standard error of the mean (n = 5 control; n = 6 diabetic). The data were analysed with Student's *t*-test ($P = 0.033$)

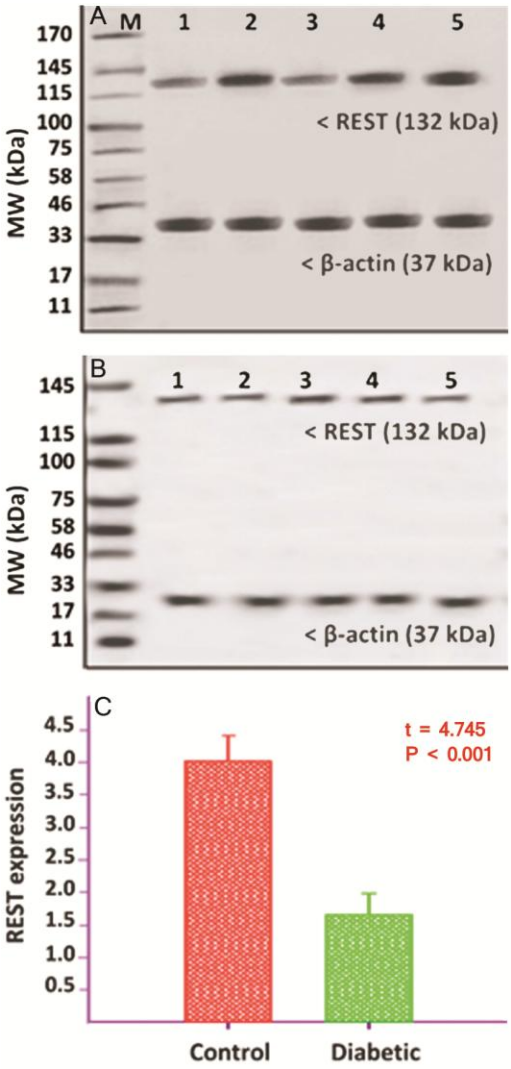

Fig. 4 — Measurement of REST protein expression in (A) control and (B) diabetic rat brain samples by western blotting. "M" refers to a molecular weight ladder. (C) Quantification of REST protein expression relative to β-actin (in intensity units). The graphs shows the mean + standard error of the mean (n = 5 per group). The *t* and *P* values are from Student's *t*-test

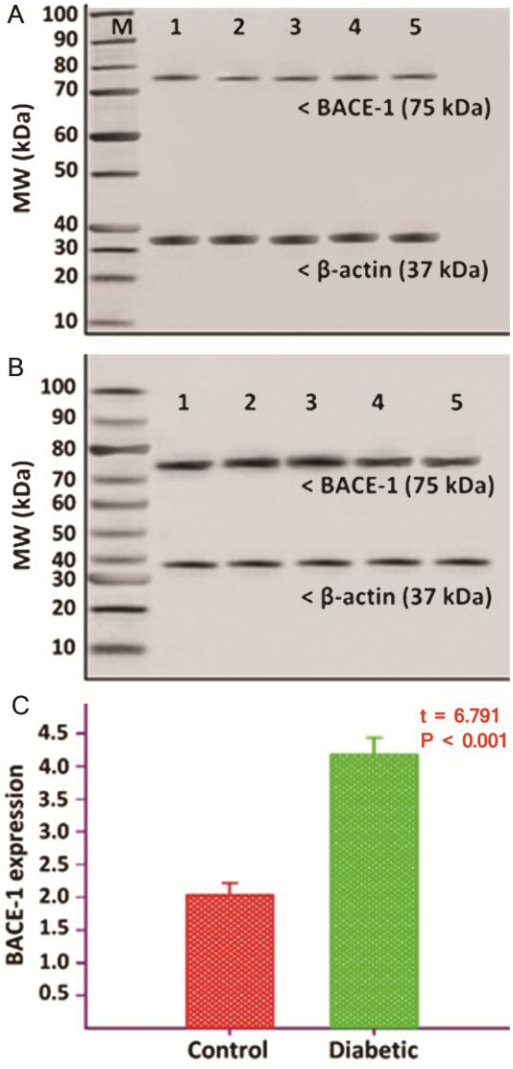

Fig. 5 — Measurement of BACE1 protein expression in (A) control and (B) diabetic rat brain samples by western blotting. "M" refers to a molecular weight ladder. (C) Quantification of BACE1 protein expression relative to β-actin (in intensity units). The graphs shows the mean + standard error of the mean (n = 5 per group). The *t* and *P* values are from Student's *t*-test

examples of imaging modalities that suffer from drawbacks such as low diagnostic accuracy and prohibitive costs. Therefore, exploration of peripheral blood markers for identifying AD risk biomarkers in diabetes would be useful to predict the problems earlier that may later lead to the onset of MCI/AD28. Researchers have attempted to use amino acid profiling to improve the diagnosis of various diseases, including neurodegenerative disorders29. Researchers have reported differential levels of several plasma amino acid profiles, including imidazole-containing free amino acids (histidine and methyl-histidine), catecholamines (L-DOPA and dopamine), and urea cycle enzymes (citrulline and ornithine), in probable AD cases compared with age-matched controls30. We found considerably higher serum citrulline and homocitrulline levels in diabetic compared with control rats (Table 2). Diabetic rats also had considerably lower serum argininosuccinate levels than control rats (Table 2). Altered urea cycle metabolites have been reported in AD20, and a recent study demonstrated that urea cycle activation clears Aβ aggregates to detoxify ammonia in astrocytes, although it impairs memory in AD31.

According to Corso *et al*.[32], patients with different types of cognitive impairment, such as probable AD,

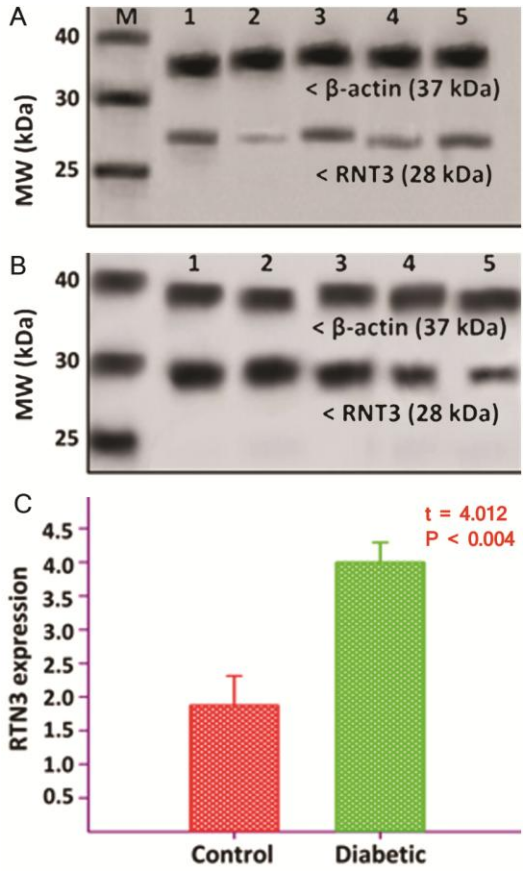

Fig. 6 — Measurement of RTN3 protein expression in (A) control and (B) diabetic rat brain samples by western blotting. "M" refers to a molecular weight ladder. (C) Quantification of BACE1 protein expression relative to β-actin (in intensity units). The graphs shows the mean + standard error of the mean (n = 5 per group). The *t* and *P* values are from Student's *t*-test

MCI, and subjective memory complaint (SMC), demonstrate elevated serum citrulline and homocitrulline levels compared with healthy subjects. The fact that patients with cognitive dysfunction display altered serum profiles that are similar to the present work implies an association between diabetes and cognition-associated diseases such as AD. Moreover, pathogenic AD mechanisms that involve perturbed cerebral glucose metabolism[9] might increase urea cycle amino acids to provide energy through the tricarboxylic acid cycle[32], a phenomenon that is consistent with the present findings. While Corso *et al.*[32] found elevated serum argininosuccinate levels during the conversion of MCI to AD, our findings in alloxan-induced diabetic rats are the opposite (Table 2). Argininosuccinate is synthesized from citrulline and aspartate in cells and used as a precursor for arginine in the urea cycle[33]. The decreased serum argininosuccinate levels in diabetic rats are quite intriguing. One study indicates that proper maintenance of vascular endothelial function requires arginosuccinate synthase, which is transcriptionally regulated by insulin to promote argininosuccinate synthesis[34]. Therefore, decreased serum insulin levels in diabetic rats might account for the decreased serum argininosuccinate levels and poor vascular endothelium, a feature in patients with AD.

We showed a reduction in blood insulin levels in diabetic rats, which may lead to hyperglycemia and an increased risk of AD[35]. However, glucokinase inhibition is the mechanism through which alloxan suppresses insulin production. Increased plasma lactate levels in diabetic rats have also been described[36]. Moreover, elevated blood lactate levels have been reported in patients with type 1 and type 2 diabete[37].

Serum metabolomic studies have shown that elevated 2,4-DHB levels are associated with hypoxia in the pathogenesis of AD[22]. Elevated serum sphinganine-1-phosphate and 7-ketocholesterol levels are also involved in the transition of MCI to probable AD[38]. Significant upregulation of other serum metabolites such as 3,4-DHA, docosapentaenoic acid, and uric acid has also been documented to substantiate progression of dementia as well as AD, which implicates hypoxia during disease progression[39]. As there are only a few studies that have reported on the relation between serum 2,4-DHB and sphinganine-1-phosphate levels and dementia/ AD, our results from diabetic rats may strengthen biomarker panel exploration. Sphingosine-1-phosphates are signalling lipids that have been described as a plasma biomarker of AD and cognitive impairment[40]. There have been recent reports of dysregulated sphingosine-1-phosphate signalling through its receptor S1PR in AD mouse models[41,42]. Sphingolipids have been linked to brain ageing and neurodegenerative diseases, including AD, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis[43]. In addition to controlling exosomes to increase neurotoxicity in brain cells, they interact with transcription factors including NF-κB, FOXOs, and AP-1 to cause cell death. However, based on some reports, the levels of many sphingolipids in AD brain, including ceramides, sphingosine, ganglioside GM1, and ganglioside GM3, are increased, while sphingomyelin and sphinganine-1-phosphate are decreased. The elevated serum lipids (Table 3) in the present study implicate similar

alterations of these markers in diabetic rats, thereby favouring a plausible link between diabetes and cognitive impairment.

Even in the context of AD pathology, an increase in REST is thought to mediate a neuroprotective response in ageing, which may be linked to the maintenance of cognitive function and an extended life[44]. REST is induced in neurons from cognitively healthy, aged individuals. While REST expression increases during normal brain ageing, it is markedly decreased, almost undetectable, in brain regions that are vulnerable to AD-mediated damage[44]. Low plasma REST levels during ageing may indicate poor protection against brain stress. Hence, researchers suggest that REST could be a candidate biomarker of cognitive decline and AD[45]. While REST expression exerts a neuroprotective function in the ageing brain[45], loss of REST is associated with MCI or severe cognitive impairment and AD[44]. REST, however, has been shown to be significantly elevated in the prefrontal cortex and hippocampal CA1 and CA3 neurons of postmortem brain samples from normal elderly individuals[46].

In agreement with previous results from neurodegenerative diseases[44,47], we discovered dramatically increased *Rest* gene expression in diabetic rat brains (Fig. 1). However, REST protein expression was lower in diabetic compared with control rat brains (Fig. 4). It is necessary to note that there is not always a direct correlation between mRNA and protein expression[48,49]. For example, alternative splicing can cause exons to be skipped, producing mRNA species that are either unstable or quickly degraded or that code for unstable proteins, thereby reducing protein expression. Alternative splicing of the *Rest* gene can be attributed in part to the difference between mRNA and protein expression profile. It may be even possible that the interaction of microRNAs (miR-124a, miR-9, and miR-132) with *Rest* mRNA suppresses its neuronal translation, leading to decreased REST protein expression[50]. It has been suggested that miRNA bind to the 3′-untranslated region (UTR) of target mRNAs to repress gene expression by triggering mRNA degradation and inhibiting translation[51].

A striking upregulation of REST in critical brain regions during normal ageing suggests its protective response to stress-induced neuronal insults like premature neural stem cell depletion, neuronal hyperexcitability, oxidative stress, neuroendocrine dysfunction, and neuropathology, while REST expression is lost in ageing neurons associated with AD in humans[44]. Thus, REST has been implicated as a marker of stress and AD, and augmenting REST expression might be neuroprotective[52]. While the importance of *Rest* mutations has not yet been resolved in patients with diabetes, in mice activation of *Rest* in beta cells during development and in adults seems to lead to the development of diabetes[53].

Sharoar *et al*.[54] found that RTN3-immunoreactive dystrophic neurites (RIDNs) accumulate RTN3. This accumulation coincides with the formation of high-molecular-weight RTN3 aggregates in the brains of people with AD and mice that express mutant amyloid precursor protein (APP). Notably, the cortical and hippocampal areas are more likely to exhibit this phenomenon. Dystrophic neurites in Tg-RTN3 mice, together with deficits in spatial learning, memory, and synaptic functioning, provide further evidence that RIDNs contribute to AD cognitive dysfunction. Therefore, RTN3 elevation is associated with impaired axonal transport of BACE-1[55], decreased dendritic spine density, and dysfunctional long-term potentiation[56]. Even a modest increase in RTN3 levels leads to accumulation of dystrophic neurites in animal brains. Therefore, the significant upregulation of RTN3 we observed in diabetic rat brains implicates cognitive and synaptic impairment risks associated with diabetes mellitus. In fact, pharmacological inhibition of RTN3 aggregation has been proposed to be an effective strategy for managing neuritic dystrophy and cognitive decline[57]. In the current study, we found that AD-related issues in the diabetic state are linked with increased RTN3 protein expression in diabetic rat brains (Fig. 6).

RTN3 can interact with BACE1 and negatively affect its activity[58]. In a recent work, Gns *et al*.[59] revealed a significant interaction between RTN3 and BACE1. Downregulation of RTN3 has been reported in AD brains, and interaction between RTN3 and BACE1 considerably hampers BACE1 enzymatic activity on APP axonal transport, which in turn stops the amyloidogenic pathway and promotes the non-amyloidogenic pathway. Because BACE1 interacts exclusively with RTN3 monomers and not with RTN3 dimers[60], RTN3 aggregation may decrease its interaction with BACE1, leading to increased Aβ formation[61]. In the present work, there was reduced *Rtn3* mRNA expression (Fig. 3) and increased RTN3 protein expression (Fig. 6) in

diabetic rat brains. These results are in contrast to the findings reported by Kume *et al.*[62], namely no difference in RTN3 expression in human AD brains compared with control samples. However, other studies have reported increased RTN3 protein expression in the brain, suggesting that RTN3 aggregate formation may induce Aβ production[63]. At the mRNA level, multiple *Rtn3* isoforms (A1-2, A2, A2-4, A3a-4a, A3b-4b, A4a, A4b, B, A3-4, and pan) have been reported in the mouse due to alternative splicing. However, there is a lack of information on the rat *Rtn3* mRNA isoforms. Therefore, the decreased brain *Rtn3* mRNA expression may be due to alternative splicing events.

RTN3 deficiency in AD brain markedly elevates BACE1 protein, and presumably activity and, consequently, increases APP processing with concomitant amyloid deposition. These data suggest that RTN3 is involved in the control of BACE1 activity and AD pathogenesis. Araki *et al.*[64] discovered that in the hippocampus and cerebral cortical areas of APP/RTN3 transgenic mice, amyloid plaques and the Aβ burden are significantly decreased compared with APP transgenic animals. These results confirm the involvement of RTN3 in regulating BACE1 activity to modulate APP processing that leads to Aβ formation and plaque formation. In the present work, suppressed *Rtn3* and elevated *Bace1* mRNA expression in diabetic rat brains overlap with AD features. These data provide further evidence for the potential link between diabetes and AD. BACE1 is considered to be the key therapeutic target to lower brain Aβ levels, and thus it is important in preclinical and clinical research[65]. Several investigations have shown that RTN3 and BACE1 are localized in human pyramidal neurons; RTN3 is expressed predominantly in these neurons. A deeper understanding of the molecular functions of RTN3 in the brain may aid the development of new treatment options for diabetes and AD. Owing to the increasing prevalence of AD with ageing[66] and the limited bioavailability of the currently used AD treatment drugs, namely rivastigmine, donepezil, and memantine, there is a need for new investigations to identify novel drugs[67,68]. A vital biomarker discovery panel would be beneficial for this endeavour.

Conclusion

In alloxan-induced diabetic rats, we found that hyperglycemia and insulin resistance in the brain may connect urate turnover and alterations in neural regulatory genes. Our findings support recent research showing that a lack of or insensitivity to insulin may play a role in the development of AD in people with chronic diabetes. While there are many similarities between the brains of individuals with diabetes or AD, and diabetes represents an AD risk factor, additional research is needed to identify the exact mechanism behind the link between diabetes and AD.

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Conflict of interest

The authors declare that they have no conflict of interest.

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