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TLR4-dependent metabolic changes are associated with cognitive impairment in an animal model of type 1 diabetes

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ABSTRACT

We investigated the role of Toll-like receptor 4 (TLR4), a major mediator of innate immune responses, on cognitive performance in a type 1 diabetes model (T1D). After administration of streptozotocin, both TLR4 knockout (TLR4 KO) and wild type (WT) diabetic mice displayed metabolic alterations similar to those observed in T1D patients, including increased levels of glucose, cholesterol, triglycerides and ketones. T1D mice exhibited cognitive impairment which was less severe in TLR4 KO mice compared to WT mice. WT mice with higher glucose and those with higher triglyceride levels exhibited significantly more anxiety and impaired memory compared to those with lower levels of glucose and triglycerides; these correlations were absent in TLR4 KO mice. Additional findings suggest roles for TLR4 signaling in modifying the expression of enzymes involved in energy metabolism in brain cells in the setting of T1D. Our data show that TLR4 contributes to the negative impact of T1D on anxiety and cognition.

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1. Introduction

Diabetes and cognitive decline are common co-morbid conditions in the older population [1,2]. Diabetes is characterized by impaired insulin signaling due to hypoinsulinemia in case of type 1 diabetes (T1D), or by insulin resistance in type 2 diabetes [3]. Once known as juvenile diabetes to distinguish it from type 2 which usually has a later onset, it is now clear that the onset of T1D occurs even more frequently in adults than in children [4]. A recent longitudinal neuroimaging study found that hyperglycemia is associated with accentuated decrease in whole brain gray matter over a 2-year time period in children with T1D [5]. Similarly, T1DM adults showed diminished total brain volume compared to control subjects [6,7]. Postmortem studies have revealed increased neuronal loss [8] and cerebral cortex degeneration [9] in T1D patients compared to age-matched controls. Cognitive deficits such as impaired learning and memory, problem solving and mental flexibility are more common in T1D subjects than in the general population [10]. The overall worldwide rise of diabetes's prevalence has increased the focus on understanding its pathophysiology, yet very little is known about the underlying mechanisms linking diabetes to neurological dysfunction.

Although the cause of T1D is not fully understood, the destruction of insulin-producing β cells in the pancreas is believed to be of immunological origin [11]. Furthermore, inflammation is known to contribute to the initiation and progression of T1D and its complications [11]. Toll-like receptor 4 (TLR4) is a prominent component of the innate immune system, mediating inflammatory responses to different self- and non-self-ligands. Recently recognized as a modulator of neuronal survival during sterile injuries, TLR4 is also thought to play a role in central nervous system plasticity [12], as well as in learning and memory [13] and cognitive dysfunction in pathological settings [14].

Notably, elevated glucose levels can drive the upregulation of TLR4 in cultured immune cells [15], and TLR4 expression and intracellular signaling are increased in monocytes of T1D diabetic patients [16], and macrophages of diabetic mice [17].

In the present study we employed TLR4-deficient mice and a model of type 1 diabetes to elucidate the roles of TLR4 signaling in the adverse effects of diabetes on brain function and metabolism.

2. Methods

2.1. Animals and diabetes model

Adult 3–4 months old male TLR4 −/− (TLR4 KO) and +/+ (WT) mice (25–30 g) were kept under 12 h-light–dark cycle and allowed free access to food and water. Each mouse was fasted for 4 h before receiving a single intraperitoneal dose of 200 mg/kg of streptozotocin (STZ) (or citrate buffer (0.1 M)). Mice were provided 10%

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sucrose in their drinking water for the first day after STZ administration. Survival rates were 80% and 73% for WT and TLR4 KO mice, respectively. Levels of glucose were monitored using a glucometer (Abbott Diabetes Care, Inc, Alameda, CA), and only mice with fasting glucose levels $\geqslant$250 mg/dL two days after STZ administration were considered diabetic and included in the study. This research was approved by the National Institute on Aging Animal Care and Use Committee and was performed according to guidelines in the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Behavioral assessments

2.2.1. Elevated plus maze

Anxiety-like behavior was evaluated by recording the movement of the mice for 5 min in an elevated (60 cm) plus-shaped maze comprised of two open arms (25 × 5 cm) with a clear 1 cm wall, and two closed arms with 30 cm high dark walls. Each mouse was placed in the center of the maze facing the open arm. Arm preference was automatically analyzed using the ANYmaze video tracking software (Stoelting, Kiel, WI).

2.2.2. Fear conditioning

For the fear conditioning tests the mice were habituated to the testing room for five consecutive days. During the training session, mice were placed in a contextual conditioning chamber (model MED-VFC-NIR-M; Med Associates, Georgia, VT, USA) and allowed to explore for 2 min. The mice were subjected to sessions of a 30 s neutral discrete stimulus (conditioned stimulus, audio tone 6 kHz, 70 dB), followed by 2 s of a motivationally significant stimulus (unconditioned stimulus; foot shock, 0.5 mA). Each session of conditioned and unconditioned stimulus pairings was separated by 30 s. In the cued session mice were placed in the chamber in a different context and allowed to explore for 5 min before being subjected to five audio tones over a 5 min period. The percentage of time freezing was recorded and used as an indices of cued memory.

2.3. Biochemistry

Triglycerides, 3-hydroxybutyrate, and cholesterol concentrations were quantified in serum using a Roche Cobas Fara II analyzer (Roche Diagnostic Systems; Montclair, NJ) as described previously [18].

2.4. RNA extraction and real-time PCR

RNA from the tissue was isolated using Trizol (Invitrogen) and purified with an RNA Micro Kit (Qiagen, Valencia, CA). Following treatment with DNase I, RNA was quantified and equal amounts were retro-transcribed using the SuperScript First Strand Synthesis System (Invitrogen Life Technologies). Real-time PCR analysis was performed with a PTC 200 Pelthier Thermo Cycler and Chromo 4 Fluorescent Detector (BioRad, Hercules,CA), and Sybr® Green PCR Master Mix according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). The comparative Ct method was used to determine the normalized changes of the target gene relative to a calibrator reference.

2.5. Statistical analysis

Results are expressed as mean and S.E.M. of the indicated number of animals or experiments. Statistical comparisons were performed using Student's *t*-test or two-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test as suitable. To assess potential correlations between the biochemical variables and the anxiety and learning and memory behaviors, a Pearson correlation test was used. All analyses were performed using a Prism software package (Graphpad Software, San Diego, CA, USA).

3. Results

3.1. Type 1 diabetes model

In T1D patients the inability to utilize glucose results in hyperglycemia, increased hunger, thirst, urination, and weight loss. We recapitulated T1D in mice by administration of streptozotocin (STZ) which selectively eliminates pancreatic beta cells. As expected, following STZ we observed significant increases in fasting glucose levels ($F_{(9,180)} = 35.6$), and weight loss ($F_{(6,139)} = 13.7$) that were similar in both WT and TLR4 KO mice (Fig. 1A and B). In addition to hyperglycemia, the STZ-treated animals also manifested other biochemical changes typically observed in T1D, including elevated levels of circulating cholesterol ($F_{(1,41)} = 13.8$), triglycerides ($F_{(1,38)} = 27.25$) and the ketone 3-hydroxybutyrate ($F_{(1,38)} = 23.2$) (Fig. 1C). No interaction was found between treatment and genotype for any of the metabolic parameters. Interestingly, in the STZ treated TLR4 KO mice the levels of triglycerides were lower compared to STZ treated WT mice (Fig. 1C), suggesting a better metabolic control of T1D in the absence of TLR4.

3.2. T1D hyperglycemia correlates with increased anxiety

While peripheral consequences of the T1D on metabolism and associated disease processes have been extensively studied, very little is known about their contribution to the psychological and cognitive abnormalities that are often a complication of diabetes. We first assessed anxiety levels by testing the mice in the elevated plus maze. We found an interaction between treatment and genotype ($F_{(1,48)} = 5.66$ $p = 0.02$), with WT diabetic mice spending less time in the open arm, a sign of increased anxiety (Fig. 2A). When we analyzed the relationship between the different metabolites and the behavioral phenotype we discovered that WT mice with higher levels of glucose ($r = -0.43$; $F_{(1,21)} = 4.68$ $p = 0.04$), and triglycerides ($r = -0.47$; $F_{(1,19)} = 5.41$ $p = 0.03$) were those with higher anxiety levels (Fig. 2B). Although not significant, a similar correlative trend was observed for 3-hydroxybutyrate, a product of triglyceride catabolism ($r = -0.34$; $F_{(1,19)} = 2.50$ $p = 0.13$), while levels of cholesterol were not correlated with anxiety behavior($r = 0.02$; $F_{(1,19)} = 0.007$ $p = 0.94$) (Data not shown). On the other hand, in TLR4 KO mice the STZ treatment did not modify the time spent in the open arm (Fig. 2A) and the correlations between metabolic parameters and anxiety seen in WT mice were absent (Fig. 2C) [glucose ($r = -0.01$; $F_{(1,20)} = 0.004$ $p = 0.95$); triglycerides ($r = 0.09$; $F_{(1,19)} = 0.17$ $p = 0.69$); ketone bodies ($r = 0.007$; $F_{(1,19)} = 0.001$ $p = 0.97$); cholesterol ($r = 0.08$; $F_{(1,20)} = 0.13$ $p = 0.72$)], suggesting that TLR4 signaling negatively modulates brain metabolism and contributes to the anxiety phenotype associated with T1D.

3.3. Evidence that cognitive impairment in T1D mice is mediated by TLR4

Learning and memory were tested using the fear conditioning paradigm. We have previously shown that TLR4 KO mice have a better spatial memory compared to WT mice [13]. This together with anxiety and generalized non associative freezing may influence the contextual portion of the text, we thus focus on amygdala-dependent memory. In the cued portion of the test, diabetic WT mice responded to the sound by freezing less than their non-diabetic siblings ($F_{(1,40)} = 43.4$ $p < 0.001$) (Fig. 3A). This memory impairment negatively correlated with levels of glucose

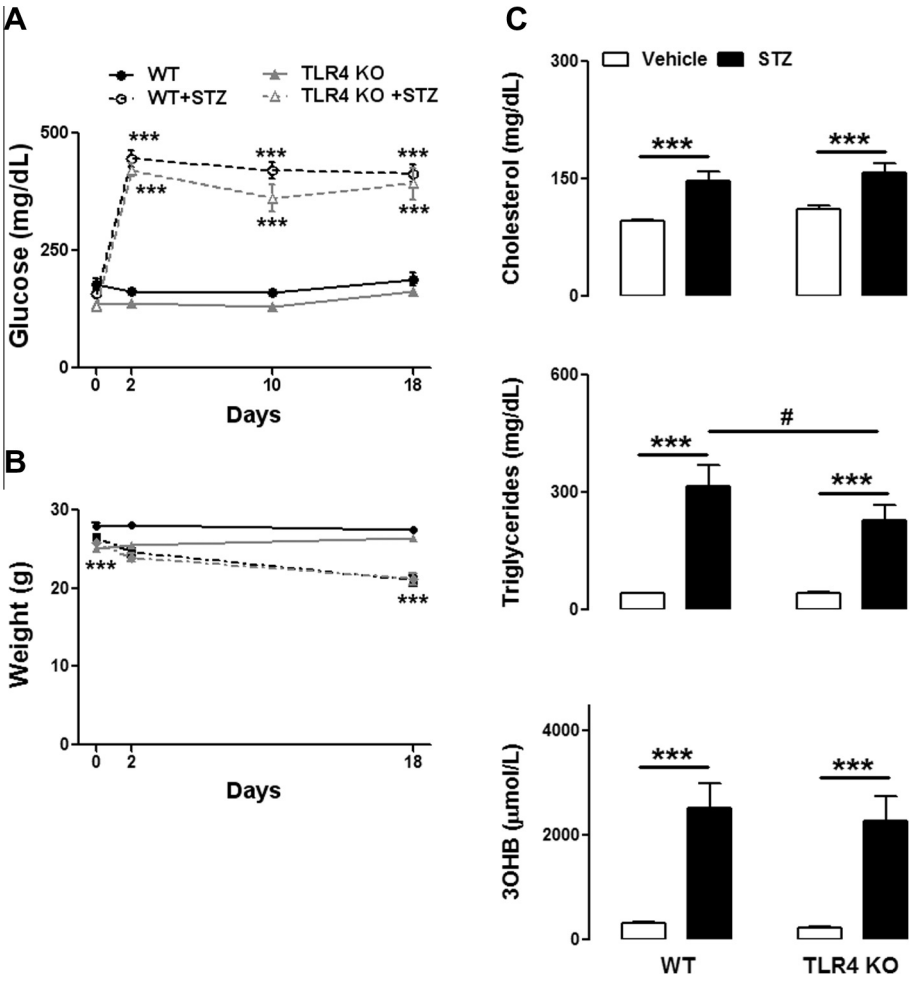

Fig. 1. Changes in metabolic parameters and body weight in STZ-induced diabetes. WT and TLR4 KO mice were injected with a single dose of STZ to induce a type 1 diabetic phenotype. (A) Glucose levels were significantly increased within two days after STZ administration and remained elevated throughout the study. In both genotypes the development of diabetes was associated with weight loss (B), and increases of circulating cholesterol (C), triglycerides (D) and ketone bodies (E). $^{***}p < 0.001$ versus Vehicle; $^{\#}p < 0.05$ versus WT + STZ ($n = 9–13$; ANOVA).

($r = -0.58$; $F_{(1,21)} = 10.69$ $p = 0.004$) and ketones ($r = -0.50$; $F_{(1,17)} = 5.76$ $p = 0.028$) (Fig. 3B). On the other hand, diabetes did not modify the behavioral response in TLR4 KO mice (Fig. 3A), and the relationships between metabolic alterations and freezing time seen in WT animals were absent [glucose ($r = -0.002$;$F_{(1,20)} = 0.0001$ $p = 0.99$), ketone bodies ($r = 0.25$; $F_{(1,18)} = 1.22$ $p = 0.28$)] (Fig. 3B).

3.4. TLR4 signaling negatively influences adaptive responses of brain cells to diabetes

Given the differences in anxiety and cognitive performance in WT and TLR4 KO mice we measured levels of mRNAs encoding proteins involved in inflammation and cellular energy metabolism in cerebral cortex tissue samples from control and diabetic WT and TLR4 KO mice. As expected, TLR4 mRNA was completely absent in TLR4 KO mice (Fig. 4A). Levels of TLR4 mRNA were unaffected by diabetes in WT mice (Fig. 4A). The mRNA levels of tumor necrosis factor α (TNFα) were similarly upregulated by T1D in WT and TLR4 KO mice (Fig. 4A). Levels of interleukin 1β and MCP-1 mRNAs were unaffected by diabetes in WT mice, and there was a trend towards higher levels of MCP-1 mRNA in diabetic TLR4 KO mice (Fig. 4A). Glucose is the predominance source of energy for neurons, and yet under particular circumstances, the brain can undergo adaptive changes that enhance its ability to utilize alternate fuel sources, such as monocarboxylic acids, lactate and ketones. We tested the mRNA levels of key enzymes for the utilization of glucose and ketones bodies. The mRNA levels of pyruvate kinase (PK) which catalyzes the formation of pyruvate in the last step of glycolysis, were slightly elevated by T1D in WT mice, but reached higher significant levels in TLR4 KO mice (Fig. 4B). Furthermore, mRNA levels of both lactate dehydrogenase (LDH) isoforms A and B were maintained in T1D TLR4 KO mice, while a significant decrease was observed for LDH-A in T1D WT mice (Fig. 4B), indicating reduced production of lactic acid in astrocytes. Regarding ketone body metabolism, in the absence of TLR4 mRNA levels of acetyl-CoA-acetyltransferase 1 (ACAT), indispensable for ketolysis, were elevated in cerebral cortex of T1D TLR4 KO mice compared to T1D WT mice (Fig. 4B). Finally, TLR4 deficiency did not impact mRNA levels of the neuronal monocarboxylate transporter 2 (MCT-2), but did prevent a significant reduction in mRNA levels of the astrocytic form 1 (MCT1) in T1D mice (Fig. 4B).

4. Discussion

We found that the metabolic alterations caused by T1D are associated with impaired memory, as well as an anxiety phenotype. Notably, our data suggest TLR4 signaling has a negative

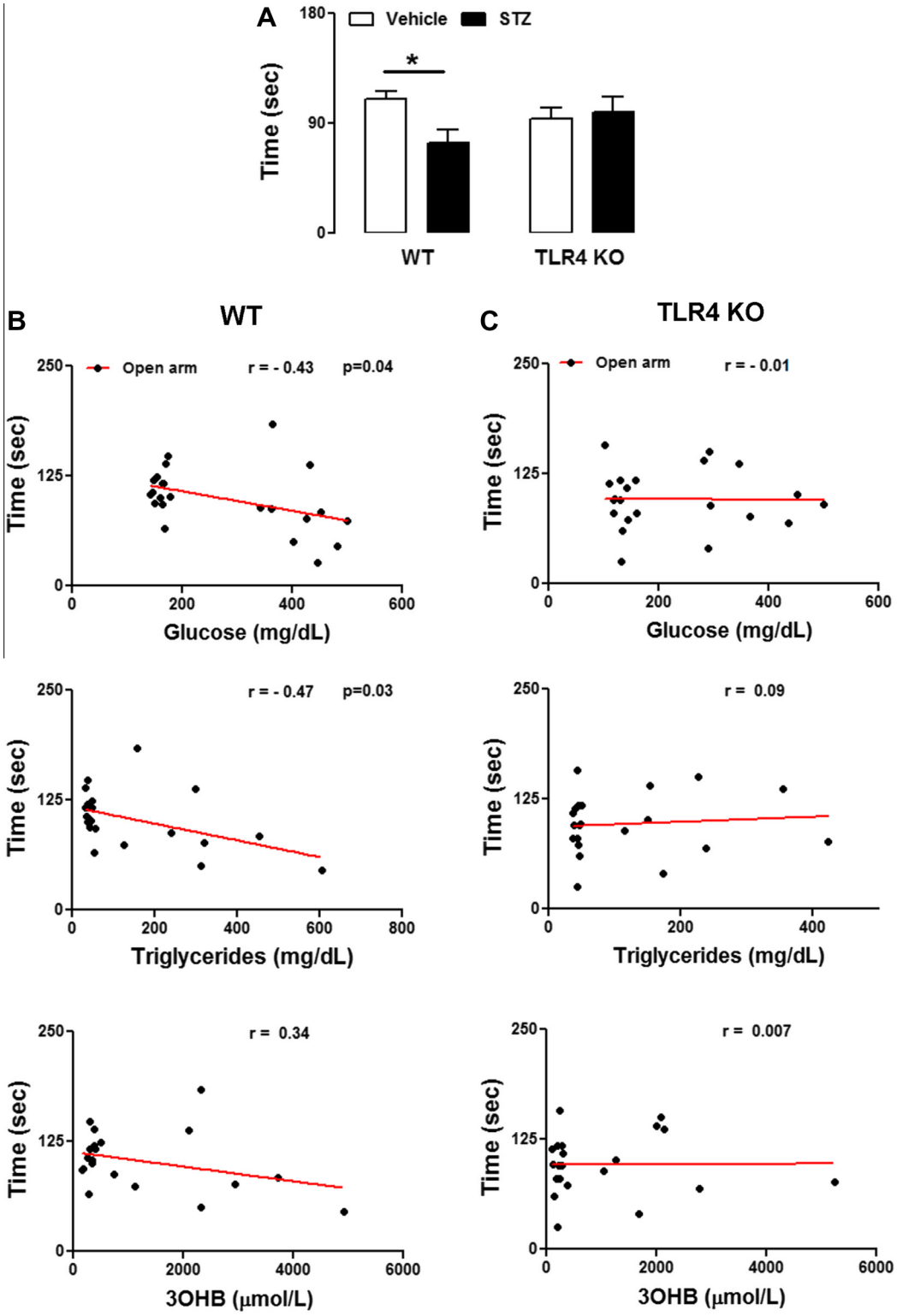

Fig. 2. Hyperglycemia and ketosis are associated with increased anxiety. Anxiety-like behavior was assessed by elevated plus maze testing. (A) STZ treated WT mice spent significantly less time in the open arm. *$p < 0.05$. In WT mice (column B) but not in TLR4 KO mice (column C) the time spent in the open arm was associated with the increased levels of glucose and triglycerides, but not ketone bodies.

influence on both cognitive outcomes in diabetic mice. In the absence of TLR4, T1D resulted in preserved expression levels of the astrocytic monocarboxylate transporter 1, increased expression of ACAT (a key enzyme in ketolysis), while the changes in glycolytic enzymes suggest a preserved ability to produce lactate in brain cells of TLR4 KO mice compared to WT mice.

The brain requires a constant and substantial energy supply to maintain its function, and glucose is the preferred energy substrate for brain cells. Several studies have demonstrated clear energy metabolism differences between neurons and astrocytes. Although neurons do exhibit glycolytic activity, their metabolism is mostly oxidative and can utilize substrates other than glucose provided

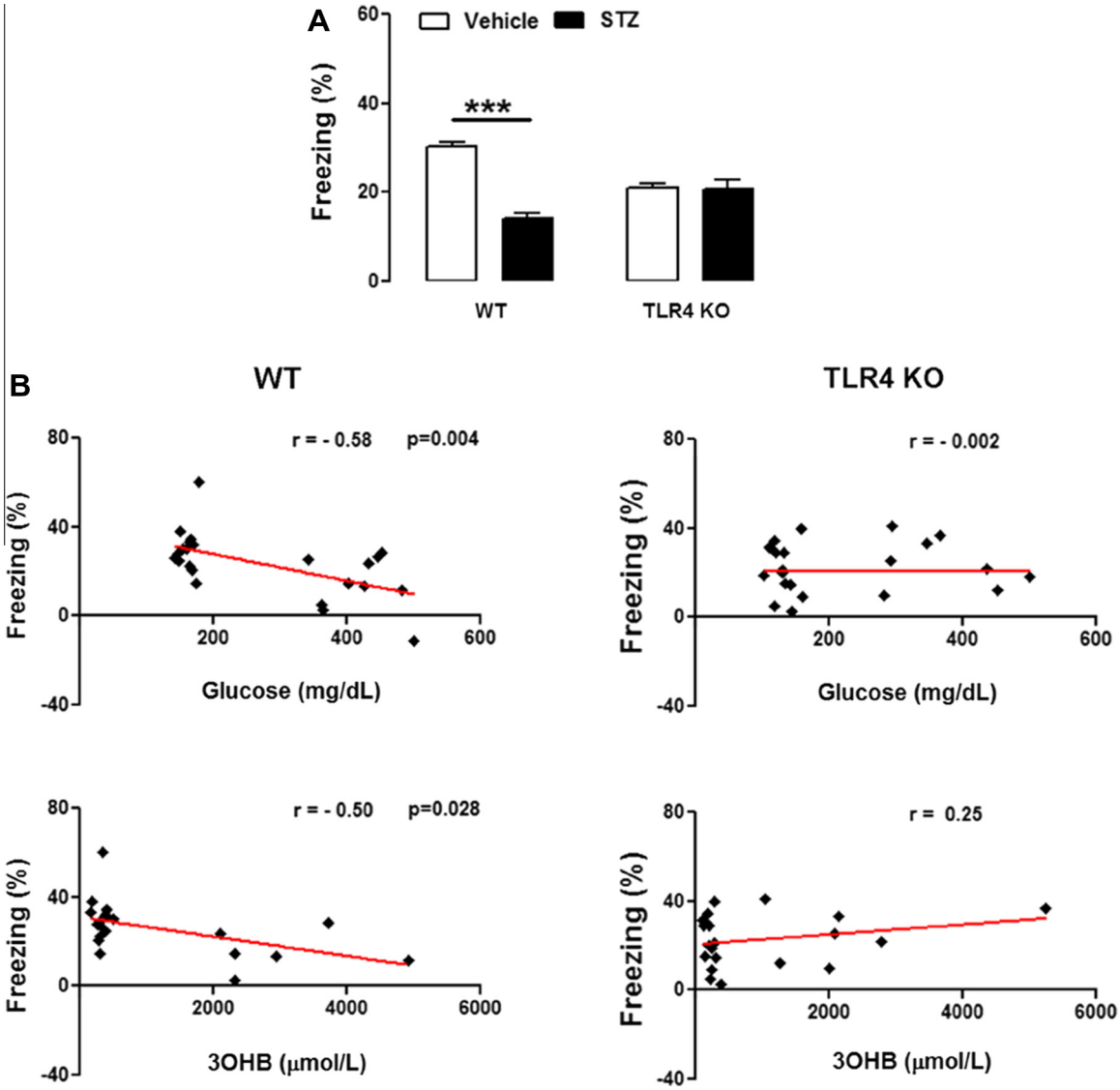

Fig. 3. Associations between cognitive function and energy metabolism involved TLR4 signaling. In WT mice diabetes decreased tone-cued fear reaction (A), and the freezing impairment was negatively correlated with glucose and ketone body levels (B) $^{***}p < 0.001$. ($n = 9–13$ ANOVA).

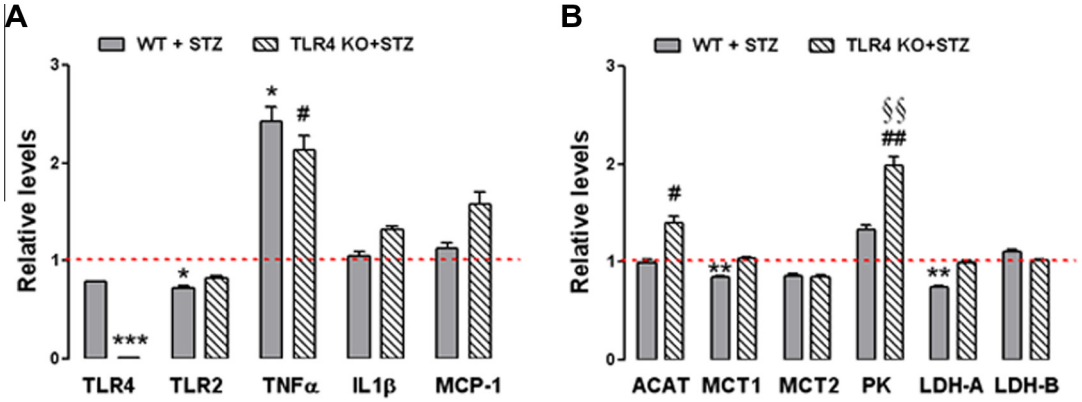

Fig. 4. TLR4 modifies T1D-induced changes in the expression of genes encoding proteins involved in cellular energy metabolism in the brain. Relative levels of mRNAs for the indicated TLRs, cyto- and chemo-kines (A), and metabolic enzymes and transporters (B) were measured in cerebral cortical tissue from normoglycemic and hyperglycemic WT and TLR4 KO mice. $^{*}p < 0.05$ versus WT; $^{**}p < 0.01$ versus WT; $^{***}p < 0.01$ versus WT; $^{\#}p < 0.05$ versus TLR4 KO; $^{\#\#}p < 0.01$ versus TLR4 KO; $^{\S\S}p < 0.01$ versus WT + STZ ($n = 5–9$; ANOVA).

by astrocytes (i.e. monocarboxylic acids, lactate, ketones) [19]. It was recently shown that enhancement of glycolysis in neurons can actually be detrimental [20]. Under hyperglycemic conditions, the increased utilization of glucose through glycolysis and the oxidative polyol pathway leads to osmolarity changes, impairment of the glutathione cycle, increased oxidative stress and non-enzymatic protein glycation, which can result in cell death [21]. On the other hand, in astrocytes glycolysis is the predominant metabolic pathway and its enhancement is favored by the low expression of an essential component of the malate-aspartate shuttle in astroglial mitochondria [22]. Furthermore, astrocytes can also oxidize fatty acids to generate ketones, and the metabolism of ketones results in lower production of reactive oxygen species and reduced mitochondrial stress compared to glycolysis [21].

Our results showing that WT mice with T1D exhibit heightened anxiety and impaired cognitive function are consistent with the idea that sustained hyperglycemia can lead to neuronal dysfunction and ultimately to neurotoxicity. They are also in agreement with previous findings showing that in humans the hippocampus is particularly vulnerable to high glucose levels [23,24]. Our finding that the negative association between glucose levels and cognitive performance depended upon the presence of functional TLR4 signaling in the absence of differences in pro-inflammatory cytokine expression, suggests that TLR4 may function as a metabolic regulator in an inflammation-independent fashion. Peripheral changes in TLR4 expression have been observed in T1D [16], T2DM [25], and metabolic syndrome patients [26]. While the mechanism by which TLR4 affects peripheral metabolism have begun to be elucidated during the past decade, nothing is known about roles of TLR4 in central nervous system metabolism and function in T1D. Peripherally, TLR4 activation decreases fatty acid ß-oxidation [27], enhances glycolysis, attenuates oxidative metabolism in mitochondria and promotes generation of ROS [28]. Our data suggest that similar mechanisms may also take place in the brain cells, leading to impaired cognitive function. Indeed, studies of cell culture and mouse models of ischemic neuronal injury suggest that TLR4 signaling renders neurons vulnerable to dysfunction and degeneration in a setting of impaired energy metabolism [29].

Our findings suggest that the effects of T1D on the brain include increased glycolysis, and that the TLR4 signaling pathway promotes increased oxidative metabolism relative to anaerobic lactate production. Neurons express LDH1 (LDH-B tetramer) which essentially converts lactate to pyruvate to fuel mitochondrial oxidation, while astrocytes express LDH5 (LDH-A tetramer) which converts pyruvate to lactate. Lactate can be taken up by neurons via MCT and is then oxidized to produce ATP [30]. When neurons are supplied both glucose and lactate they preferentially oxidize lactate due to the fact that the competition between LDH and glycolytic glyceraldehyde-3-phosphatase for cytosolic NAD^+ favors LDH [31]. Both memory formation and long-term plasticity are impaired by interference with MCTs, and such impairments can be reversed by administration of lactate but not glucose [32]. Notably, we found that levels of MCT-1 expression in the cerebral cortex were preserved in T1D in absence of TLR4 signaling. MCT-1 is expressed in endothelial cells and astrocytes [33], while MCT-2 is present in neurons [34]. MCT-1 is fundamental for the transfer of lactate from astrocytes to neurons [19], for long term memory formation [32], and for astrocytic uptake of ketone bodies from the blood stream [35].

Ketone bodies are an alternate neuronal fuel source associated with negligible reactive species formation [21]. In ketotic states resulting from starvation, diabetes, fat-feeding or ketogenic diets, cerebral utilization of ketones is increased in direct proportion to the degree of ketosis [36]. Ketogenic diets can afford neuroprotection in various neurodegenerative conditions, most notably severe epileptic seizures [37]. Interestingly, administration of a TLR4 antagonist in mouse epileptic models has also been shown to decrease acute and chronic seizure recurrence [38]. Oral administration of medium-chain triglycerides increases ketone production and improves cognition function in T1D patients [39]. In addition to the TLR4-dependent modification of MCT-1 corroborating a diminished ability to uptake and possibly shuttle ketones to neurons, we also observed modifications in ACAT levels. ACAT catalyzes the last step of fatty acid and ketone catabolism by converting acetoacetyl-CoA into two molecules of acetyl-CoA which in turn feeds into the Kreb's cycle and oxidative phosphorylation to generate ATP in mitochondria. ACAT is highly expressed in hippocampus, amygdala and cortex, areas that play major roles in cognition and anxiety. Although present in neurons ACAT is predominantly expressed in astrocytes, a cell type capable of β-oxidation of fatty acids, and to convert fatty acids to ketones that are then exchanged with neurons [40].

In conclusion, our findings suggest that activation of the TLR4 signaling pathway in T1D suppresses the ability of the brain to utilize alternate fuel sources resulting in impaired cognitive functions. Therefore, therapeutic strategies aimed at decreasing TLR4 signaling may not only reduce diabetic peripheral complications, but may also ameliorate its neurological complications.

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