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Hyperglycemia-driven neuroinflammation compromises BBB leading to memory loss in both diabetes mellitus (DM) type 1 and type 2 mouse models

Slava Rom^{1,2}, Viviana Zuluaga-Ramirez¹, Sachin Gajghate¹, Alecia Seliga¹, Malika Winfield¹, Nathan A. Heldt¹, Mikhail A. Kolpakov³, Yulia V. Bashkova³, Abdel Karim Sabri³, and Yuri Persidsky^{1,2}

¹Department of Pathology and Laboratory Medicine, Temple University, Philadelphia, PA 19140, USA

²Center for Substance Abuse Research, Temple University, Philadelphia, PA 19140, USA

³Cardiovascular Research Center, Lewis Katz School of Medicine, Temple University, Philadelphia, PA 19140, USA

Abstract

End organ injury in diabetes mellitus (DM) is driven by microvascular compromise (including diabetic retinopathy and nephropathy). Cognitive impairment is a well-known complication of DM types 1 and 2; however, its mechanism(s) is(are) not known. We hypothesized that blood brain barrier (BBB) compromise plays a key role in cognitive decline in DM. Using a DM type 1 model (streptozotocin injected C57BL/6 mice) and type 2 model (leptin knockout obese db/db mice), we showed enhanced BBB permeability and memory loss (Y-maze, water maze) that are associated with hyperglycemia. Gene profiling in isolated microvessels from DM type 1 animals demonstrated deregulated expression of 54 genes related to angiogenesis, inflammation, vasoconstriction/vasodilation, and platelet activation pathways by at least 2-fold (including eNOS, TNF α , TGF β 1, VCAM-1, E-selectin, several chemokines and MMP9). Further, the magnitude of gene expression was linked to degree of cognitive decline in DM type 1 animals. Gene analysis in brain microvessels of DM type 2 db/db animals showed alterations of similar genes as in DM 1 model, some to an even greater extent. Neuropathologic analyses of brain tissue derived from DM mice showed microglial activation, expression of ICAM-1 and attenuated coverage of pericytes compared to controls. There was a significant upregulation of inflammatory genes in brain tissue in both DM models. Taken together, our findings indicate BBB compromise in DM *in vivo* models and its association with memory deficits, gene alterations in brain endothelium and

Corresponding authors: Yuri Persidsky, MD, PhD, 3500 N. Broad St., Department of Pathology & Laboratory Medicine, MERB, room 840, Philadelphia PA, 19140. Phone: (215) 707-4353; Fax: (215) 707-2781, yuri.persidsky@tuhs.temple.edu, Slava Rom, PhD, 3500 N. Broad St., Department of Pathology & Laboratory Medicine, MERB, room 880A, Philadelphia PA, 19140. Phone: (215) 707-2543; Fax: (215) 707-5255, srom@temple.edu.

Author contributions

VZR, SG, AS, MW, NAH, MAK, YVB, AKS - data acquisition and analysis, drafting, revising and final approval article. SR and YP - conception and design, data acquisition, analysis and interpretation, drafting and revising article, and final approval.

Disclosure/Conflict of Interest

None

neuroinflammation. Prevention of BBB injury may be a new therapeutic approach to prevent cognitive demise in DM.

Keywords

BBB; endothelial dysfunction; diabetes; dementia; inflammation

Introduction

Development of cognitive impairment/dementia and its links to various pathologic conditions remains elusive. A number of underlying mechanisms/causes has been proposed including genetic defects in Alzheimer's disease (AD) and Parkinson's disease, metabolic dysfunctions (diabetes, homocysteinemia, toxic environmental factors, effects of drugs, etc.), hypertension, chronic neuroinflammatory conditions (autoimmune or infectious origin), and traumatic brain injury. Yet, up to 40% of these conditions converge on defects in the brain microvasculature[1] and its coupling to neuronal functions resulting in hypo-perfusion and eventual neuronal demise[2]. Rapid progress with brain imaging, allowing evaluated perfusion in conjunction with structural changes and neuronal function, speed such studies. Understanding of the mechanisms of such changes is of high significance due to potential of harnessing novel therapeutic interventions. It is fully applicable to cognitive demise in patients suffering from diabetes mellitus (DM) type 1 or type 2[3–5]. DM is a metabolic disease related to the inability to produce insulin and proper utilization of glucose (type 1) or insulin resistance when this hormone is produced but cannot interact with its receptors (type 2). Both conditions result in high glucose levels in blood, enhanced generation of reactive oxygen species (ROS) and end organ injury stemming from microvascular abnormalities[6]. Examples of such well-documented DM complications are cardiovascular disease, nephropathy and retinopathy all stemming from microvascular injury. The relationship between microvascular changes and cognitive decline in DM had not been substantiated until very recently, including blood perfusion, neuronal function, white matter microstructure, and metabolic function[7]. Overall small vessel abnormalities documented in DM include white matter lesions, microbleeds, silent brain infarcts and lacunar abnormalities, indicative of cognitive decline[8].

Besides changes in blood supply, another important factor in DM is increased permeability of the blood brain barrier (BBB)[9]. The BBB is composed of highly specialized brain microvascular endothelial cells (BMVEC) connected by tight junctions (TJ) with very low pinocytotic activity and uniquely expressed transporters. The function of BMVEC is supported by pericytes enveloping the abluminal side of brain endothelium[10]. Proper function of the BBB ensures protection of the central nervous system (CNS) unique milieu and restricts penetration of toxic substances across the barrier, while providing an adequate supply of nutrients. Disruption of the BBB and its association with cognitive decline has been recently demonstrated in DM patients[11] and was associated with increased levels of vascular dysfunction markers in cerebrospinal fluid (CSF). Indicators of BBB injury have better predictive power for dementia development than genetic hallmarks of AD. Further, memory deficits have been documented in animal models of DM[12], which showed BBB

dysfunction[13] paralleling a loss of pericytes and neuronal dysfunction. Causes for vascular dysfunction in DM are related to heightened generation of ROS due to enhanced glucose metabolism, subsequent buildup of glycolytic intermediates, advanced glycation end products (AGEs) and activation of protein kinase C (PKC)[14,15]. These events eventually may result in NF- κ B activation, overexpression of adhesion molecules (VCAM-1, ICAM-1), activation of the small GTPase, RhoA and diminished expression of TJ proteins[16]. The relationship between glucose levels and cognitive decline is complex. In humans, higher blood glucose levels were associated with worse cognitive performance[17,18], while a recent report suggested that a history of severe hypoglycemic episodes was associated with a greater risk of dementia[19]. However, the exact mechanisms of injury, differences between DM type 1 and 2, and the therapeutic potential of BBB protective strategies in cognitive decline are currently unknown. Here, we explored the hypothesis that DM types 1 and 2 decrease BBB integrity directly (via effects on brain endothelium and pericytes) and promote a pro-inflammatory phenotype of brain endothelium (resulting in low level inflammation) that further exacerbates barrier injury. We used animal models of DM types 1 and 2 and showed increased BBB permeability that are associated with hyperglycemia. Importantly, these animals demonstrated cognitive decline paralleling BBB injury. Profiling of genes in CNS microvessels isolated from these animals showed upregulation of pro-inflammatory and barrier destabilizing molecules. The magnitude of their expression interrelated with cognitive abnormalities. Neuroinflammatory responses in both animal models were associated with a decrease of pericyte BBB coverage. Our report provides evidence from animal models for the causes of vascular dementia, and might lead to development of future therapeutics to diminish its burden.

Materials and Methods

Animals

C57BL/6 mice (10 week old male) as well as leptin deficient mice db^{+}/db^{+} (further db/db) and leptin heterozygous db^{+}/db^{-} (further $db/hemi$) were acquired from the Jackson Laboratory (Bar Harbor, ME). To achieve statistical significance in each experiment, mice were divided into groups of 6 to 10 animals (exact numbers for each experiment are indicated in figure legends).

All *in vivo* experiments were approved by the Temple University Institutional Animal Care and Use Committee in accordance with guidelines based on the National Institutes of Health (NIH) guide for care and use of laboratory animals and ARRIVE (Animal Research: Reporting *In Vivo* Experiments) guidelines (www.nc3rs.org.uk/arrive-guidelines).

Induction of diabetes

Diabetes type 1 was induced as described[20]. In short, C57BL/6 mice (10 week old, 25–30 g body weight) were randomly divided into groups. One group received once daily intraperitoneal (ip) injection of streptozotocin (STZ) for five consecutive days (50 mg/kg in citrate buffer, pH 4.5, freshly made every day). Control group mice received citrate buffer only. The first day of STZ injection was assigned as the starting time for diabetes. Serum glucose concentrations were monitored on 7 days, 4, 8 and 12 weeks after the start. Blood

glucose levels (BGL) were determined by glucose analyzer (Bayer Contour®, Ascensia Diabetes Care, Parsippany, NJ), according to manufacturer's instructions.

Y maze exploratory memory test

Spontaneous exploratory behavior was investigated by using a Y-maze as described[21–23]. The Y-maze apparatus consists of 3 arms, each 21 cm long, 4 cm wide, and 40 cm tall with a central entry zone. It is made of plastic and placed at an angle of 33°. Control and STZ-injected mice or db/db mice were placed individually in the central entry zone and allowed to freely explore the apparatus for 5 minutes while being video-taped. The total number of arm entries and alterations between the arms are recorded. Frequent exploration in all 3 arms indicates normal spontaneous exploration whereas restricted arm entries indicate deficits in spontaneous exploration. Alternations were defined as successive entries into each of the three arms as on overlapping triplet sets (i.e., ABC, BCA, ...). Percent of spontaneous alternation was defined as the ratio of actual (total alternations) to possible (total arm entries – 2) number of alternations $\times 100$. Alternate arm returns (AARs) and same arm returns (SARs) were also scored for each animal in order to assess aspects of attention within spontaneous working memory[23]. Total entries were scored as an index of ambulatory activity in the Y-maze. Mice showing scores below six entries were excluded. A global analysis of data was made using ANOVA with the Dunnett test post-hoc comparisons, whereas comparisons between two groups for individual time points were done using the unpaired Student *t*-test.

Morris Water Maze

The Morris Water Maze (MWM) was first established by neuroscientist Richard G. Morris in 1981 in order to test hippocampal-dependent learning, including acquisition of spatial memory and long-term spatial memory[24]. The theory behind it is that the animal must learn to use local cues to navigate the shortest path to the hidden platform when started from different, random locations around the perimeter of the water tank. Most protocols use four start locations: N, S, E and W. Animals are given a series of daily trials using a semi-random set of start locations. Semi-random start position sets are most common, such that the four positions are used, with the restriction that one trial each day is from each of the four positions. Mice were randomly selected to control (n=8) and STZ groups (n=20). The MWM test was performed 12 weeks after STZ injections. On the first day of MWM test, each mouse was allowed to swim freely in water for 2 min where the platform had been removed, observing the swim action and speed of the mouse in order to eliminate unqualified animals. On the next day, each rodent was discharged into the water with a platform placed inside from a marked starting point in one of the four quadrants, facing the tank wall, encompassing training for 3 days. Mice were assessed in the probe trial 24 hours after the last training session that consisted of a 60-second free swim in the pool without the platform. Each animal's performance was monitored using the Any-Maze™ Video Tracking System (Stoelting Co., Wood Dale, IL), which provided data for the acquisition parameters (latency to find the platform and distance swam) and the probe-trial parameter (number of entries to the target platform zone and time in quadrants). For the better assessment of learning ability of the mouse, a statistical analysis was made of the latency to find the platform and the number of repeated entries after first entry.

In Vivo Permeability Assay

Changes in BBB permeability were assessed using the fluorescent tracer, sodium-fluorescein (Na-F) as described[25,26]. Briefly, to prevent blood clotting in the vessels, animals were injected ip with heparin (20 U) followed intravenous (iv) injection of 50 μ l of 2% sodium fluorescein (NaF) in PBS. The tracer was allowed to circulate for 30 min. The mice were anesthetized and then transcardially perfused with PBS until colorless perfusion was visualized. The animals were then decapitated and the brains quickly isolated. After removal of the olfactory bulbs, cerebellum, and brain stem, the tissue was weighed and homogenized in 10 times volume of 50% trichloroacetic acid. The homogenate was centrifuged for 10 min at $13000 \times g$ and the supernatant was neutralized with 5 M NaOH (1:0.8). The amount of Na-F was measured using a Synergy 2 plate reader (BioTek, Winooski, VT). Fluorescent dye content was calculated using external standards; data are expressed as amount of tracer per mg of tissue.

Mouse brain tissues

Microscopic examination of the brains used a standardized protocol with sections from the neocortex (frontal and parietal), basal ganglia, hippocampus, midbrain, pons, medulla, and cerebellum. Paraffin sections (5 μ m) were stained with hematoxylin-eosin. One section from the frontal cortical lobe was used for the evaluation of neuroinflammation and BBB structure by immunohistochemistry using the following antibodies: IBA (1:100, Wako Chemicals USA, Richmond, VA) for microglia activation, claudin-5 (1:50, Thermo Fisher Scientific, Waltham, MA) for endothelial cells, occludin (1:50, Novus Biologicals, Littleton, CO) and CD13 for pericytes (1:1000, R&D Systems, Minneapolis, MN). Primary antibodies were detected by Dako Envision Kit (Dako, Carpinteria, CA) or Vectastain Elite Kit (Vector Laboratories, Burlingame, CA) with either DAB (single stain) or Vector Red substrate (for double stains). Samples were imaged under 20 $\times$ objective magnification using a DS-Fi1c camera (Nikon Corporation, Tokyo) configured to an upright microscope (i80 Eclipse, Nikon). Immunostains for CD13/Iba1 were assessed in blinded fashion using a semi-quantitative scoring protocol: 0 - negative staining, 1 - mild positivity (<10% of cells), 2 - moderate positivity (10–75%), 3 – strong positivity (75%) [27]. CD13 assessment was limited to capillaries (no more than 6 μ m in diameter). For each animal, 4–6 randomly selected fields in the cortex were analyzed. Immunostains for claudin-5 and occludin were evaluated in blinded fashion using semi-quantitative scoring: 0 – no staining, 1 – weak staining (<10% of capillaries), 2 – moderate, variable labeling (10–75% capillaries), 3 – strong, variable staining (>75% capillaries) and 4 – strong, uniform staining (95%).

Brain microvessel isolation

Mouse brain microvessels (BMVs) were isolated using a modified protocol based on previously published studies[28,29,25]. In short, mice were overdosed with CO₂ and their brains harvested. All following steps were carried out on ice (or at 4°C). Following a wash in phosphate-buffered saline, the brains were homogenized using a dounce homogenizer (0.25 mm clearance) (whole brain is defined as the S0 fraction; the nomenclature of S0, S1, and S5 describes the BMVs fractionation steps consistent with the BMVs isolation procedure as described below and previously by Yousif[29]. Overall, 15 mL of 30% Ficoll was added to

10 mL of the homogenate and mixed thoroughly. The resulting density gradient was centrifuged at $5,800 \times g$ for 20 minutes (the pellet is defined as S1 fraction). The pellet was resuspended in 1 mL phosphate-buffered saline with 1% bovine serum albumin and passed through a glass bead column, with 100 μ m nylon mesh filter on the top and a 40- μ m nylon mesh filter at the bottom. The glass beads were gently agitated in phosphate-buffered saline with 1% bovine serum albumin to obtain BMVs. The resulting sample was washed with bovine serum albumin-free phosphate-buffered saline before use in experiments and is defined as the S5 fraction.

PCR array and qPCR

Total RNA was extracted from FFPE paraffin-embedded (FFPE) cortical tissues (same area as for IHC) using RecoverAll™ Total Nucleic Acid Isolation Kit[30–33] and from BMVs using PARIS™ RNA Isolation Kit (both ThermoFisher Scientific). Total RNA (200 ng) was converted to cDNA using RT² PreAMP cDNA Synthesis Kit (Qiagen, Hilden, Germany). A PCR-based microarray assay for evaluating the expression of genes involved in inflammatory response and autoimmunity used RT²-profiler PCR Array (PAMM-077Z) and endothelial cell biology RT²-profiler PCR Array (PAMM-015Z) (SA Biosciences Corp., Frederick, MD)[26]. Profiling was performed for cDNA from two separate experiments. Data were scrutinized using a web-based analysis tool (SA Biosciences/Qiagen). Specific primers and probes for LT α , CD40lg, Myd88, and SLC2A1 (Glut1) genes were obtained from ThermoFisher Scientific and analyses were executed using the StepOnePlus real-time PCR system (ThermoFisher Scientific). Amplification was examined using the Ct method, using a web-based data investigation tool (SABiosciences/Qiagen) by normalization to housekeeping genes and fold-change calculated from the difference between experimental condition and untreated control. Fold changes of <2 or >2 were considered significant. Data are presented from two independent experiments and each group is comprised of 3–4 individual mice. To eliminate biased data, two people performed analysis in blinded fashion. Pathway analysis was performed by commercially available Ingenuity® Pathway Analysis (IPA®) system tools (Qiagen Bioinformatics, Redwood City, CA).

Soluble receptor for Advanced Glycosylation Endproducts (AGEs) detection

Soluble receptor for AGEs (sRAGE) was measured in mouse serum utilizing a commercially available ELISA kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. Data are presented as triplicates for each mouse and groups were comprised of 3–14 individual mice.

Statistical Analysis

Results are expressed as the mean $\pm$ SEM of experiments conducted multiple times. Multiple group comparisons were performed by one-way analysis of variance with Dunnet's posthoc tests. Statistical analyses were performed utilizing Prism v5 software (GraphPad Software Inc., La Jolla, CA). Differences were considered significant at P values < 0.05 .

Results

High blood glucose is associated with memory impairment in both type 1 and type 2 DM models

A summary of body weights and glucose readings is provided in Table 1. C57-STZ treated mice displayed a 3-fold increase in fasting blood glucose levels (BGL) and a significant decrease in body weight, mimicking body weight decline in humans with diabetes type 1[34]. Db/db mice presented much higher BGL, 4.9- and 1.35- fold *vs.* C57-STZ-treated mice, respectively. Db/db mice also showed significant obese phenotype, being 1.55-fold heavier than their heterozygous counterparts, characteristic of DM type 2 patients[35].

In order to evaluate cognitive function in DM models, we used two well-established tests, Y-maze and Morris water maze (MWM). The Y-maze spontaneous alternation paradigm is based on the ordinary tendency of rodents to explore a novel environment[23]. When positioned in the Y-maze, mice will explore the least recently visited arm, and thus tend to alternate visits between the three arms. An animal with an impaired working memory cannot remember which arm it has just visited, and thus shows decreased spontaneous alternation[21–23]. In Y-maze tests, mice treated with STZ (12 weeks prior to test) showed significant ($p<0.001$ *vs.* NT) reduction of $17\% \pm 4\%$ in alternations (Fig. 1a). Type 2 DM db/db mice featured even greater memory loss with a reduction of $25\% \pm 6.5\%$ ($p<0.001$ *vs.* hemizygous) (Fig. 1a). Regression analysis showed that there was strong correlation between high BGL and memory deficits (low percent in alterations) in STZ-treated wild type (WT) C57BL/6 and db/db mice (Fig. 1b,c).

C57BL/6 mice (with or without STZ treatment) were also tested for spatial memory function using the MWM evaluating hippocampal-dependent learning, including acquisition of spatial memory and long-term spatial memory[24]. In these experiments, we performed visible platform training followed by hidden platform testing with four probe trials per day[36,37,24]. All mice in each group were able to reach the training criterion within 4 days and were similarly proficient swimmers. However, only mice with the highest BGL (400–500 mg/dL) showed significant ($p=0.007$) latency in time to reach the hidden platform for the first time on the test day (Fig. 2a) and longer distance travelled until first entry in the target zone (data not shown). Mice with BGL of 250–350 mg/dL or 400–500 mg/dL, presented significantly diminished memory as measured by the number of repeated entries to the hidden platform (Fig. 2b). Interestingly, within the group of mice with high BGL, there were mice that showed mild or worse memory phenotypes. We decided to assign labels to these sub-groups as “semi-impaired” for mild or “impaired” for worse memory phenotype, respectively (Fig. 2). In total, these results confirm our hypothesis that high BGL are associated with memory loss.

Increased BBB permeability in DM type 1 and 2 models

We evaluated BBB permeability in animals with STZ-induced hyperglycemia using the fluorescent tracer, NaF, as described[26]. The amount of NaF in the cerebrum was elevated as compared to untreated controls and regression analysis showed a correlation between permeability level and BGL with $R^2=0.4428$ in STZ-treated mice (Fig. 3a,b). Similarly, we

also found 2.2-fold increased BBB permeability in db/db mice as compared to heterozygous controls with normal BGL (Fig. 3c). Next, regression analysis revealed strong correlation between memory loss (measured by percent of alternations in Y maze) and permeability level with $R^2=0.5689$ in STZ-treated mice and $R^2=0.9460$ in db/db mice, with $p=0.0073$ and $p=0.0054$, respectively (Fig. 3d,e).

Diabetes causes inflammation in brain BMVs

It has been suggested that sRAGE serve as decoys for AGEs, thus reducing RAGE signaling and inflammation[38–41]. We evaluated sRAGE levels in serum of DM type 1 and 2 mice using commercially available ELISA and discovered that DM mice had significantly ~1.4–1.6-fold lower levels of sRAGE than their control counterparts (Fig. 3f), suggesting a higher potential for inflammation in DM mice. The state of systemic inflammation that impairs endothelial function and contributes to atherosclerosis has been associated with DM[42]. DM patients are believed to acquire endothelial pathological phenotype due to the high levels of circulating inflammatory markers, including tumor necrosis factor- α (TNF α), C-reactive protein, interleukin-6 (IL-6), and intercellular adhesion molecule 1 (ICAM-1) [43–47]. To evaluate relevant molecule expression in brain endothelium of diabetic mice, we isolated BMVs and profiled the expression of genes commonly involved in regulation of endothelial functions and inflammation. Utilizing a commercially available qPCR-based array, 84 genes were analyzed. All gene expression data were then analyzed by IPA® system tools. Fifty-four genes related to pathways regulating angiogenesis, inflammation, vasoconstriction/vasodilation, and platelet activation were deregulated by at least 2-fold. Since we found differences in memory phenotypes (Fig. 2), we explored whether there would be any dissimilarity between semi-impaired and impaired mice in deregulated gene expression. Indeed, many genes had significant fold-change differences between these two groups (Fig. 4a). Among them were eNOS, TNF α , TGF β 1, Tymp and MMP9, that have been shown to affect microvessel functions[48–53]. Next, we wanted to see if genes that were significantly up-regulated in mice with impaired memory would be affected in BMVs isolated from db/db mice. qPCR analysis revealed that many of the genes up-regulated in STZ-treated mice with memory deficits also showed enhanced expression in BMVs from db/db mice, some of these genes to a much greater extent (Fig. 4b).

Other genes upregulated in brain BMVs included several chemokines promoting monocyte and lymphocyte attraction (CCL2, CCL5, CXCL2, CXCL3), adhesion molecules facilitating leukocyte adhesion (Pecam1, E-selectin), β -integrins (mediating adhesion of endothelial cells to basement membranes) and prostaglandin synthase. Such changes in gene expression are overall indicative of a pro-inflammatory phenotype, which directly or indirectly (enhanced interactions with white blood cells) can contribute to enhanced BBB permeability in diabetic animals. Of interest is that some of these genes showed significantly higher expression in type 2 DM as compared to type 1 DM animals. Despite our assumption that the TJ protein, occludin, or adherent junction protein (AJ), cadherin 5, would be down-regulated in DM animals with enhanced BBB permeability, we found 8–16-fold and 4–22-fold increases in expression of these TJ and AJ protein genes, suggesting improper folding or incorporation in the cell membrane that may reflect a compensatory phenomenon in DM.

Signs of neuroinflammation in DM animal models parallel loss of pericyte coverage and decrease in TJ proteins expression

To assess inflammatory status in the brain tissue, we performed expression profiling of the genes regulating inflammatory pathways. mRNA was extracted from the cortex area in contralateral hemisphere from the same mice that were used for BMV gene profiling (Fig. 5). Many genes were significantly affected in STZ-treated mice (Fig. 5a). Two of these genes, $LT\alpha$ ($TNF\beta$) and CD40lg, have been described in the literature to be up-regulated in AD or other types of dementia [54–57], and Myd88 to be down-regulated[58]. We evaluated expression of these genes in db/db mice and found that $LT\alpha$ and Myd88 had a similar regulation profile, while CD40lg did not change in db/db mice vs. controls (Fig. 5b). Glucose transporter, Glut1 (SLC2A1) gene has been shown to be up-regulated in blood vessels in mice with diabetic retinopathy[59]. Therefore, we decided to assess Glut1 levels in microvessels isolated from diabetic mice; indeed both DM model mice showed higher Glut1 gene expression than their control counterparts (Fig. 5c). Interestingly enough, we found that in STZ-treated mice there was a wide distribution in fold changes in gene expression. When we matched the gene expression data with our Morris Water Maze data (Fig. 2), we found a significant difference in fold changes between mice that showed impaired (marked in red) or semi-impaired phenotypes ($p<0.05$) (Fig. 5c).

Immunohistochemical evaluation of the cortex showed 1.50 ± 0.09 -fold increase in ICAM-1 expression in STZ-treated mice vs non-treated mice (Fig. 6a,b,c,h), whereas db/db mice displayed 2.00 ± 0.13 -fold increase in ICAM-1 expression (Fig. 6d,e,f,h).

Next, we evaluated microglia reaction (Iba1), pericyte coverage (CD13) and TJ (claudin-5) in type 1 and type 2 DM mice models, STZ-diabetic mice and db/db mice by immunohistochemistry. We found that STZ-diabetic mice showed a significant decrease in pericyte presence at the BBB and enhanced microglia reaction in diabetic mice as compared to controls with normal BGL (Fig. 7a,c). Similar immunohistochemical assessment of db/db mice displayed enhanced microglial reaction and diminished pericyte coverage in diabetic vs control animals (Fig. 7b,d). Then, we appraised expression of the TJ proteins in the cortex area and found a significant decrease in claudin-5 expression in both DM models: $16.2\%\pm5.4\%$ decrease in STZ-treated mice (Fig. 8a,c) and $33.7\%\pm6.6\%$ in db/db mice (Fig. 8b,d). Evaluation of occludin expression on serial sections showed $28.8\pm8.6\%$ diminution in the STZ diabetic model and $51\pm4.5\%$ decrease in db/db mice when compared to respective controls (Fig. 8e,f).

Taken together, our results suggest a causative link between BBB dysfunction and cognitive deterioration in diabetic conditions.

Discussion

Cerebrovascular pathology is often found in a wide variety of cognitive impairment or dementia disorders. One of the indicators of cerebral vascular disease is BBB dysfunction[60,61]. The BBB serves as a selective diffusion barrier at the level of the cerebral microvascular endothelium to maintain homeostasis in the CNS by regulating ion balance, aiding in nutritional transport, and blocking influx of potentially neurotoxic

molecules from the circulation[60,61]. DM is a metabolic disorder characterized by hyperglycemia leading to end-organ injury in various organs due to microvascular compromise (cardiovascular disease, nephropathy and retinopathy) and inflammation. Learning abilities and memory deficits have been documented in DM type 1 or type 2 patients[3–5], which might be due to cerebral vascular dysfunction. Association between microvascular changes and cognitive decline in DM has not been substantiated until very recently, with defects noted in blood perfusion, neuronal function, white matter microstructure, and metabolic function[7,62].

BBB breakdown has been suggested as one of the causes of dementia in DM and AD[63]. However, the exact mechanisms of injury, relationship between enhanced permeability and memory loss, differences between DM types 1 and 2, and therapeutic potential of BBB protective strategies in cognitive decline are currently unknown. Here, we investigated the idea that DM types 1 and 2 decrease BBB integrity directly (via effects on brain endothelium and pericytes) and promote a pro-inflammatory phenotype of brain endothelium (resulting in a low level inflammation) that further exacerbates barrier injury. We used animal models of DM types 1 and 2 and found that both models mice displayed a significant increase in BGL vs control counterparts, mimicking hyperglycemia in DM patients[34,35]. DM mice exhibited memory decline, confirmed by two well-established tests, Y-maze and MWM[23,24]. DM mice showed deterioration in their abilities in acquisition and long-term spatial memory similar to recent study[64]. STZ-injected mice displayed a broad range of BGL, but only mice with the highest BGL showed significant latency in spatial memory acquisition (time to reach hidden platform for the first time) and to memorize platform location (number of repeated entries). Mice that showed mild or worse memory phenotypes (marked as semi-impaired or impaired, respectively) demonstrated significantly different gene profiling in BMVs isolated from these animals. Amongst deregulated genes, there were many genes involved in inflammation and barrier destabilizing molecules (MMP9, eNOS, TGF β , TNF α , Tymp)[48–53]. Several studies have suggested that sRAGE serves as a decoys for AGEs, thus reducing RAGE signaling and inflammation[38–41]. We discovered that DM mice had significantly lower levels of sRAGE than control animals, suggestive of a higher potential for inflammation in DM mice.

We showed increased BBB permeability in DM type 1 and 2 animals that was significantly associated with hyperglycemia and memory deficits. These changes paralleled deregulation of genes in brain endothelium associated with BBB injury and inflammation. In spite of our assumption that in DM animals with enhanced BBB permeability, TJ protein occludin or AJ protein cadherin 5 would be down-regulated, we found their levels highly increased, implying improper folding or incorporation in cell membranes that may reflect a compensatory phenomenon in DM. Interestingly, we found significant reduction in TJ expression (protein) by immunohistochemistry (occludin and claudin-5) in both DM animal models as compared to controls (Fig. 8). Similarly, Li *et al.* demonstrated a decrease in the amount of occludin detected by western blotting of isolated CNS microvessels in STZ-diabetic mice[65]. They also admitted that post-translational oxidative modifications or phosphorylation might be responsible for increased barrier permeability[66]. Differences might be explained as due to mRNA vs. protein detection for TJ proteins associated with adaptive mechanism, and further studies are needed to clarify this phenomenon.

Gene profiling resulted in significant up-regulation of pro-inflammatory pathways in the brain tissues of the DM mice, which resulted in microglia activation, reduced pericyte coverage, shown by immunohistochemical assessment. Salameh *et al.* recently showed pericyte loss in STZ-induced diabetic mouse brains[67]. Pericytes are essential for the viability and function of the blood-retinal barrier and BBB [68,67,10,69] and their loss, which is caused by hyperglycemia-induced oxidative damage, is associated with diseases such as diabetic retinopathy and Alzheimer's disease[70,71]. Mice in both DM types displayed a significant decrease in TJ protein (claudin-5) expression. Takechi *et al.* recently showed that in a high-fat and high-fructose diet-induced DM model, reduction in expression of TJ proteins, occludin and ZO-1, was also associated with increased BBB leakiness, astrogliosis, neuroinflammation and memory loss (as shown by MWM latency)[72]. Immunohistochemical evaluation showed significant increase in ICAM-1 expression in DM mice. DM patients are believed to acquire endothelial pathological phenotype due to the high levels of circulating inflammatory markers and ICAM-1[44,72]. Previously, diminution in pericyte presence has been described in DM both in the blood retinal barrier and BBB[73]. Pericytes provide functional support to the brain endothelium and their loss leads to enhanced permeability and tissue injury in DM[74] and neuroinflammatory conditions[10]. Of interest, DM retinopathy is a predictor of decline, presumably due to CNS microvasculature demise[75]. All these factors together might explain the abnormal BBB permeability observed in DM mice.

We found several pro-inflammatory genes to be up-regulated in DM type 1 animals, including chemokines. Among deregulated genes, we identified a few genes known to be associated with cognitive deficit; LT α (TNF β) and CD40lg were found to be up-regulated[54–57] in AD or other type of dementia, and Myd88 to be down-regulated[58]. Recently, the Glut1 gene has been shown to be up-regulated in blood vessels in mice with diabetic retinopathy[59]. Recent studies recognized diabetic retinopathy to be one of the prognosticators for mental decline[75]. Our results showed that mice with the highest Glut1 levels were the mice with memory deficits. Glut1 is also critical for the maintenance of appropriate brain capillary networks, cerebral blood flow, and BBB integrity, as demonstrated in humans with SLC2A1 mutations and Slc2a1 transgenic mice, which contributes to neurodegeneration and behavioral deficits in a murine model of AD and GLUT1 deficiency[76–78]. Targeting Glut1 in diabetes might provide future therapeutic directions in alleviating retinopathy or memory deficits.

The current study presents a strong association between hyperglycemia, BBB permeability and cognitive dysfunction in both DM type animal models. Hyperglycemic animals showed a pro-inflammatory phenotype both in BMVs and brain tissue. Disruption of the BBB was associated with and present in animals with cognitive decline, similar to recently demonstrated BBB dysfunction association with cognitive decline in DM patients[11], and was associated with increased levels of vascular dysfunction markers in CSF. Ample work remains to be done regarding the understanding of mechanisms of BBB changes and its involvement in dementia development in DM, and is of high significance due to the potential of employing novel therapeutic interventions protecting BBB and preventing cognitive impairment.

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Figure 1. High glucose levels lead to memory deficits in types 1 and 2 diabetes mice

a. Percentage of alternations measured by Y-maze test in type 1 DM (STZ-treated or non-treated mice) or type 2 DM (db/db or heterozygous db/hemi) mice. Regression analysis of percent of alternations vs. glucose levels in WT mice, with or without STZ-induced diabetes (b) or in db/db mice (c). p values indicate significance between NT control (n=14) and STZ-treated animals (n=14), db/db mice (n=5) or db/hemi mice (n=8).

Figure 2. High glucose levels diminish memory in type 1 diabetes mice

Morris water maze results are presented as time to reach (latency) the target platform zone for non-treated (BGL=80–110 mg/dl) and STZ-treated mice with different glucose levels (a) and the number of repeated entries to the target platform zone for STZ-treated mice with different glucose levels (b). Results are shown as mean±SEM (6 to 9 animals per group). P values indicated on figures indicate significance vs. non-treated (BGL=80–110 mg/dl).

Figure 3. Hyperglycemia driven BBB permeability triggers memory loss in DM mice
 Regression analysis of permeability (measured by NaF accumulation in the brain) vs. glucose levels in mice with (a) or without (b) STZ-induced diabetes. c. Enhanced BBB permeability in db/db mice (measured by NaF accumulation in the brain). Regression analysis of memory loss (alternation %) vs permeability (measured by NaF accumulation in the brain) in WT mice with STZ-induced (DM type 1) (d) or db/db (DM type 2) (e). f. Decreased levels of sRAGE measured by ELISA. ** $p<0.05$ or *** $p<0.01$ vs control [untreated (NT) control (n=14), STZ-treated animals (n=14), db/db mice (n=5) or db/hemi mice (n=5)]. Results for both permeability and sRAGE are presented as the mean $\pm$ SEM from triplicate serum samples.

Figure 4. High glucose levels cause inflammation in brain microvessels

qPCR analysis of inflammatory response array for mRNA extracted from microvessels isolated from STZ-treated or db/db mice or their controls. IPA® analysis of differentially expressed genes (a). Fold regulation of the genes in STZ-treated vs. NT control (b) or db/db mice vs. hemizygous controls (c). ns indicates no significance, **p<0.01 indicates significance between mice with impaired vs. semi-impaired memory. Arrows point to genes described in the literature to be involved in BBB permeability.

Figure 5. Hyperglycemia causes inflammation in brain tissue and increased Glut1 expression in microvessels

qPCR analysis of an inflammatory response array for mRNA extracted from brain tissue isolated from STZ-treated or non-treated WT mice (a). qPCR data for LT α , Myd88 and CD40lg in brains of db/db mice (b). qPCR data for Glut1 gene in cerebral microvessels in DM types 1 and 2 mouse models (c). qPCR was done in triplicate and results are shown as mean $\pm$ SEM (3 animals per group). ns indicates no significance, * p <0.05 or ** p <0.01 indicates significance between diabetic mice and their respective controls. # p <0.05 indicates significance between mice with impaired vs. semi-impaired memory.

Figure 6. Enhanced expression of ICAM-1 in brain endothelium in DM types 1 and 2 mouse models

(a,b,c) Brain microvascular endothelial cells showed increased staining for ICAM-1 (b,c) in STZ-treated vs. control untreated mice (a). (d,e,f) Db/db homozygous mice (when compared to control heterozygous animals, panel d) demonstrated enhanced expression of ICAM-1 in endothelial cells of capillaries and venules (e,f). (g) LPS-injected mouse used as a positive control demonstrated very high levels of ICAM-1 expression[79]. Original magnification, A–G $\times 400$. (h) Semi-quantitative evaluation of ICAM-1 staining was performed as described[27]. ** $p < 0.002$

Figure 7. Microglial activation and decreased pericyte coverage of the BBB in DM type 1 and 2 models

Enhanced microglial staining (Iba1, red) was associated with diminished pericyte coverage (CD13, brown) in STZ-treated (a) or db/db (b) vs. respective control mice. Original magnification was $\times 400$, and $\times 1000$ in marked areas. (c,d) Semi-quantitative evaluation of double Iba1/CD13 staining was performed as described[27]. * $p < 0.05$, ** $p < 0.002$ represent significance vs control.

Figure 8. Claudin-5 and occludin protein expression is decreased in BMVs in DM type 1 and 2 models

Claudin-5 immunostaining is diminished (cl-5, brown) in STZ-treated (a) or db/db (b) vs. respective control mice. Original magnification was $\times 400$. Semi-quantitative evaluation of claudin-5 (c,d) and occludin (e,f) staining was performed as described[27].

Table 1

Body mass and blood glucose levels in mice.

Group/treatment	Body weight (g)	Glucose levels (mg/ml)
NT	28.06±2.08	105.7±21.54
STZ	24.42±1.5 *	365.78±138.42 **
db/hemi	30.71±0.53	137.13±6.95
db/db	43.99±2.28 **	491.91±27.11 ***

*
p<0.05,

**
p<0.01 or

p<0.001 indicate significance vs. control (NT for STZ-treated group, and db/hemi for db/db mice).