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RESEARCH

Altered Expression of Large Amino Acid Transporter (LAT1) and Branched-Chain Metabolic Enzymes in the Type 1 Diabetic Rat Retina

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Abstract
Diabetic retinopathy (DR) is a significant complication of diabetes that results in retinal damage and the risk of blindness. Branched-chain amino acids (BCAAs) are essential amino acids that fulfill physiological roles in energy balance and neurotransmitter regulation within the retina. While high levels of BCAAs are associated with DR, their metabolism in DR remains poorly understood. This study investigated the mechanisms underlying increased BCAA levels and their role in DR using STZ-diabetic rats (4 and 8 weeks). We measured mRNA (qPCR), protein (immunoblotting/immunofluorescence), and biochemical markers (BCAA and oxidative stress). Results showed elevated BCAA levels in both the serum and the retinas of diabetic rats at 4 and 8 weeks versus controls. Large amino acid transporter (LAT1), branched-chain amino acid transferase (BCATs), and branched-chain keto dehydrogenase α subunit (BCKDE1α) mRNA levels remained significantly unchanged, while BCKDE1β subunit was notably affected by diabetes. The protein expression of BCAT1 significantly decreased in the diabetic retina after 4 and 8 weeks of diabetes. LAT1 expression increased after 8 weeks compared to 4-week diabetic rats, but this change was insignificant. The protein expression of BCAT2 and BCKDE1 subunits did not change significantly due to diabetes. Additionally, the expression levels of neurotrophic factors (BDNF), antioxidant (GSH), and glutamine synthase (GS) were lower in diabetic retinas compared to controls. In contrast, TBAR and apoptosis factors (caspase 3 and BAX) were increased in diabetic retinas. These findings suggest that increased LAT1 expression and decreased BCAT1 protein in the diabetic retina contribute to elevated BCAA levels. Together with reduced GS expression, increased oxidative stress and apoptosis may lead to neuronal damage and retinal neurodegeneration in DR, indicating the significance of altered BCAA metabolism in DR progression.

Keywords Diabetic retinopathy · Neurodegeneration · BCAA · LAT1 · BCATs · BCKDE1 · BDNF

Introduction

Diabetic retinopathy (DR) is the most severe complication of diabetic eye disease, causing damage to the retinal cells, eventually leading to irreversible blindness in diabetic patients. According to the last report published by the International Diabetes Federation Diabetes (IDF), approximately 1 in 5 diabetic patients have some degree of DR, and nearly 1 in 10 have a severe form of the condition and are at risk of losing vision [1]. The prevalence of DR has reached alarming levels, severely affecting the lifestyle of patients and society, and imposing a huge economic burden on healthcare systems. The most common risk factor related to DR development is the duration of diabetes; almost all patients with type 1 diabetes and more than 50% of type 2 diabetes are diagnosed with some clinical grades of DR [2]. Within the diabetic retina, microvascular damage and macular edema are the major clinical hallmarks of DR, but they occur at the advanced stage of the disease. However, it has been established that retinal neuronal dysfunction and neurodegeneration are early events in the progression of the disease, which

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may cause later vascular damage, the pathogenesis of DR [3, 4].

The pathophysiology of DR is complex, but growing evidence suggests that alterations in branched-chain amino acids (BCAAs; leucine (leu), isoleucine (ile), and valine (val)) homeostasis may contribute to its development and progression [5, 6]. BCAAs are essential amino acids necessary for protein synthesis and cellular energy needs, and they also function as signaling molecules. They are crucial for glucose and lipid homeostasis and immunological responses. They serve as nutrient signals and nitrogen donors for neurotransmitter synthesis and glutamate/glutamine cycling in the brain and retina [7]. Individuals with obesity, insulin resistance, and diabetes have been found to have elevated levels of BCAAs [8]. In type 2 diabetes, BCAAs levels are now regarded as predictive markers of disease development. Furthermore, several studies have reported an excess of BCAAs in the serum and retina of diabetic rats [6, 9]. However, further studies are needed to fully determine the impact of altered BCAAs metabolism on neuronal damage in DR.

The regulation of BCAAs levels is controlled by the expression of their transporter and catabolic enzymes. Normally, BCAAs are taken up into the brain and retina through the large neutral amino acids transporter (LAT1) at the blood barrier [10]. After absorbing the BCAAs, they are catabolized by cytosolic and mitochondrial branched-chain aminotransferases (BCAT1 and BCAT2), followed by irreversible oxidative decarboxylation of the α-keto acid products, catalyzed by the branched-chain α-keto acid dehydrogenase complex enzymes (BCKD), which are the rate-limiting enzymes in BCAAs degradation. Recent studies have shed light on the role of BCAAs metabolism and its dysregulation in various neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease, Huntington's disease, and maple syrup urine disease (MSUD) [11]. MSUD is characterized by a build-up of both BCAAs and their neurotoxic metabolites, resulting in neurological dysfunction, seizures, and death. Additionally, the antiepileptic drug gabapentin, known to inhibit BCAT1, has proven effective in treating behavioral alterations in AD [12]. In a previous study by our research group, gabapentin treatment in diabetic animal models showed retinal neuroprotection by decreasing caspase-3 activity and reducing oxidative stress in the diabetic retina [5]. Furthermore, the correlation between BCAAs and DR is supported by observations of elevated BCAAs levels in the retinas of diabetic animals and the vitreous of diabetic patients with proliferative diabetic retinopathy, the advanced stage of the disease [5, 13].

The reasons for increased BCAAs in the retina and whether the excess BCAAs play a role in diabetic retinal pathology are not yet fully understood. Furthermore, the link between BCAAs regulatory protein expression and DR remains unknown. Additionally, a large body of research has studied BCAAs metabolism in diabetic complications using type 2 diabetes models [14–16]. However, the type 1 diabetic model, such as the STZ-induced rat, which replicates the human condition of type 1 diabetes, presents unique metabolic and vascular challenges that are important for understanding the entire range of diabetes complications. Therefore, we employed the STZ-induced rat model that reflects the acute onset of insulin deficiency seen in type 1 diabetes, offering a more precise mechanism for exploring the specific BCAAs metabolic disruptions involved in retinopathy. This study elucidated the expression of the BCAAs transporter LAT1, the expression of the two isoforms of BCAT, and the rate-limiting enzyme BCKDE1 in the retina of type 1 diabetic rats and their implication in the neurodegeneration of DR.

Methods

Experimental Animals

Twelve-week-old male Sprague Dawley rats ($n = 50$), weighing 250–290 g, were obtained from the Experimental Surgery and Animal Laboratory at the College of Medicine, King Saud University in Riyadh, Saudi Arabia. The rats were housed in individually ventilated cages, with 3 to 4 rats per cage, and were allowed to acclimate for 1 week. The rats were maintained under controlled environmental conditions (25 °C with a 12-h light/dark cycle). They had free access to Purina rat chow (manufactured by Grain Silos and Flour Mills Organization, Riyadh, Saudi Arabia) and drinking water. After 1 week of acclimation, the rats were divided randomly into two groups: normal healthy rats (control), containing 20 rats, and diabetic rats, containing 30 rats. Diabetes was induced with a single intraperitoneal injection of 50 mg/kg freshly prepared streptozotocin (STZ: Cat. Number SC-200719, Santa Cruz, USA) in a 0.1 mol/L citrate buffer (pH 4.5). Diabetes induction was confirmed by measuring fasting blood glucose levels from tail vein blood samples using an ACCU-CHEK Compact Plus glucometer. Rats with blood glucose levels above 250 mg/dL were classified as diabetic and included in the study, while non-diabetic and dead rats were excluded. The study focused on two time points: 4 and 8 weeks post-diabetes induction. Therefore, we further divided rats into four groups. At the end of the 4 weeks of diabetes induction, 10 diabetic and 10 age-matched control rats were fasted for 7 h, then deeply anesthetized with a high dose of sevoflurane (Tabuk Pharmaceutical, KSA) to induce euthanasia followed by blood samples that were obtained by cardiac puncture and they were intracardially perfused with 100 mL normal saline to wash out blood from tissues and confirm death. All rats in the remaining group were kept for 8 weeks, and then, 10

diabetic rats and 10 age-matched control rats were fasted for 7 h and then sacrificed. Blood samples were collected after fasting, and serum was separated and stored at −80 °C until analysis. Eyeballs were enucleated for immunofluorescence, and retinal tissues were isolated for mRNA and Western blot analyses. The sample size of rats/experiments used in this project is statistically accepted according to the "resource equation" methods [17]. All experimental procedures, including anesthesia, adhered to the ARVO guidelines for the Care and Use of Experimental Animals and the guidelines of the Experimental Surgery and Animal Laboratory, College of Medicine, King Saud University. The study was approved by the Animal Ethics Committee at King Saud University (Reference No: KSU-SE-21-04).

BCAAs Concentration in the Rat Serum and Retina Lysates

Serum branched-chain amino acids (BCAAs) were quantified using a BCAA kit (ABIN5067587, antibodies-online, USA) following the manufacturer's instructions. Briefly, 20 µL of serum was diluted with 80 µL phosphate-buffered saline (PBS) and centrifuged at 10,000 rpm for 5 min to remove insoluble particles. The supernatant was collected for analysis. Isolated rat retinas (4–5 per group) were sonicated in 130 µL of PBS on ice. The samples were then centrifuged at 10,000 g to eliminate undissolved debris, and the supernatant was collected. A 25 µL aliquot of the lysate was used to measure the total BCAAs concentration, which was normalized to protein concentration. BCAAs levels in each sample were determined using a standard curve of known BCAAs concentrations. Color intensity was measured with a microplate reader (Autobio Labtech Instruments Co., Ltd., China). Protein concentrations in each sample were estimated using the Lowry method.

qPCR of BCAT and LAT1 in the Normal and Diabetic Rat Retinas

RNA was extracted from rat retinas using the RNA Quick-RNA MiniPrep Kit (Zymo Research, Irvine, USA) following the manufacturer's protocol. Briefly, isolated rat retinas (5 per group) were sonicated in 150 µL of RNA lysis buffer, then centrifuged at 10,000 g for 2–3 min to remove undissolved debris. The supernatant, containing the total RNA, was processed according to the kit instructions. RNA purity was assessed using the A260/A280 ratio measured by a spectrophotometer. One microgram of total RNA was converted into complementary DNA (cDNA) using the advanced cDNA synthesis kit (Molequle-On, Auckland, New Zealand). Primers for quantitative PCR (qPCR) were designed and obtained from Integrated DNA Technologies (Coralville, USA), with sequences listed in Table 1.

Table 1 Primer sequences for qPCR analysis

Target	Primers sequences (5′3′)
BCAT1	F: GGG CTG CAA CTA TGG ATC TT
	R: ATC GTG CCC ACT TCA GTT ATC
BCAT2	F: GGC AAT GGA ACA AGC CTC TA
	R: GCA CAG AAT GAC GAA CAG GA
BCKDE1α	F: ACT TCG TCA CCA TTT CCT CTC
	R: CCC TCG CCA AAG TAA CAG AT
BCKDE1β	F: CCC AGG AAT CAA GGT GGT TAT
	R: CTG CCC GGT AAA GTA TCT TAG G
LAT1	F: ACC TCT GTC CAC CTC CAT AA
	R: GAA GGG TAG TTG GTG CCT ATT
β-Actin	F: GAC AGG ATG CAG AAG GAG ATT AC
	R: GCC AAG AGA GTT GGC TAC AA

qPCR was performed using Power SYBR Green Master Mix (Applied Biosystems, California, USA). Each reaction contained 5 µL of PCR master mix, 0.5 µM of each primer, and 2 µL of diluted cDNA. The qPCR protocol included an initial 10-min denaturation at 95 °C, followed by 40 cycles of amplification (95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s). A dissociation program (melting curve analysis) was conducted with one cycle at 95 °C for 15 s and 60 °C for 1 min to verify the specificity of amplification and the absence of primer dimers. Amplification data were collected using the 7500 Fast Real-Time PCR System (Applied Biosystems, USA). All samples were run in duplicate under consistent conditions, and Ct values were calculated using the baseline threshold method. Relative mRNA expression levels were normalized to β-actin and determined using the 2-ΔΔCt method [18].

Western Blot Analysis

The Western blotting experiments were performed as described previously [4]. Total retinal proteins were extracted using 10 mM HEPES lysis buffer, pH 7.4 buffer, containing 100 mM NaCl, 1% Triton X-100, 0.2% SDS, and a protease inhibitor cocktail. An equal amount of proteins (30 µg) was separated via 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked in a blocking solution (5% non-fat milk in TBS-T) and incubated with primary antibodies and overnight at 4 °C. After washing the membranes, horseradish peroxidases (HRP)-conjugated secondary antibodies were incubated for 2 h at room temperature (RT), followed by chemiluminescent detection of protein using an ECL kit (Biorad, CA, USA). Western blot band intensity was quantified using Image Lab software (LI-COR C-DiGit Blot Scanner from Biosciences, USA) and verified with Image J. Band intensities were normalized to the corresponding loading control

(β-actin & Tubulin). All experiments were independently repeated at least two to three times. The following primary antibodies were used: polyclonal rabbit antibody to BCAT1 and BCAT2 (1:10,000 dilution, antibodies-online, Aachen, Germany), monoclonal mouse BCKDE1α and BCKDE1β (1:10,000, Santa Cruz, Dallas, USA), and monoclonal mouse anti-LAT1 (1:10,000, Santa Cruz, Dallas, USA). HRP-conjugated antibodies to rabbit IgG (1:4000, Santa Cruz, Dallas, USA) or HRP-conjugated antibodies to mouse IgG (1:4000, Santa Cruz, Dallas, USA) were used as secondary antibodies.

Immunofluorescence

The whole eyes of the rats were fixed in 4% paraformaldehyde buffer for 24 h. Then, they were preserved in 70% ethanol for 24 h until embedded in paraffin blocks. The blocks were cut into 3-µm-thick sections for immunostaining and placed on coated slides, with two to three sections used per eye. Histological slides of normal and diabetic rat eye sections were prepared by dewaxing and rehydration, followed by washing twice with distilled water and once with PBS. The deparaffinized sections underwent antigen retrieval by treatment in 10 mM sodium citrate buffer containing 0.05% Tween-20. To block nonspecific binding sites, sections were incubated with 10% normal goat serum for 1 h. They were then incubated overnight at 4 °C with primary antibodies: rabbit anti-BCAT1 (1:300 dilution; antibodies-online, Aachen, Germany), mouse anti-BDNF, mouse anti-glutamine synthetase, mouse anti-BAX, and rabbit anti-caspase 3 (1:300 dilution; Santa Cruz Biotechnology, USA). After primary antibody incubation, sections were rinsed with PBS and incubated with the appropriate secondary antibodies: goat anti-rabbit IgG (Alexa Fluor® 594, 1:500; Abcam, Cambridge, UK) and/or goat anti-mouse IgG H&L (Alexa Fluor® 488, 1:500; Abcam, Cambridge, UK) for 45 min at room temperature in the dark. Following this, slides were washed with PBS and incubated in DAPI solution (Abcam, Cambridge, UK) for 3 min, protected from light. Finally, coverslips were applied, and the sections were examined and photographed using a ZEISS spinning disk confocal microscope (Zeiss, Germany). Fluorescent intensity was quantified using ZEN microscopy software, following the method described in [19].

Glutathione Assay

Reduced GSH levels were measured in the retinas of diabetic and non-diabetic rats using a modified method based on Sedlak and Lindsay [20]. Retinal homogenates were prepared as described in the Western blot section and then deproteinized with trichloroacetic acid. After a 5-min incubation, the mixture was centrifuged at 10,000 rpm, and the supernatant was collected. To determine GSH levels, 80 µL of the supernatant was mixed with 30 µL of Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid), DTNB). The resulting yellow color was measured at 412 nm using a spectrophotometer within 5 min. A standard curve of GSH, ranging from 0 to 50 µM, was used to calculate the unknown GSH concentrations in the retinal samples. Protein concentrations in each sample were estimated using the Lowry method [21].

Estimation of Thiobarbituric Acid Reactive Substances (TBARS) Levels

The lipid peroxidation was used as an indicator of oxidative stress. The levels of lipid peroxidation products (TBARS) were quantified in the retina using a commercially available assay kit (ZeptoMetrix kit). Retinal tissues were lysed by applying a short burst of ultrasonication in the lysis buffer. The homogenates were centrifuged at 11,000 g for 20 min at 4 °C. The supernatants were used for TBAR quantification. Fifty microliters of the supernatant was mixed with 1.25 mL of the reaction buffer provided in the kit. The mixture was then heated at 95 °C for 60 min. After cooling and centrifugation, the absorbance of the supernatant was measured using a spectrophotometer.

Statistical Analysis

Statistical analysis was performed using SPSS version 29 software and Microsoft Excel 365. All data are reported as means ± SEM. A one-way ANOVA following the LSD test was used to compare multiple groups, and an unpaired *t*-test was applied to compare the two groups. p values < 0.05 were considered statistically significant.

Results

Body Weight, Blood Glucose, and BCAAs Concentration in Control and Diabetic Rat Serum and Retina

After 4 and 8 weeks of STZ-induced diabetes, body weights, blood glucose levels, and BCAAs concentration were measured in both diabetic and age-matched control rats. As shown in Fig. 1, the body weights of the diabetic rats were significantly lower compared to the control groups at both 4 and 8 weeks ($p < 0.001$). Additionally, as expected, fasting blood glucose levels were markedly higher in all diabetic rats compared to the controls ($p < 0.001$). The total serum concentration levels of BCAAs in 4-week diabetic rats (121.4 ± 15.4 µmol/mL)

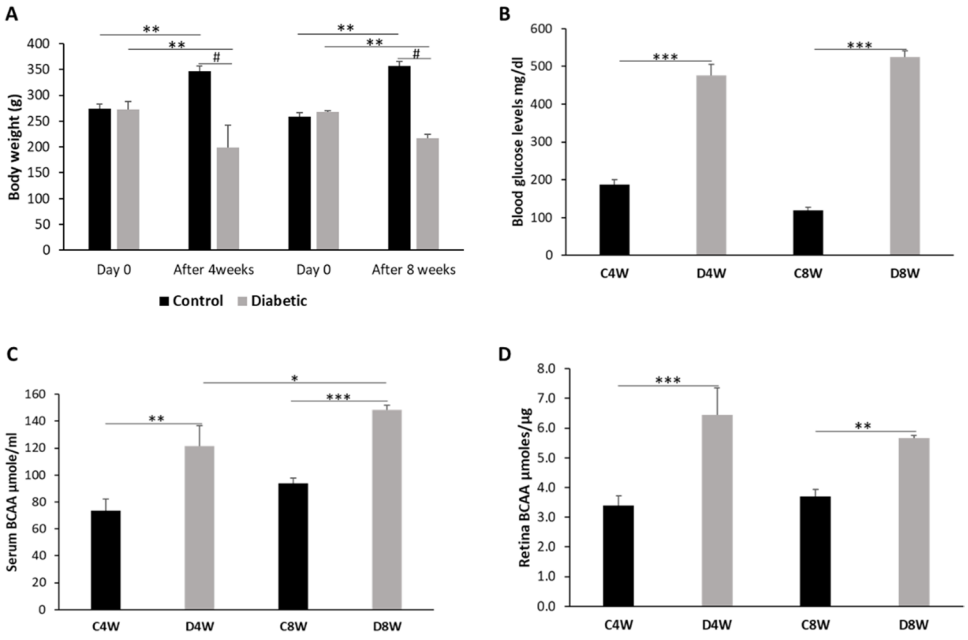

Fig. 1 Body weight, blood glucose, and BCAAs concentration in control and diabetic rat serum and retina. (**A**) Body weight and (**B**) blood glucose in the control and diabetic rats. (**C**) Concentration of BCAAs in serum and (**D**) BCAAs levels in the retina. The values are means ± SEM. $^{**}p < 0.01$, $^{***}p \leq 0.001$, and $^{*}p < 0.05$, significant. C4W represents a control for 4 weeks; D4W is diabetic for 4 weeks; C8W represents a control for 8 weeks, and D8W represents diabetic for 8 weeks

were significantly higher than in controls (73.6 ± 8.58 µmol/mL; $p < 0.01$). This increase was even more evident in 8-week diabetic rats, where BCAAs levels rose to 148.6 ± 3.08 µmol/mL compared to 93.9 ± 3.65 µmol/mL in age-matched controls ($p < 0.001$). Additionally, BCAAs levels in the serum of 8-week diabetic rats were significantly higher than those in 4-week diabetic rats (154.6 vs. 121.4 µmol/mL; $p < 0.05$). Similarly, BCAAs concentrations in the retinas of diabetic rats at both 4 and 8 weeks were significantly elevated compared to their respective control groups ($p < 0.001$). However, there are no significant differences in retinal BCAAs levels between the diabetic groups.

mRNA and protein Expression of the LAT1 Transporter in the Control and Diabetic Rat Retina

LAT1 is the primary transporter responsible for moving BCAAs from the bloodstream into the retina and retinal cells. To examine LAT1 expression, we analyzed its mRNA levels in control and diabetic rat retinas using qPCR. As shown in Fig. 2A, LAT1 mRNA expression in the retina was initially reduced after 4 weeks of diabetes, followed by a slight increase at 8 weeks. However, these changes were not statistically significant compared to their respective age-matched control rats. Additionally, after 8 weeks, the mRNA was higher than that of 4-week diabetic rats, but did

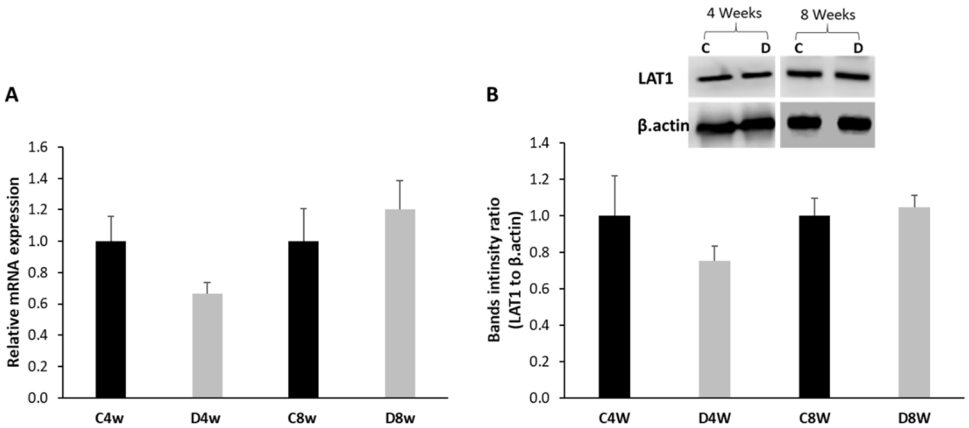

Fig. 2 LAT1 mRNA and protein expression in the control and 4- and 8-week diabetic rat retinas. (**A**) Relative expression of mRNA of LAT1 in 4- and 8-week diabetic and non-diabetic control rat retinas. (**B**) The ratios of LAT1 protein bands to β.actin as an internal control. Values are means ± SEM ($n = 4$). C4W represents a control for 4 weeks; D4W is diabetic for 4 weeks; C8W represents a control for 8 weeks, and D8W represents diabetic for 8 weeks

not reach statistical significance. We further examined the effect of diabetes on LAT1 protein expression in the retinas of 4- and 8-week diabetic rats compared to their age-matched controls using Western blot analysis (Fig. 2B). A single LAT1 protein band (~45 kDa) was observed when retinal homogenates were separated by gel electrophoresis and immunoblotted with a specific antibody. Densitometric analysis revealed no significant difference in LAT1 protein expression between the groups. However, after 8 weeks of diabetes, LAT1 protein expression elevated compared to 4 weeks of diabetes, but no significant change compared to 8-week age-matched controls.

mRNA of BCAAs Metabolic Enzymes in Control and Diabetic Rat Retina

BCATs are the primary isozymes responsible for initiating the catabolism of BCAAs. The product branched-chain keto acids (BCKA) is completely oxidized by BCKD complex enzymes, initiated by binding to the α- and β-branched-chain keto-acid decarboxylase (E1), which is encoded by the BCKDHA and BCKDHB genes. Using specific primers, we evaluated the impact of diabetes on the retinal mRNA expression of BCAT1, BCAT2, and BCKDE1 subunits in the normal retina. Figure 3 illustrates that although the mRNA levels of BCAT1, BCAT2, and BCKDE1α are decreased in diabetic retinas, the changes were not statistically significant compared to age-matched controls. The gene expression of BCKDE1β was significantly reduced in the retina after 4 weeks of diabetes compared to the controls. However, after 8 weeks of diabetes, the level also decreased, but not significantly ($p = 0.1$).

Protein Expression of BCAAs Metabolic Enzymes in the Retina

Next, we performed Western blotting experiments to investigate the effect of diabetes on the protein expression levels of BCAT1, BCAT2, BCKDE1α, and BCKDE1β in the retinas of diabetic rats at 4 and 8 weeks compared to their age-matched controls. Figure 4 reveals a single major protein band, approximately 44–45 kDa, corresponding to all proteins in the rat retina when probed with specific primary antibodies. Notably, BCAT1 protein levels were significantly reduced in the retinas of diabetic rats at both 4 and 8 weeks compared to controls (Fig. 4A). In contrast, BCAT2 (Fig. 4B) and BCKDE1 subunits (Fig. 5) levels remained unchanged with diabetes.

Protein Expression of BCAT1 in Control and Diabetic Rat Retina Layers

We employed immunofluorescence for BCAT1 to determine which layers and cells are affected by diabetes (Figs. 6 and 7). Fluorescence intensity measurements indicated a significant decrease in BCAT1 protein expression in almost all retina layers: the ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), and outer nuclear layer (ONL) of diabetic retinas at both 4 and 8 weeks.

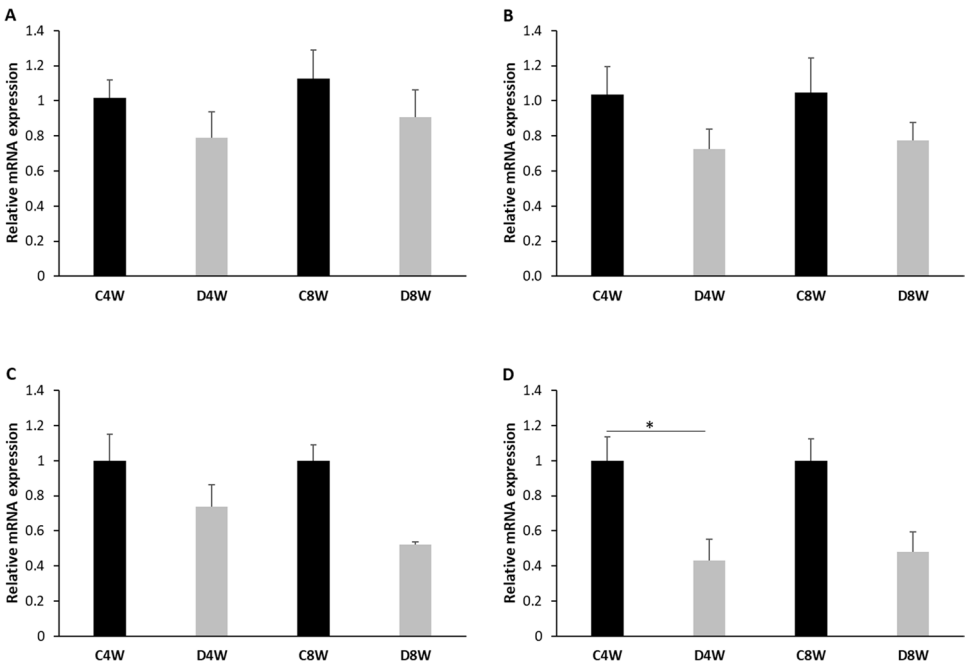

Fig. 3 Diabetes effects on the mRNA expression of BCAAs metabolic enzymes. Relative mRNA expression of (**A**) BCAT1, (**B**) BCAT2, (**C**) BCKDE1α, and (**D**) BCKDE1β in the 4- and 8-week diabetic group against control. All experiments were carried out in duplicate. Values are means ± SEM; *$p < 0.05$, significant ($n = 4$). C4W represents a control for 4 weeks; D4W is diabetic for 4 weeks; C8W represents a control for 8 weeks, and D8W represents diabetic for 8 weeks

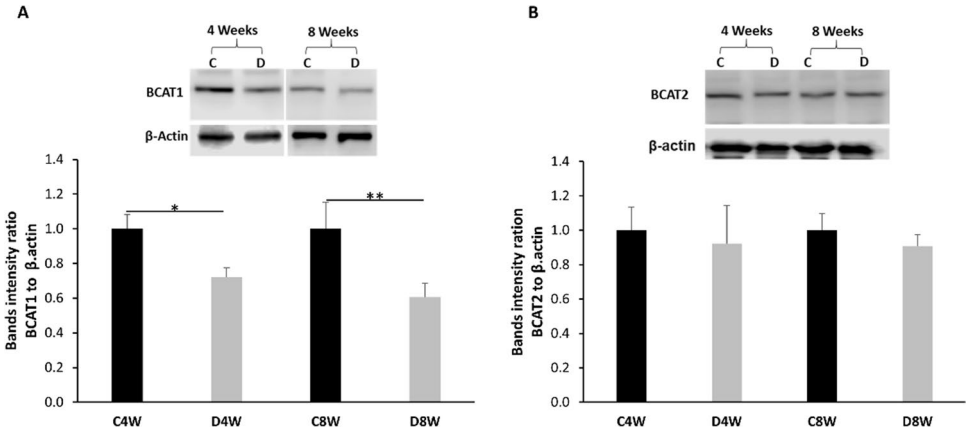

Fig. 4 BCAT1 and BCAT2 protein expression in the control and diabetic rat retinas. (**A**) Protein expression of BCAT1 in control and diabetic rat retinas. (**B**) BCAT2 expression in control and diabetic rat retinas. Data presented as ratios of BCAT1/2 protein band density to β-actin. Values are means ± SEM for six determinations ($n = 6$). $^{**}p < 0.01$ and $^{*}p < 0.05$, significantly different from their controls; C4W represents a control for 4 weeks; D4W is diabetic for 4 weeks; C8W represents a control for 8 weeks, and D8W represents diabetic for 8 weeks

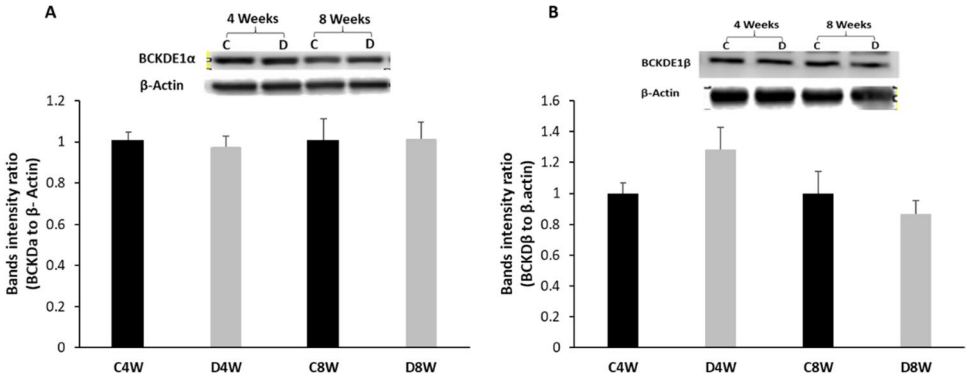

Fig. 5 BCKDE1 subunit protein expression in the control and diabetic rat retinas. (**A**) Protein expression of BCKDE1α in control and diabetic rat retina. (**B**) BCKDE1β expression in control and diabetic rat retina. Data presented as ratios of BCKDE1 protein band density to β-actin. Values are means ± SEM for six determinations ($n = 6$). C4W represents a control for 4 weeks; D4W is diabetic for 4 weeks; C8W represents a control for 8 weeks, and D8W represents diabetic for 8 weeks

Brain-Derived Neurotrophic Factors (BDNF) in Control and Diabetic Rat Retina

BDNF (brain-derived neurotrophic factor) plays a crucial role in neuronal survival and growth and is important in the protein expression of BCAT1. Therefore, the expression levels of BDNF protein were measured in the retinas of non-diabetic control rats and diabetic rats using Western blotting and immunofluorescence techniques. In our previous work, the expression of BDNF was decreased after 4 weeks of induced diabetes [4], and here in this study, we focused on 8 weeks. Densitometric analysis of the protein bands revealed a significant decrease in BDNF expression levels in diabetic rats compared to controls ($p < 0.05$) (Fig. 8A). Furthermore, reduced BDNF levels were observed in all layers of the retina, with the most significant decreases occurring in the GCL, IPL, and outer plexiform layer (OPL) (Fig. 8C).

Oxidative Stress in Control and Diabetic Rat Retina

BCAT protein activity is affected by oxidative environments. Therefore, oxidative stress was analyzed in diabetic rat retinas by measuring the levels of both glutathione and TBARS as markers of oxidative stress in the retinal tissues. Glutathione is a critical endogenous antioxidant that protects cells from oxidative stress. The reduced glutathione (GSH) levels were determined in the retinas of 8-week diabetic rats and age-matched controls. The GSH level in the diabetic retinas was significantly decreased ($p < 0.01$) in the diabetic retina compared to controls (Fig. 9A). Conversely, the level

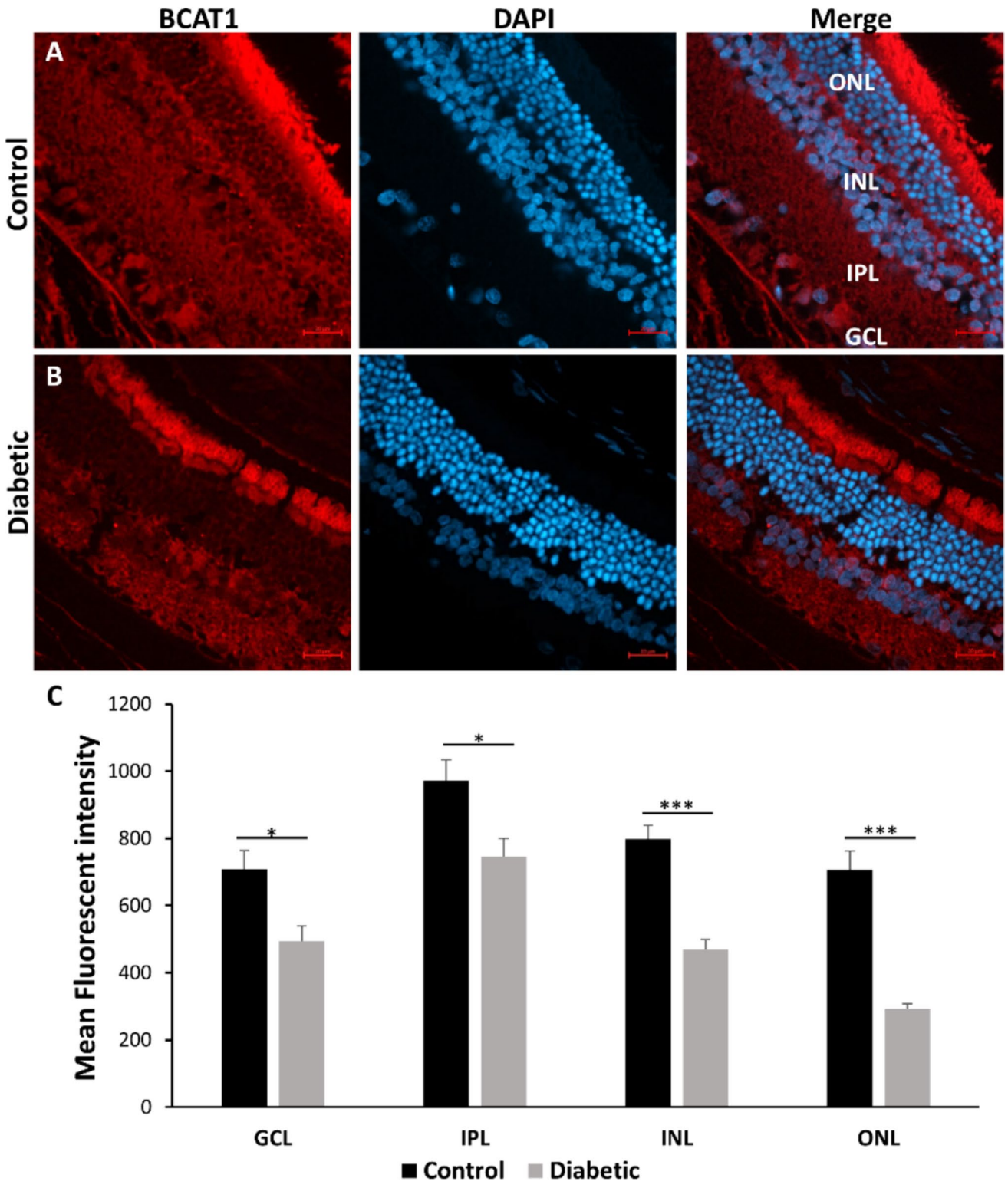

Fig. 6 Immunofluorescence staining of BCAT1 in control and 4-week diabetic rat retina. (**A**) IF staining image showed the localization and the intensity of BCAT1 in the normal rat retina. (**B**) The image showed the intensity of BCAT1 in the 4-week diabetic rat retinas. (**C**) Mean fluorescence intensity of BCAT1 in the control and diabetic retina layers. Values are means ± SEM. $^{**}p < 0.01$, significantly different from controls

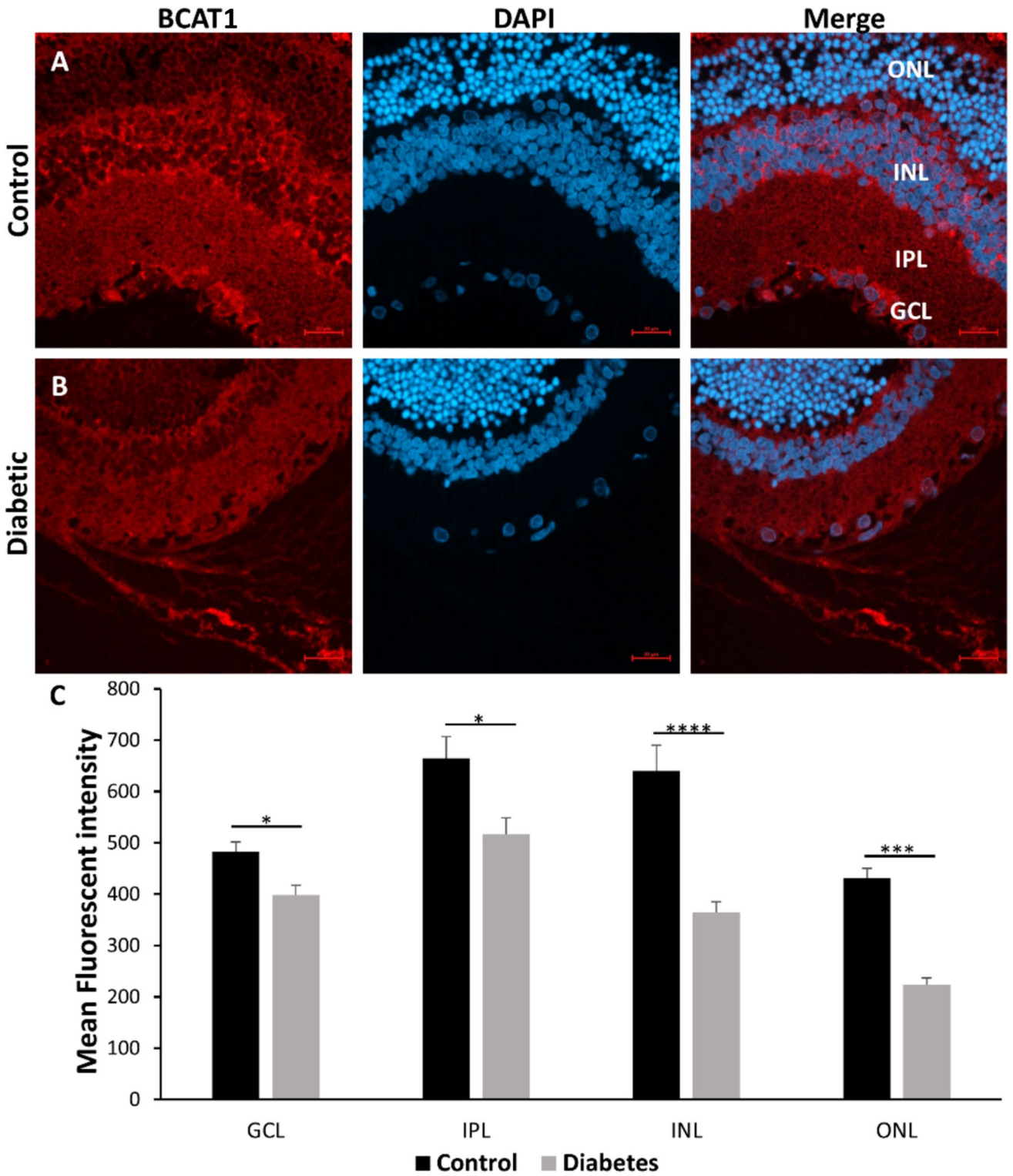

Fig. 7 Immunofluorescence staining of BCAT1 in control and 8-week diabetic rat retina. (**A**) IF staining image showing the location and the intensity of BCAT1 in the control rat retina. (**B**) The image showed the intensity of BCAT1 in the 8-week diabetic rat retina. (**C**) Mean fluorescence intensity of BCAT1 levels in the control and diabetic retina layers. Values are means ± SEM ($n = 4$). $^{**}p < 0.01$, significantly different from controls

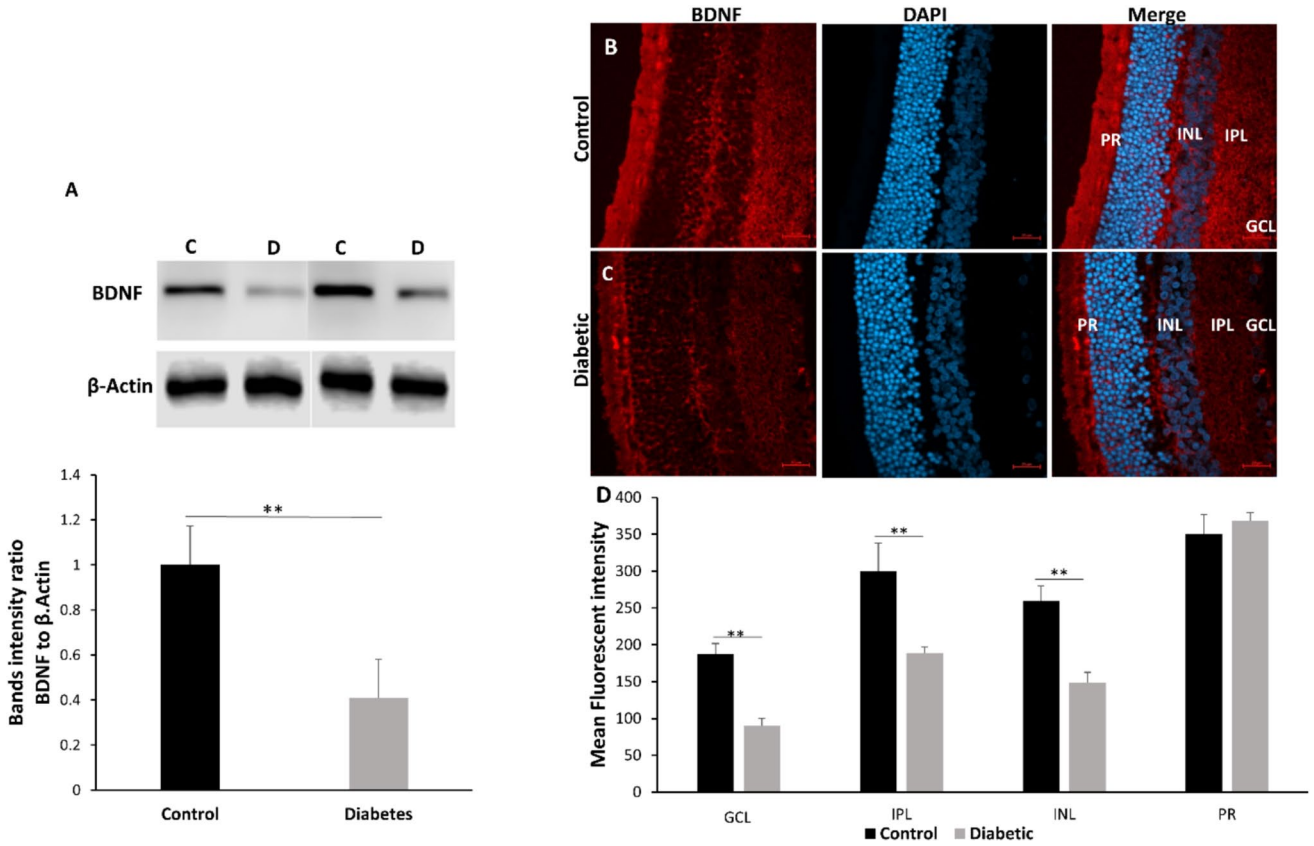

Fig. 8 Expression of BDNF in the normal and diabetic rat retinas. (**A**) Representative immunoblots of BDNF and β-actin bands. (**B**) Immunofluorescence staining of BDNF in control and (**C**) 8-week diabetic rat retina. (**D**) Mean fluorescence intensity of BDNF levels in the control and diabetic retina layers. Values are means ± SEM. $^{**}p < 0.01$, significantly different from controls. Abbreviations: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; PR, photoreceptors. Scale bar: 20 μM

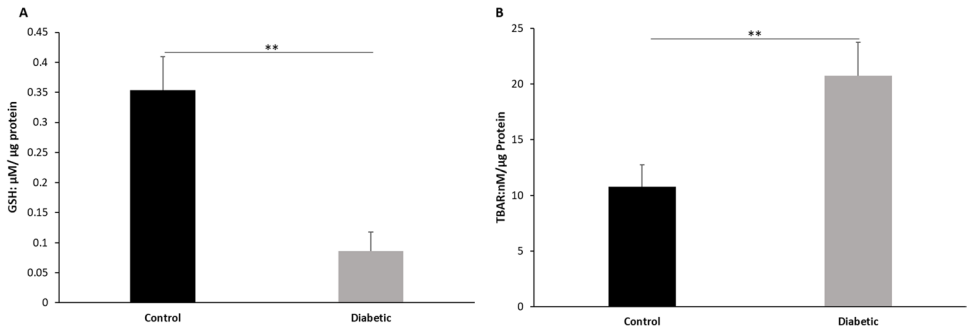

Fig. 9 Oxidative stress in the control and diabetic rat retina. (**A**) Glutathione and (**B**) TBARS levels in the control and 8-week diabetic rat retina. Values are expressed as means ± SEM ($n = 4$); $^{**}p < 0.01$, significantly different from control

of TBARS increased at least twofold in diabetic retinas as compared to control ($p < 0.01$).

Glutamine Synthetase Expression in Control and Diabetic Rat Retina

Glutamine synthetase (GS) is predominantly expressed in the retina's glial cells, including Müller cells and astrocytes. We investigated GS expression in the retinas of both control and diabetic rats (Fig. 10). Our data indicate a significant decrease in GS expression in all retina layers of diabetic rats compared to controls.

Pro-Apoptotic Factors Expression in Control and Diabetic Rat Retina

The expression of pro-apoptotic proteins, including cleaved caspase-3 and Bax, was also examined in the retinas of

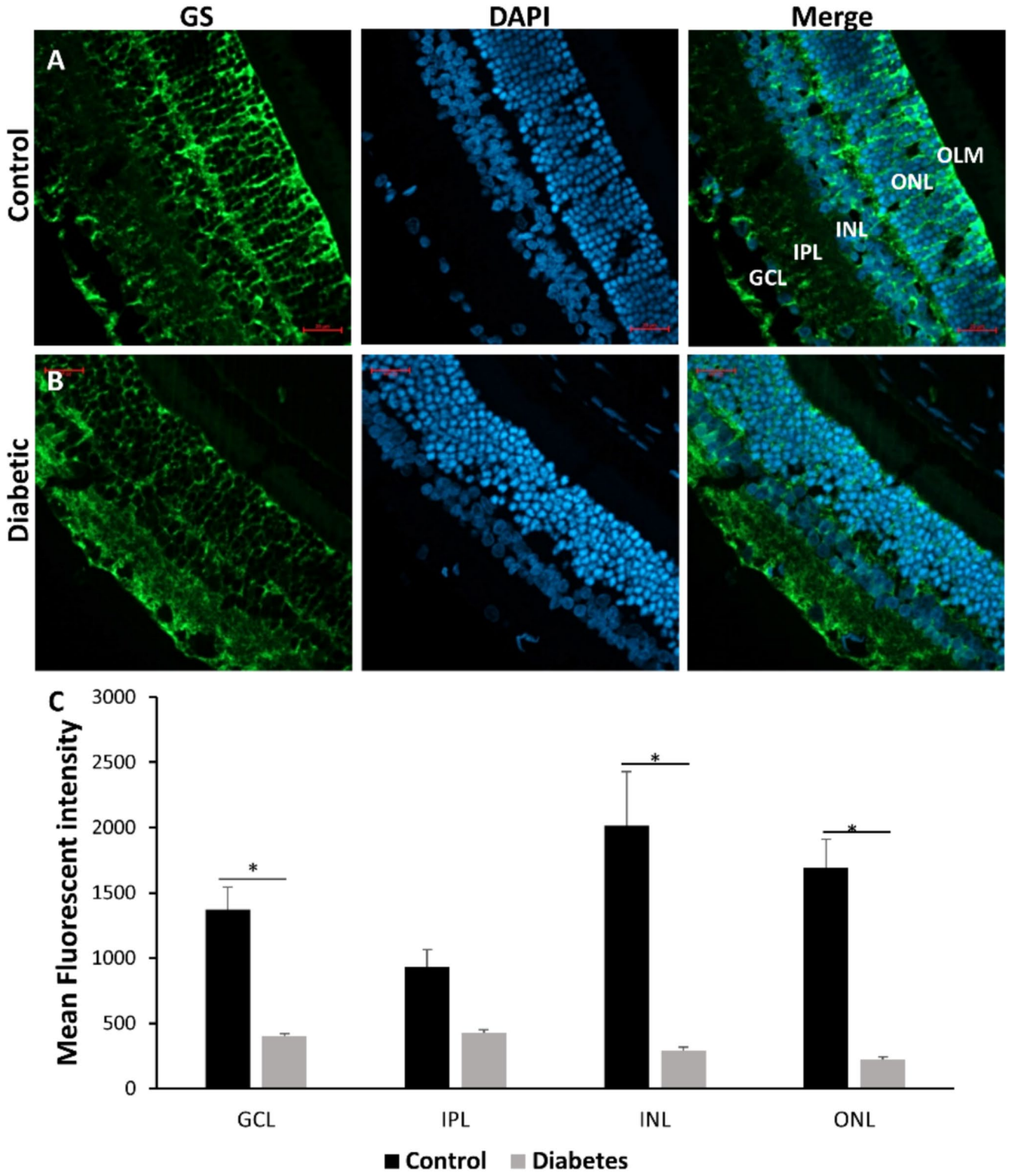

Fig. 10 Immunofluorescence staining of glutamine synthetase (GS) in control and 8-week diabetic rat retina. (**A**) Image panels of IF staining showed GS localization and intensity in the control rat retina. (**B**) The image panel showed the intensity of GS in the retina of an 8-week diabetic rat. DAPI was used as a nuclear stain. (**C**) Mean fluorescence intensity of GS levels in the control and diabetic retina layers. Values are means ± SEM. $^{**}p < 0.05$, significantly different from controls

control and diabetic rats using immunofluorescence techniques (Figs. 11 and 12). Bax protein levels showed a significant increase in the GCL, IPL, and INL of the diabetic retinas compared to the controls ($p < 0.001$) (Fig. 11). Furthermore, caspase-3 was observed as distinct fluorescent dots in the GCL and other layers of the diabetic retina, indicating active apoptosis (Fig. 12).

Discussion

The retina is a high-energy-demand tissue supported by a dense network of blood vessels that ensures a continuous supply of energy metabolites and oxygen essential for mitochondrial ATP production and retinal function [22]. Diabetes, an endocrinological disorder, disrupts metabolite levels systemically and locally in various organs, including the retina. This disruption is a critical factor in the progression of diabetic retinopathy. Among the altered metabolites in diabetes, branched-chain amino acids (BCAAs) are notable, as elevated levels may contribute to the pathogenesis of diabetic complications, including diabetic retinopathy. Elevated BCAA levels have been reported in the serum and retina of both diabetic animals and patients [5, 9, 13]. In this study, we investigated the impact of diabetes on the expression of specific BCAA transporter and key BCAAs catabolic enzymes: transaminases (BCAT isozymes) and BCKA dehydrogenase enzymes that may influence BCAAs metabolism in the diabetic rat retina. Initially, we measured BCAAs levels in both blood and retinal tissues under normal and diabetic conditions. We then assessed the expression of BCAAs transporters, including LAT1, in the retina. Additionally, we examined the expression of BCATs, critical enzymes in BCAAs metabolism that also serve as nitrogen donors, and the expression of BCKDE1, which plays a crucial role in energy biosynthesis in the rat retina.

The STZ-induced animal model of type 1 diabetes is widely used to replicate the human condition of decreased insulin production, which leads to altered metabolic pathways. As anticipated, STZ-induced diabetic rats exhibited weight loss and elevated blood glucose levels over time. Additionally, these diabetic rats showed increased serum and retinal levels of BCAAs compared to age-matched control rats. Our findings align with previous studies in type 1 and type 2 diabetic patients and experimental rodents [8, 23, 24]. In diabetes, insulin deficiency reduces protein synthesis and increases whole-body protein breakdown, contributing to elevated serum BCAAs levels. In addition, Karusheva et al. (2021) reported that insulin deficiency in individuals with type 1 diabetes impairs postprandial BCAAs clearance, leading to elevated plasma concentrations of leu, Ile, and val [25]. These increased BCAAs are proposed to contribute to insulin resistance and further β-cell dysfunction, potentially creating a self-perpetuating metabolic disturbance in the early stages of the disease, even when glycemia remains well controlled [22]. Elevated BCAAs levels in diabetes are associated with reduced glycolysis, increased fatty acid oxidation, and impaired BCAAs catabolism in skeletal muscle, the primary site of BCAAs metabolism [23]. Chronic exposure to high BCAA levels has also been linked to diabetic complications [24]. Lim et al. reported that high serum BCAAs levels in type 2 diabetes are associated with decreased glomerular filtration rate and impaired renal function [26]. Furthermore, several studies have indicated that elevated BCAAs levels have detrimental effects on vascular endothelium, leading to atherosclerosis and cardiovascular complications [16, 27]. Based on our data and that of others in the field, we conclude that insulin deficiency in type 1 diabetes or insulin resistance in type 2 diabetes disrupts BCAAs metabolism in a way that may both reflect and contribute to the metabolic defects associated with diabetic complications such as retinopathy.

BCAAs are transported from the blood to the retina across the blood-retinal barrier by the L-type amino acid transporter 1 (LAT1) [10]. In the retina, LAT1 is expressed in photoreceptors, the inner nuclear layer, and the ganglion cell layer (data not shown), suggesting that LAT1 plays a crucial role in maintaining BCAAs and large neutral amino acid levels in the neural retina. In this study, LAT1 expression was not significantly different in diabetic rat retinas compared to those of control rats. However, this transporter also carries other large neutral amino acids such as histidine, phenylalanine, tryptophan, and tyrosine, leading to competition among these amino acids [28]. Therefore, excess BCAAs under diabetic conditions could reduce the retinal uptake of essential amino acids, such as tryptophan, potentially impairing the synthesis of neurotrophic serotonin and kynurenic acid derived from tryptophan, which may contribute to neurodegeneration [29–31]. Additionally, the increased leucine levels in the diabetic retina, driven by LAT1, may promote neurodegeneration and neovascularization through mTOR activation [32, 33].

The first two steps of BCAAs catabolism, transamination and oxidative decarboxylation, are common to all three BCAAs, involving the BCAA aminotransferase (BCAT) and branched-chain α-keto acid dehydrogenase (BCKD) enzymes. Our recent research identified the expression and distribution of BCAT isozymes across nearly all retinal layers [34]. BCAT1 is found in ganglion cells, as well as in the inner and outer nuclear layers and the plexiform layers, where it accounts for about 30% of the total retina's neurotransmitter glutamate production. It serves as a key precursor not only for the excitatory neurotransmitter glutamate but also for the inhibitory neurotransmitter GABA, thus playing a direct role in maintaining neurotransmitter balance in neuronal cells [35, 36]. In contrast, BCAT2 is

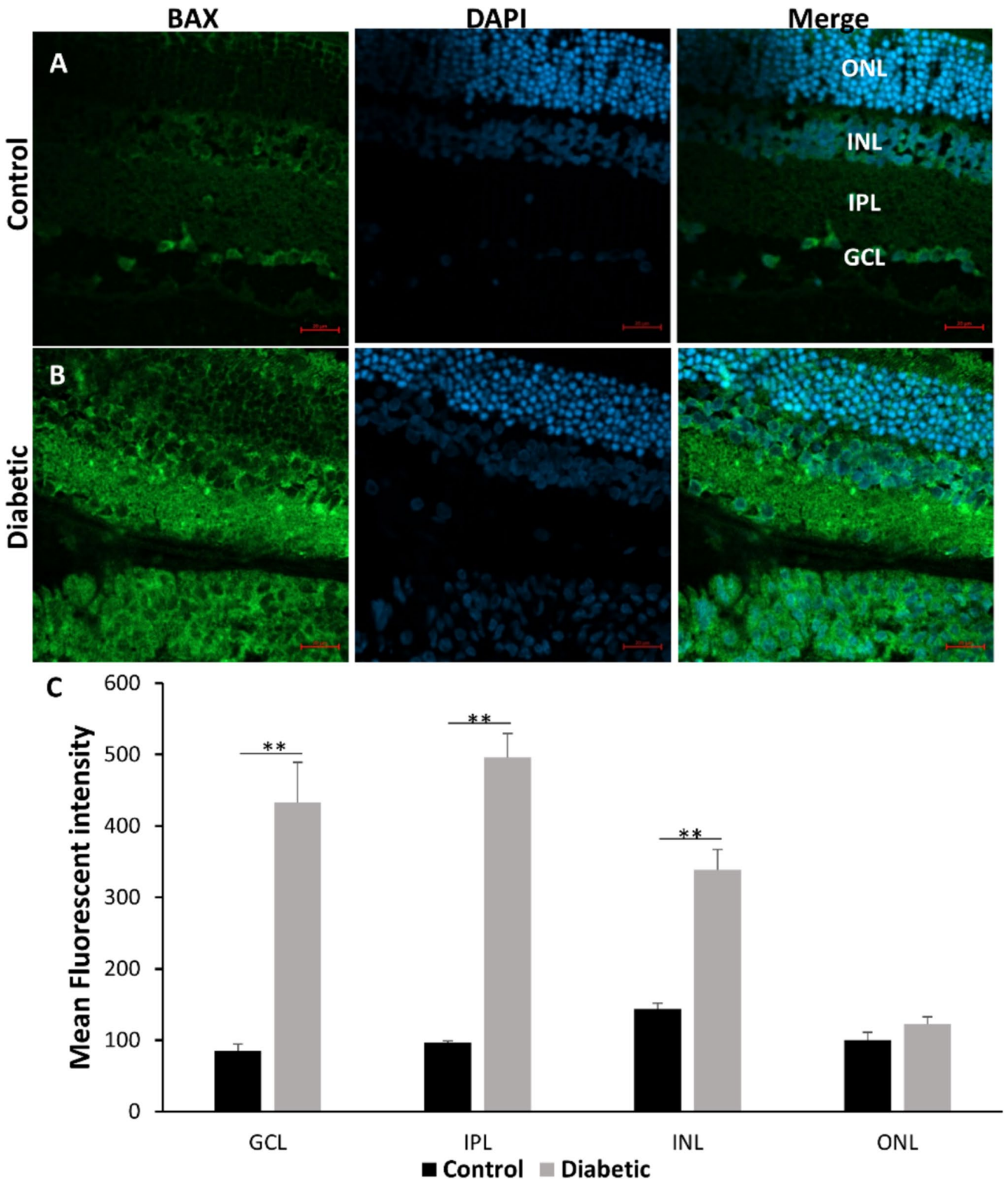

Fig. 11 Immunofluorescence staining of BAX in control and 8-week diabetic rat retina. (**A**) IF staining image showed the location and the intensity of BAX in control rat retina. (**B**) The image showed the intensity of BAX in the 8-week diabetic rat retina. (**C**) Mean fluorescence intensity of BAX levels in the control and diabetic retina layers. Values are means ± SEM. $^{**}p < 0.05$, significantly different from controls. Abbreviations: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar: 20 μM

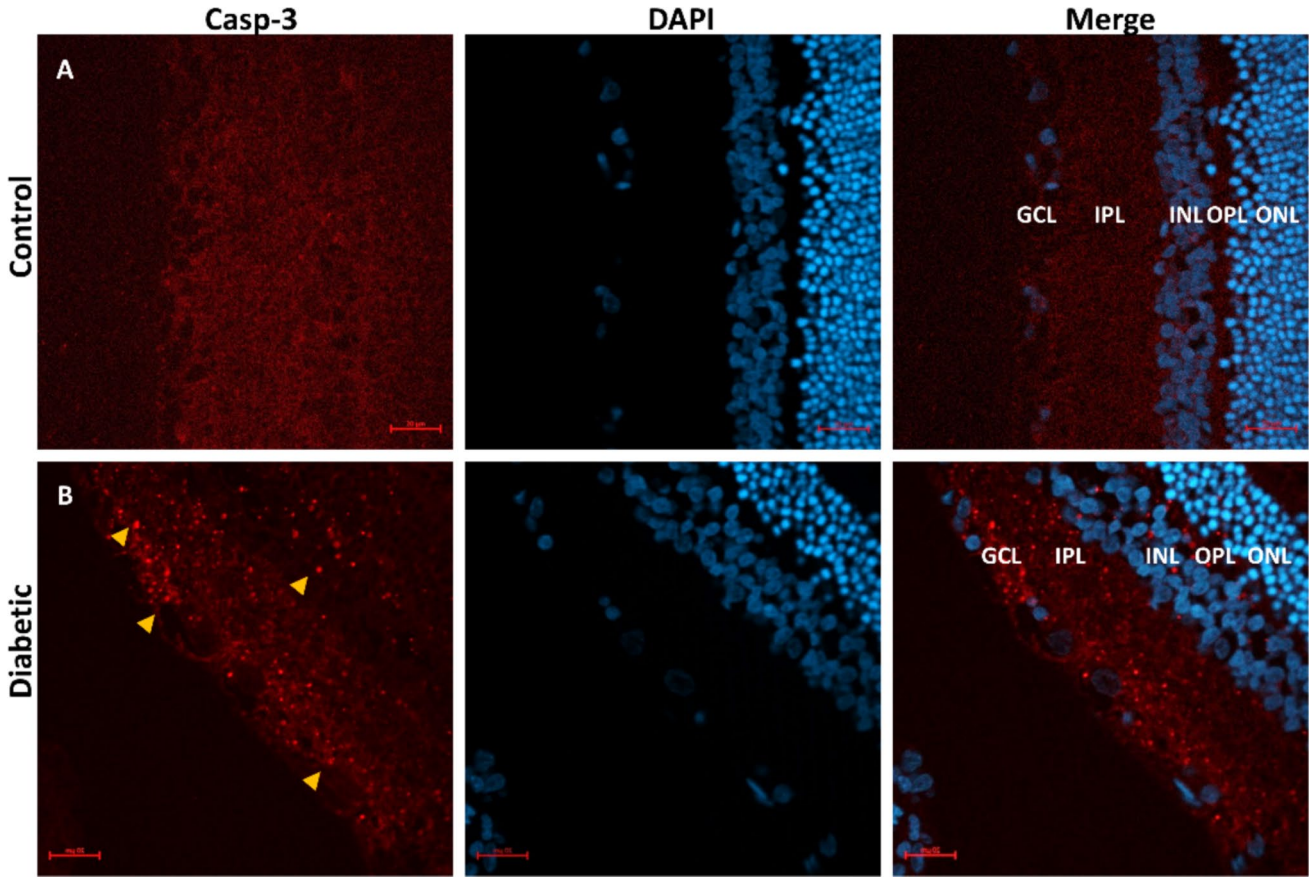

Fig. 12 Immunofluorescence staining of caspase-3 in control and 8-week diabetic rat retina. (**A**) The IF staining image showed Casp-3 in the control rat retina. (**B**) The image showed the signal of Casp-3 in the 8-week diabetic rat retina. Abbreviations: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Scale bar: 20 μM

localized to Müller cells, contributing to nitrogen recycling and supporting neuronal glutamate synthesis. BCKDE1 is predominantly expressed in retinal neurons (data not published yet), supporting its proposed role in neuronal BCAAs metabolism. The differential distribution of BCAA catabolic enzymes in the retina layers suggests that BCAAs play crucial roles in shuttling and regulating metabolites and maintaining glutamate homeostasis between neurons and glial cells, akin to their roles in the brain [34–36]. Deficiencies in BCAAs catabolic enzymes' expression have been associated with neurodegenerative diseases, cancer, and inflammatory conditions [37]. Additionally, our group has demonstrated that gabapentin, an inhibitor of BCAT1, mitigates neurodegeneration in diabetic retinopathy, highlighting BCAT's potential role in disrupting glutamate homeostasis in the diabetic retina [5]. This study evaluated the mRNA and protein expression of BCAT isozymes and BCKDE1 subunits in the diabetic retina. While mRNA levels of both BCAT1, BCAT2, and BCKDE1α were reduced at 4 and 8 weeks of diabetes, these changes were not statistically significant compared to controls. In contrast, the expression of BCKDEβ mRNA significantly decreased after 4 weeks of diabetes, suggesting that the transcriptional process may respond to the pathological condition. However, the reduction was no longer statistically significant by 8 weeks compared to the control rats' retinas.

The protein levels of BCAT1 were significantly decreased in the neuronal layers of the diabetic retina, including the GCL, INL, and ONL. The significant decrease observed at both 4 and 8 weeks, with no further decline over time, indicates that disruptions in BCAT1 expression within the diabetic retina start early in the disease and do not progressively worsen. This downregulation of BCAT1 may hinder BCAAs clearance and lead to their buildup in the retina, which can disturb normal retinal function and contribute to neurological complications. These findings are consistent with previous research indicating reduced BCAAs catabolism due to lower BCAT expression and activity in the adipose tissue of obese and insulin-resistant individuals [38]. Additionally, dysregulation of BCAT proteins has been associated with impaired cell differentiation, altered epigenetic profiles, and

metabolic dysfunctions related to various diseases such as Alzheimer's disease, heart failure, asthma, and cancer [39]. These findings underscore the importance of understanding the regulatory mechanisms of BCAT proteins in the early stages of the disease. Future research should focus on uncovering the mechanisms underlying this early downregulation and exploring strategies to maintain or restore BCAT1 expression during the initial phases of DR, thereby reducing long-term neurological complications. Furthermore, future studies should examine a wider range of time points to better understand the temporal dynamics of this molecular change.

Neurotrophic factors are important in neuronal survival, growth, and maintenance. Reduced levels of BDNF have been correlated with impaired insulin function and dysregulation of metabolism in the diabetic retina. Previous studies have reported a significant decrease in the expression of BDNF in the retina of diabetic rats [4, 40]. Consistent with these findings, we also observed decreased levels of BDNF expression in the diabetic retina. The reduction of BDNF levels was clearly observed in the GCL, INL, and IPL, as shown in Fig. 8. Interestingly, in this study, a decrease in the levels of BCAT1 was observed in the same retinal layers where BDNF expression was reduced. This is supported by previous studies indicating that BDNF regulates BCAT1 expression in the brain [39, 41]. Therefore, the expression of BCAT1 may vary in different retinal layers based on the differences in BDNF expression.

Oxidative stress is a significant risk factor in the progression of DR. It is characterized by an imbalance between the production of free radicals and their elimination by antioxidants. Previous studies have indicated that dysregulation of BCAAs contributes to increased oxidative stress, potentially leading to neuro-retinal damage in DR [5]. Consistent with these findings, we observed lower levels of GSH and higher levels of lipid peroxidation, along with elevated BCAAs in the serum and retinas of diabetic rats compared to controls. Research has shown that the increased oxidative stress in the retinas of ob/ob mice may be associated with elevated BCAAs levels and changes in metabolites [42]. An in vitro study reported that brain cells cultured in a medium containing high levels of BCAAs generated more free radicals and exhibited decreased neuroprotective functions [43]. Furthermore, BCAT proteins have a redox-active CXXC center that plays an important role in the enzymatic regulation of BCAT proteins. Previous studies have found that the oxidation environment significantly inhibits the second half-reaction of the BCAT2 protein (deamination of Glu). Therefore, the elevated BCAAs levels and increased oxidative stress in the diabetic retina may drive the BCAT2 reaction equilibrium toward increased glutamate synthesis in Muller cells. Our findings are consistent with Gowda et al.(2011), who reported that BCAAs inhibit the conversion of glutamate to α-ketoglutarate by BCAT in the diabetic rat retina [9].

Muller cells are responsible for converting glutamate into non-toxic compounds such as glutamine. We further assessed the expression of glutamine synthetase (GS), the enzyme responsible for synthesizing glutamine from glutamate in glial cells. Our findings and those of other studies indicate a significant depletion of GS in the diabetic retina compared to the controls [44, 45]. Notably, GS protein expression was reduced throughout the entire retinal layers, from the outer limiting membrane to the GCL, reflecting the location of Müller cells. Excess glutamate within Muller cells could hinder the uptake of extracellular glutamate, resulting in higher extracellular glutamate levels. This accumulation may contribute to neurodegeneration through glutamate excitotoxicity.

Apoptosis, or programmed cell death, is regulated by various proteins within cells, such as Bax and the caspase protease family. A decrease in the expression of anti-apoptotic factors, along with an increase in pro-apoptotic factors in the diabetic retina, correlates with accelerated neuronal cell death and reduced axonal regeneration, which are reliable markers of apoptosis [46]. Consistent with previous studies, we observed increased levels of pro-apoptotic factors Bax and caspase-3 in the diabetic retina. These apoptosis factors were elevated in the same retinal layers where BCAT1 expression was reduced. Several studies have reported that reduced expression of BCAT1 leads to increased mitochondrial respiration and neuronal damage, contributing to neurodegeneration [47, 48]. Additionally, research by Lai et al. (2021) indicates that the reduction of BCAT1 significantly exacerbates cardiomyocyte apoptosis [49]. These findings suggest that decreased BCAT1 expression in the retina may also play a role in inducing apoptosis and neurodegeneration in DR. Therefore, targeting BCAT1 could potentially alleviate the toxic effects of BCAAs accumulation on retinal cells in DR.

Taken together, our data indicate that diabetes causes a slight increase in LAT1 expression and a significant decrease in BCAT1 protein levels in the early stage of the diabetic retina. This may lead to elevated BCAAs levels both systemically and within the retina. The combination of increased BCAAs, decreased BCAT1, GS expression, GSH, and increased TBARs could contribute to neuronal damage in the diabetic retina by raising apoptosis and glutamate levels, which may promote neurodegeneration as shown in Fig. 13. However, further research is needed to examine the activities of these transporters and enzymes to better understand their metabolic roles and implications in neuronal damage during the early stages of the diabetic retina.

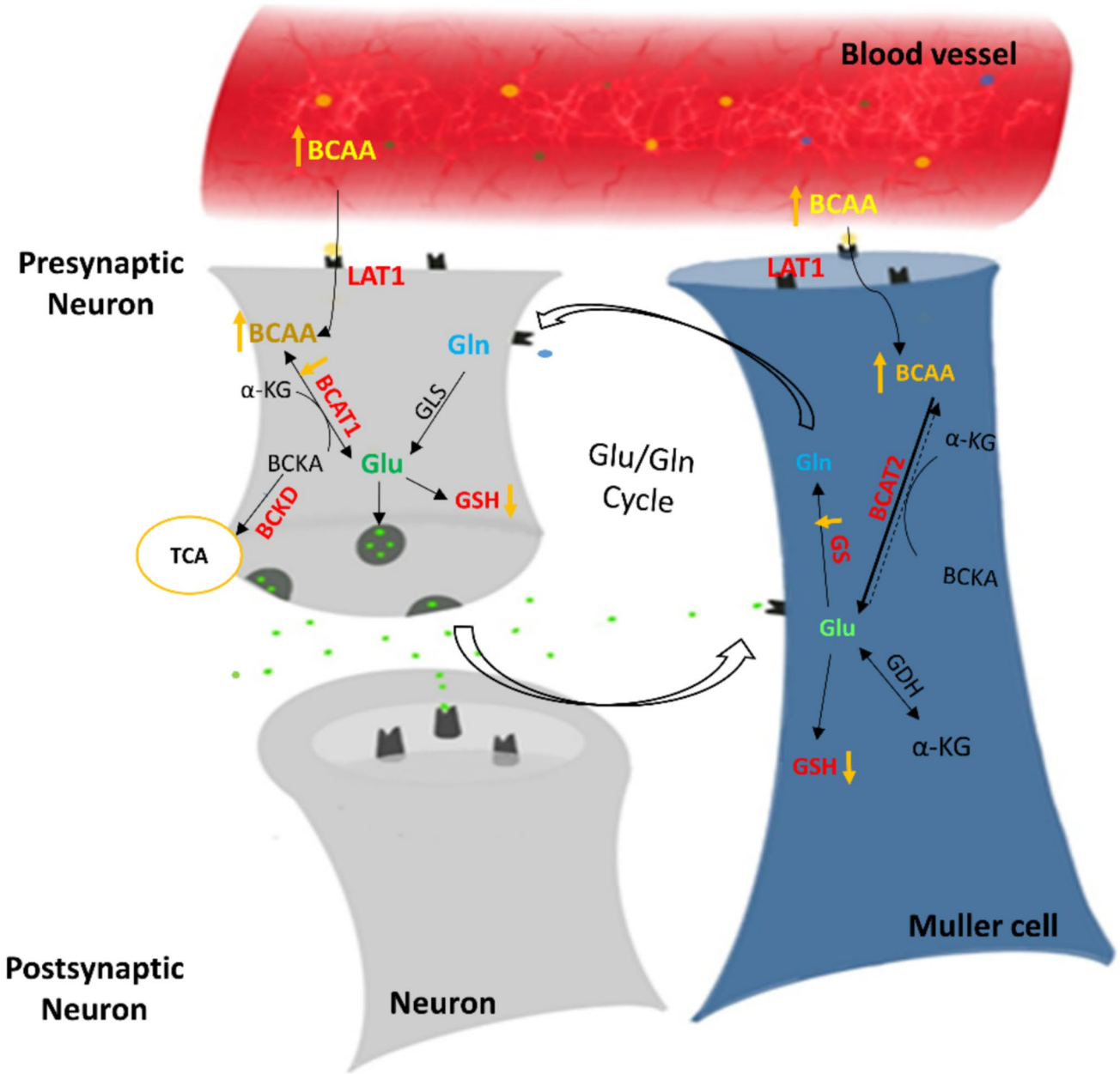

Fig. 13 Flow chart of retinal BCAAs metabolism and disruption of glutamate homeostasis under diabetic conditions. BCAAs are transported from blood vessels to the inner retina by the LAT1 transporter and are then catabolized by BCAT1 in neuronal cells or by BCAT2 in Muller cells, producing glutamate. During neurotransmission, glutamate is taken up by Müller cells and converted into glutamine and GSH. Our study reveals that in diabetes, the expression of BCAT1 in neuronal cells is decreased, potentially leading to increased BCAA concentrations in the retinal neurons. The expression of BCAT2 remains unchanged, which suggests that the high BCAA levels and oxidative stress may shift the BCAT2 reaction equilibrium towards increased glutamate synthesis. Additionally, the expression of GS, which converts glutamate to glutamine in Muller cells, is reduced in diabetes, leading to elevated glutamate levels within Muller cells. Consequently, the elevated glutamate levels within Muller cells may impair glutamate uptake from the synaptic cleft, potentially causing glutamate excitotoxicity and contributing to neurodegeneration in the diabetic retina

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Author Contribution D.I.A and M.S.O. conceived and designed the experiments. D.I.A and Y.A.A performed the experiments. M.S.O., A.A., and A.S.A. contributed reagents/materials tools. D.I.A wrote the paper, and M.S.O. revised and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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