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VITAMIN D_3 AUTO-/PARACRINE SYSTEM IN RAT BRAIN RELATING TO VITAMIN D_3 STATUS IN EXPERIMENTAL TYPE 2 DIABETES MELLITUS

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Growing evidence suggests that vitamin D_3 (D_3, cholecalciferol) deficiency and impaired signaling of the hormonally active form of D_3, 1α,25(OH)$_2$D$_3$ (1,25D$_3$), through its cellular receptor (VDR) can be significant risk factors for the development of numerous multifactorial diseases, including diabetes. Our investigation was aimed at researching the D_3 status in relation to the state of the D_3 auto-/paracrine system in the brain and clarifying the effectiveness of the therapeutic use of D_3 as a neuroprotective agent in experimental type 2 diabetes mellitus (T2DM). T2DM was induced in male Wistar rats by a combination of a high fat diet and a low dose of streptozotocin (25 mg/kg BW). Diabetic animals were treated with or without cholecalciferol (1,000 IU/kg BW, 30 days). The content of 25-hydroxyvitamin D_3 (25D$_3$) in blood serum and brain tissue was determined by ELISA. Analysis of mRNA expression of CYP24A1 and CYP27B1 genes was performed by RT-PCR. Protein levels of VDR, vitamin D_3 binding protein (VDBP), CYP27B1 and CYP24A1 were investigated by Western blotting. A significant T2DM-associated decrease in the content of 25D$_3$ in the blood serum was revealed, which correlated with a reduced content of this metabolite in the brain tissue. Impaired D_3 status in animals with T2DM was accompanied by an increase in the levels of mRNA and protein of both 25D$_3$ lα-hydroxylase (CYP27B1) and 1,25-hydroxyvitamin D_3-24-hydroxylase (CYP24A1), which, respectively, provide local formation and degradation in the nervous tissue of the hormonally active form of D_3 – 1,25D$_3$. At the same time, a significant T2DM-induced down-regulation of the brain content of VDBP was shown. In addition, diabetes caused a slight increase in the protein expression of the VDR, through which the auto-/paracrine effects of 1,25D$_3$ are realized in the brain. We have established a complete or partial corrective effect of cholecalciferol on D_3 status, its bioavailability in the CNS and the level of protein expression of CYP27B1 and CYP24A1 in the brain of rats with T2DM. Abnormal D_3 status in animals with T2DM was accompanied by compensatory changes in the expression of key components of the auto-/paracrine vitamin D_3 system. Cholecalciferol was demonstrated to be partially effective in counteracting the impairments caused by T2DM.

K e y w o r d s: vitamin D_3, 25-hydroxyvitamin D_3, vitamin D_3 auto-/paracrine system, brain, type 2 diabetes.

Diabetes mellitus is a widespread endocrine and metabolic disease that is characterized by chronic hyperglycemia caused by defects in insulin secretion and/or action with subsequent disturbances in carbohydrate, lipid and protein metabolism [1]. Damage to the nerve tissue occupies a leading place in the clinical picture of both type 1 and type 2 diabetes; however, the mechanisms of pathobiochemical changes in the central nervous system (CNS) remain the least studied at the moment [2]. It is known that the long-term impact of diabetes on the brain is manifested at the structural, neurophysiological and neuropsychological levels and is characterized by cognitive disorders, in particular psychomotor slowing and reduced mental flexibility, not related to other causes. Collectively, these deleterious alterations can be considered as a symptom complex of diabetic encephalopathy [3]. According to the literature, diabetic encephalopathy in its pure form occurs only in patients with type 1 diabetes (T1DM; about 80% of cases) since its development is mainly due to ineffective glycemic control. Mixed encephalopathy is diagnosed in patients with type 2 diabetes mellitus (T2DM) because of the predomi-

nance of the pathogenetic influence of hyperlipidemia and arterial hypertension against the background of insulin resistance and hyperglycemia [3, 4]. Numerous factors are involved in the pathogenesis of cerebral dysfunction in diabetes, such as hypoglycemic episodes, impaired cerebral blood circulation [5], the regulatory role of insulin in the brain [6], mechanisms of cell damage mediated by the effect of chronic hyperglycemia as a result of the formation of advance glycation end products [7], the development of oxidative and nitrosative stress [8, 9], mitochondrial dysfunction [10], endoplasmic reticulum stress [11], impaired neurotrophism [12], neuroinflammation [9], etc.

Environmental factors can also provoke the onset of the disease in genetically predisposed individuals. Among environmental factors, vitamin D_3 (D_3; cholecalciferol) insufficiency/deficiency is of particular interest. D_3 is known to be a pivotal modulator of metabolism and cellular functions. Most D_3 enters the blood circulation and binds to D_3 binding protein (VDBP) or chylomicron to be transported to tissues and organs. The D_3 auto-/para-/endocrine system provides the metabolism of hormonally active forms of D_3 in cells and the implementation of its pleiotropic biological effects [13]. The hormonally active metabolite of D_3 (calcitriol; 1α,25$(OH)_2D_3$) exerts its cellular effects by two mechanisms – non-genomic and genomic – similar to the action of other steroid hormones. Implementation of both mechanisms of action of 1α,25$(OH)_2D_3$ (1,25D_3) is mainly mediated by the vitamin D_3 hormone receptor (VDR) [14]. VDR is a ligand-driven regulator of gene transcription, a component of a dynamic regulatory network that directly or indirectly controls the expression of more than a thousand genes. Activated VDR is responsible for maintaining calcium and phosphate homeostasis, and is required for proper cellular growth, cell differentiation and apoptosis as well as innate and acquired immunity [15, 16]. Hydroxylases of the cytochrome P450 family are responsible for converting D_3 into its active forms: 25-hydroxylases CYP27A1 (mitochondrial isoenzyme) and CYP2R1 (microsomal isoenzyme), which are localized mainly in liver cells and convert vitamin D_3 to 25(OH)D_3 (25D_3) by attaching a hydroxyl group in the 25th position. Subsequently, 25D_3 1α-hydroxylase (CYP27B1), which hydroxylates carbon in the 1st position, converts 25D_3 into 1,25D_3 – hormonally active vitamin D_3. In addition, the D_3 auto-/para-/endocrine system includes 24-hydroxylase (CYP24A1), which is involved in the catabolism and elimination of 1,25D_3 [17].

In recent years, D_3 has been considered not only as a regulator of bone tissue remodeling processes but also as a compound that has a significant role in neurological diseases [18]. CNS dysfunction and behavioral disorders may be closely associated with impaired cholecalciferol metabolism and lowered D_3 status. Due to the ability to modulate gene transcription and involve non-genomic mechanisms of cell signaling regulation, vitamin D_3 – through 1,25D_3 and VDR – affects the differentiation and maturation of neurons [19], the formation of growth factors [20] as well as synthesis regarding a number of neurotransmitters, such as acetylcholine, dopamine and gamma-aminobutyric acid [21]. D_3 is involved in maintaining the expression level of dopaminergic and cholinergic receptors in the brain [22]. It was also shown that 1,25D_3-VDR modulates mitochondrial function by maintaining respiratory chain activity and enhances autophagy of dysfunctional mitochondria [23]. Additionally, D_3 is able to suppress the expression of NF-κB-associated proinflammatory cytokines, such as tumor necrosis factor alpha, interleukin-6 and interleukin-1 beta, thereby inhibiting neuroinflammation [24]. It downregulates the expression of L-type voltage-sensitive Ca^{2+} channels [25] and counteracts oxidative stress by enhancing antioxidant synthesis and reducing reactive oxygen species [26]. D_3 also plays a role in regulating epigenetic changes, particularly those associated with the remodeling of DNA structure. They are influenced on the one hand by oxidative stress regulated by D_3 as described previously, and on the other hand by histone methylation, which is also modulated by D_3 due to its effect on the transcription of key DNA demethylases [27].

Given its multiple effects on the nervous system as a neurosteroid prohormone, D_3 deficiency is thought to be a universal risk factor for the development of various neurodegenerative diseases, such as multiple sclerosis, Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, diabetic neuropathy, autism, depression and schizophrenia [19, 21, 28]. On experimental models of T1DM and T2DM, we previously demonstrated that D_3 deficiency can exacerbate the manifestations of numerous pathological processes in the tissues of various organs (liver, kidney, bone) [29, 30]. Despite clear associations between D_3 deficiency and T2DM, a causal relationship is not established. It is hypothe-

sized that reduced D_3 status in diabetic animals may be accompanied by changes in the expression of key components of D_3 auto-/paracrine system (VDBP, CYP27B1, CYP24A1 and VDR) in the brain tissue. As of yet, it has not been sufficiently clarified to what extent abnormal functioning of the D_3 auto-/paracrine system in nervous tissue correlates with structural and functional disruption and is accompanied by cellular dysfunction.

Given the importance of cholecalciferol as a neurosteroid in the functioning of the CNS, in this animal study we aimed to examine the D_3 status depending on the changes in the expression of key components of the D_3 auto-/paracrine system in the brain of rats with experimental T2DM as well as to determine the effectiveness of therapeutic application of cholecalciferol.

Materials and Methods

Animals and general experimental design. Three-month-old white male Wistar rats weighing 230 ± 12 g in a total number of 55 were used in the research. In the one-week acclimatization period and during the entire experiment, the animals were kept at a temperature of +18–22°C, humidity of 50–60% and natural light regime “day-night” in plastic cages with free access to water and food. The animals were divided into two groups: the group 1 (10 animals) were control rats that received a standard diet (purchased from REZON-1, Ukraine), the group 2 (45 animals) were rats with experimental type 2 diabetes induced by feeding the animals for 60 days with a self-made, high-calorie diet consisting of 54% standard vivarium diet (REZON-1, Ukraine), 25% fat (pork lard made from visceral fat), 20% fructose (Golden Pharm, Ukraine) and 1% bile (Pharma Cherkas, Ukraine) as well as a single injection of streptozotocin (STZ; Sigma-Aldrich, USA) [31]. A freshly prepared solution of STZ (25 mg/kg in 0.1 M citrate buffer, pH 4.5) was administered intraperitoneally to animals of the group 2 on the 61st day of the experiment after a 12-hour fast. Two weeks later (on the 75th day), the experimental rats were monitored for glucose levels using OneTouch Select glucometer (LifeScan Europe GmbH, Switzerland). Notably, STZ caused an animal mortality rate of 22% over a six-week period following its administration. Seven animals did not develop diabetes and were excluded from the experiment. The following experimental groups were formed: control (10 rats), diabetic (T2DM; 14 rats) and diabetic animals that received vitamin D_3 (cholecalciferol; Sigma-Aldrich, USA) orally once a day in a dose of 1,000 IU per 1 kg of body weight for 30 days (T2DM + D_3; 14 rats). Selection of the dose of cholecalciferol that maintained the optimal level of 25D_3 (about 90 nmol/l) in blood serum of diabetic animals was carried out in our previous experiments [29, 30]. In the 30-day period after the formation of groups, all animals were on standard vivarium diet. On the 105th day from the start of the experiment, the animals were subjected to an insulin tolerance test and then sacrificed under ether anesthesia.

Ethical statement. We performed all animal procedures in accordance with the protocol approved by the Animal Care Ethics Committee of the Palladin Institute of Biochemistry (Protocol N 8, 05.05.2021), adopted on the basis of national and international directives and laws relating to animal welfare: European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, France; 1986); Bioethical expertise of preclinical and other scientific research conducted on animals (Kyiv, Ukraine; 2006).

Insulin tolerance testing. Before testing, rats were deprived of food for 12 hours. After measuring the initial (basal) glucose level (0 min), the animals were injected intraperitoneally with an insulin solution (Actrapid HM Penfill, Novo Nordisk, Denmark; the stock solution contained 100 units of insulin in 1 ml) at a dose of 0.7 units per 1 kg of body weight. With the help of an intravenous catheter, blood samples were taken, and the glucose concentration was determined 30, 45, 60, 90 and 120 min after the injection of insulin. The total area under the curves in response to insulin administration was calculated using Microsoft Excel.

Determination of 25-hydroxyvitamin D_3 content in blood serum and brain tissue extract. The availability of D_3 in animals was estimated by the serological level of 25D_3, which was determined by the immunoenzymatic method developed at the Palladin Institute of Biochemistry, using polyclonal antibodies against 25D_3 and the biotin-streptavidin visualization system [32].

To determine the level of 25D_3 in the nervous system, an extract of brain tissue was prepared. Pre-homogenized brain tissue in liquid nitrogen was transferred to an Eppendorf tube and extraction buffer (phosphate-buffered solution containing 1% Tween-20) was added at a ratio of 1:1, pH 7.4.

The sample was additionally treated with an ultrasonicator on ice and incubated at +4 ˚C for two hours. Further, the sample was centrifuged at 14,000 g for 20 min, +4 ˚C. Supernatant was carefully removed, and aliquots were stored at -20 ˚C, avoiding thawing/freezing of samples. The content of $25D_3$ in the homogenate of rat brain tissue was determined according to a protocol for immunoenzymatic analysis for the determination of $25D_3$ in serological samples.

RNA isolation and real-time polymerase chain reaction (PCR). Total RNA was isolated from rat brain using the innuPREP RNA Mini Kit (Analytik Jena AG, Germany). The mRNA concentration was measured on a DS-11 Series Spectrophotometer/Fluorometer (DeNovix, USA). We used the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., USA) to synthesize cDNA samples for subsequent RT-PCR on a standard real-time PCR Thermal Cycler (Analytik Jena AG, Germany). Specific primer sequences for CYP27B1, CYP24A1, and the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) reference gene were designed using Primer-BLAST software and used at a working concentration of 10 µM: CYP27B1 (5′-TGGGTGCTGGGAACTAACCC-3′ sense, 5′-TCGCAGACTGATTCCACCTC-3′ antisense), CYP24A1 (5′-TGGGTGCTGGGAACTAACCC-3′ sense- TTCGCTCATCTCCCATTCGG-3′ sense, 5′-TTGCTGGTCTTGATTGGGGT-3′ antisense), GAPDH (5′-TGGGTGCTGGGAACTAACCC-3′ sense - TGAACGGGAAGCTCACTGG-3′ sense, 5′-TCCACCACCCTGTTGCTGTA-3′ antisense). Target genes were amplified for 60 cycles using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific Inc., USA). Relative mRNA expression calculations were performed according to the ΔΔCt comparison method. The expression level of each gene was normalized for GAPDH in the same samples and then calculated as a fold change compared to the control [33].

Western blot analysis. We measured the relative protein levels using Western blotting. Total protein extracts were prepared from frozen brain tissue according to a standard protocol using RIPA buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% Triton X-100; 1 mM EGTA; 0.1% SDS; 1% sodium deoxycholate; 10 mM sodium pyrophosphate) and a protease inhibitor cocktail (Sigma-Aldrich, USA), and then the samples were sonicated and centrifuged. The supernatants were stored at -80°C until required. Lysate samples containing 60 µg of protein (measured by Lowry method) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 12.5% gel followed by protein transfer to a nitrocellulose membrane. The membrane was then blocked for one hour with 5% skimmed milk diluted in tris-buffered saline with Tween-20 (TBST, 150 mM NaCl, 10 mM Tris-HCl, and 0.1% Tween 20; pH 7.5) and incubated overnight at +4°C with one of the following primary antibodies: rabbit monoclonal VDR (1:500, NBP2-66778, Novus Biologicals, USA), rabbit polyclonal VDBP (1:1000, NBP1-88027, Novus Biologicals, USA), rabbit polyclonal CYP27B1 (1:500, PA5-26065, Invitrogen, Thermo Fisher Scientific, USA) and rabbit polyclonal CYP24A1 (1:500, PA5-79127, Invitrogen, Thermo Fisher Scientific, USA). Subsequently, the membrane was thoroughly rinsed and incubated for one hour with goat anti-rabbit IgG (H+L)-HRP conjugate (1:4000, #1721019, Bio-Rad, USA) secondary antibody at room temperature. Thereafter, the membrane was developed with chemiluminescent agents: p-Coumaric acid (Sigma-Aldrich, USA) and luminol (AppliChem GmbH, Germany). Target protein immunoreactive signals were adjusted for corresponding β-actin levels (mouse monoclonal anti-β-Actin Peroxidase conjugate, 1:10000, A3854, Sigma-Aldrich, USA). Immunoreactive bands were quantified using Gel-Pro Analyzer v3.1 software.

Statistical analysis. We repeated all the experiments three times, with representative results presented. Data were collected from at least six rats each group and presented as means ± SEM. The Shapiro-Wilk test was used for testing on normal distribution. Statistical differences were determined by one-way ANOVA with the following Tukey's post-hoc test. Differences were considered significant when $P \leq 0.05$. All statistical analysis was performed using OriginPro 8.5 (OriginLab Corporation, Northampton, MA, USA).

Results and Discussion

The development of T2DM and impairment of carbohydrate homeostasis in rats under the conditions of our experiment was confirmed by a 2.4-fold increase in the concentration of blood glucose at the time of sacrifice (Table). Significant hyperglycemia in diabetic animals indicates decompensation of insulin resistance of peripheral tissues by increased insulin secretion from β-cells of the pancreas or exhaustion of the insular apparatus. Such disorders

T a b l e. Blood glucose level and $25D_3$ content in serum and brain tissues

Control	T2DM	T2DM + Vitamin D_3
	Blood glucose, mmol/l	
5.60 ± 0.14	13.10 ± 1.70*	11.00 ± 0.81
	Serum $25D_3$, nmol/l	
88.54 ± 7.3	27.92 ± 4.2*	85.63 ± 7.5#
	Brain tissue $25D_3$, nmol/g	
55.83 ± 3.0	44.50 ± 2.4*	63.21 ± 3.1*#

Note. All data are presented as mean ± SEM, $n = 6$; *$P \leq 0.05$ denotes significance compared with control, #$P \leq 0.05$ denotes significance compared with diabetes

usually characterize the presence of a later stage of T2DM development.

Before termination of rats, we conducted an intraperitoneal test of tolerance to exogenous insulin, which demonstrated a significant abnormality in the sensitivity of peripheral tissues to the action of the hormone in comparison with the control group. In diabetic animals, the basal level of glucose in the blood after overnight fasting increased significantly in all investigated time intervals after the injection of insulin (0.7 U/kg), Fig. 1, *A*. The level of glucose in the blood of rats of the control group under the influence of insulin reached minimum values in 45 min (2.63 mmol/l), while in animals with T2DM – in 120 min (7.50 mmol/l). The area under the curve, calculated on the basis of the insulin tolerance test, increased almost four times in rats with experimental diabetes compared to control animals, suggesting a significant slowing down of glucose absorption by peripheral tissues due to the development of insulin resistance (Fig. 1, *B*).

Maintaining a normal D_3 status prevents the development of numerous diseases, including neurodegenerative [28]. With this in mind, we evaluated the level of 25-hydroxyvitamin D_3 ($25D_3$) in blood serum as the main marker of vitamin D_3 bioavailability [34]. Type 2 diabetes was found to be accompanied by a threefold decrease in serum $25D_3$ compared to control rats (Table 1). Therapeutic administration of D_3 to rats with T2DM normalized the level of $25D_3$

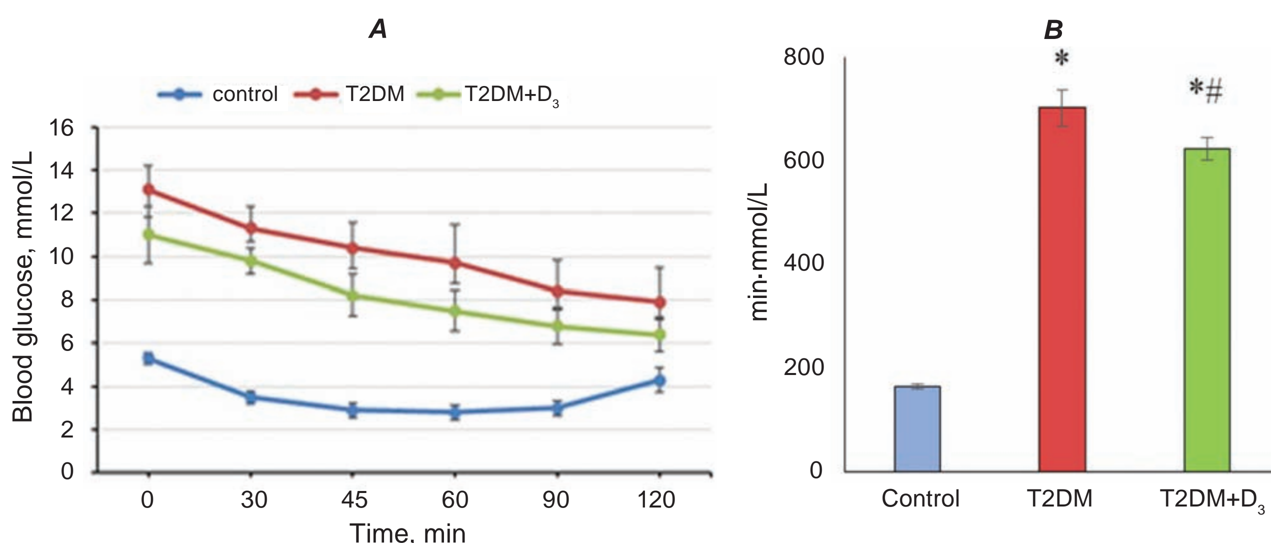

*Fig. 1. Insulin tolerance test in male Wistar rats treated with a high-calorie diet and a single injection of streptozotocin (25 mg/kg): (**A**) dynamics of changes in the concentration of glucose in the blood of rats after intraperitoneal injection of insulin (0.7 units/kg); (**B**) the area under the "time-glucose concentration" curve. All data are presented as mean ± SEM, n = 6; *P < 0.05 denotes significance compared with control, #P < 0.05 denotes significance compared with T2DM*

in blood serum without a noticeable effect on blood glucose concentration (Table).

The presence of pronounced D_3 deficiency can probably be explained by inhibition of synthesis and/or increased catabolism of the hydroxylated form of D_3. In our previous work, we found significant diabetes-induced changes in the functioning of enzymes metabolizing D_3 in liver tissue [30].

The D_3-deficient state of animals with T2DM was expected to be accompanied by a decrease in the level of $25D_3$ in rat brain tissue homogenate. The level of $25D_3$ in the brain of diabetic animals decreased by 1.25 times compared to the intact control. Administration of cholecalciferol caused a 1.42- and 1.13-fold increase in $25D_3$ content compared to diabetic and control rats, respectively. The effect of cholecalciferol may indicate a redistribution of the circulating pool of $25D_3$ in favor of the most physiologically active organs, thereby emphasizing the exceptional importance of maintaining the bioavailability of the D_3 prohormone for nervous tissue. As evidenced by previous reports, the D_3 metabolite $25D_3$ is also present in cerebrospinal fluid (CSF) and there is a correlation between brain tissue, CSF and serum $25D_3$ levels [18]. Thus, our data confirm that in T2DM the $25D_3$ level decreased not only in blood serum, but also in the brain.

A decrease in the content of VDBP can be referred to as one of the probable mechanisms, which, along with the alteration of the first step of hydroxylation of cholecalciferol in the liver, could partially explain the detected D_3 deficiency in type 2 diabetes mellitus. Since VDBP belongs to the albumin family and is known to be the main transporter of active metabolites of D_3 (85–90% of the total pool), it can effectively influence the stability, bioavailability and biological efficacy of D_3 [35]. In addition, it is known that VDBP is able to prevent disseminated intravascular coagulation by neutralizing serum globular actin and is considered as an activator of macrophages due to the enhancement of C5a-mediated chemotaxis of macrophages involved in the implementation of the immune response [36]. In fact, due to the aforementioned functions, VDBP may be associated with the pathophysiology of neurological complications of diabetes. One of the mechanisms of D_3 bioavailability for the CNS is based on the "free hormone" hypothesis, according to which unbound $25D_3$ and $1,25D_3$ from blood serum enter brain cells, overcoming the blood-brain barrier [37]. Based on this opinion, the $25D_3$-VDBP complex is considered a vital systemic reservoir in the bloodstream, which does not directly participate in the entry of active metabolites of D_3 into cells. However, some recent studies have shown that the $25(OH)D_3$-VDBP complex is also able to be internalized by megalin-dependent transport to pass across the blood-brain barrier [38]. Thus, serum VDBP deficiency may limit the availability of D_3 metabolites to the CNS. At the same time, changes in the level of VDBP protein in nerve tissue associated with T2DM have not been studied to date. We found an almost two-fold decrease in the content of VDBP protein in the brain tissue of diabetic rats compared to controls (Fig. 2). Therapeutic administration of D_3 did not affect T2DM-induced changes in VDBP expression. The data obtained correlate with a decrease in the level of $25D_3$ in the blood serum and are consistent with a reduced content of this metabolite in brain tissue.

Changes in tissue expression of cytochromes belonging to the CYP/CYP450 superfamily of monooxygenases, which are responsible for the forma-

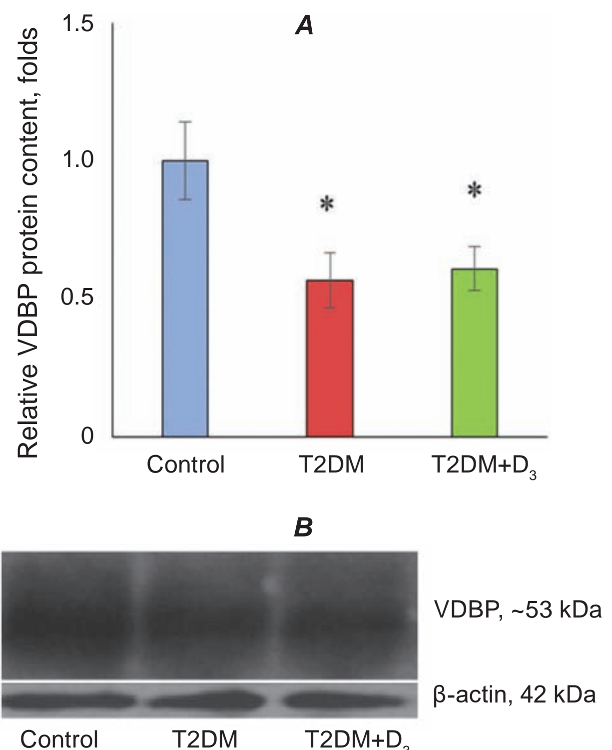

*Fig. 2. The level of VDBP protein in the brain of rats with type 2 diabetes and after supplementation of vitamin D_3. Quantification of VDBP level in the brain (**A**) and representative immunoblot (**B**) are shown. All data are presented as mean ± SEM, n = 6; *P < 0.05 denotes significance compared with control*

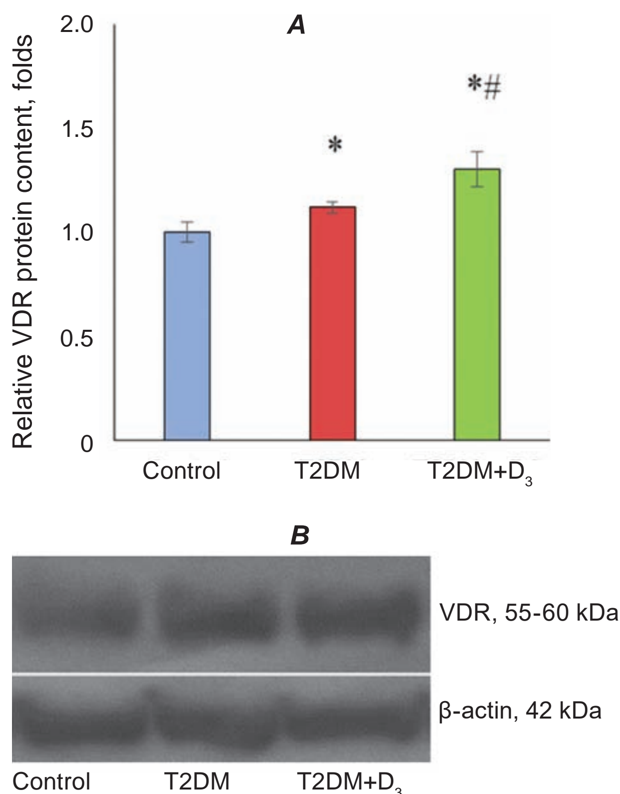

*Fig. 3. Relative content of VDR protein in the brain of rats with type 2 diabetes and after supplementation of vitamin D_3. Quantification of VDR level in the brain (**A**) and representative immunoblot (**B**) are shown. All data are presented as mean ± SEM, n = 6; *P < 0.05 denotes significance compared with control, #P < 0.05 denotes significance compared with T2DM*

tion of hydroxylated derivatives of cholecalciferol, may play a significant role in disrupting its bioavailability and VDR-mediated signaling in cells in neurodegenerative diseases caused by T2DM as well as in the mechanism of the neuroprotective impact of D_3. However, the alterations of the expression of VDR and D_3-related enzymes in normal and impaired D_3 bioavailability, associated with the development of T2DM, is currently one of the least studied issues. In light of this, our next task was to investigate the changes in the expression of key components of the D_3 auto-/paracrine system in the CNS in experimental type 2 diabetes and to find out the effectiveness of D_3 in correcting the identified abnormalities.

Since it has now been clearly shown that the hormonally active form of D_3 in various cell types exerts its biological effects through a specific receptor for $1,25D_3$ VDR, we first investigated changes in VDR protein expression. It was shown that in T2DM there was a slight upregulation of VDR protein level in the brain tissue (Fig. 3). We demonstrated the ability of D_3 to increase brain VDR expression levels compared to both diabetic and control animal groups (by 30 and 17%, respectively).

Our next task was to study the expression of 25-hydroxyvitamin D_3 1α-hydroxylase (CYP27B1), the rate-limiting enzyme responsible for the synthesis of the D_3 hormone, $1,25D_3$, which through VDR receptors provides the realization of the biological functions of vitamin D_3 cholecalciferol [24, 39]. It is possible that CYP27B1, being expressed in the kidneys and many other extrarenal tissues of the body, can locally convert $25D_3$ to $1,25D_3$ in the brain of rats and contribute to the involvement of D_3-hormone in the regulation of numerous cellular functions by auto-/paracrine mechanism. As shown in Fig. 4, *A*, diabetes elicited a significant (1.7-fold) elevation of CYP27B1 mRNA expression and an almost 1.5-fold increase in the protein content of this hydroxylase (Fig. 4, *B*) in brain tissue compared to control animals that may reflect a compensatory response to impaired VDR signaling in nerve cells and/or lowered brain $25D_3$ levels in T2DM. Administration of D_3 further upregulated CYP27B1 mRNA expression to a level nearly two-fold and 3.5-fold higher than in diabetic and control animals, respectively. At the same time, the content of CYP27B1 protein under the influence of cholecalciferol returned to the level of control values. This discrepancy between CYP27B1 gene expression and protein synthesis was unexpected and requires further research.

25-Dihydroxyvitamin D-24-hydroxylase (CYP24A1), as reported, plays an important role in the biological action of D_3 by providing the catabolism of cholecalciferol in various tissues of the body. This mitochondrial monooxygenase converts active vitamin D_3, $1,25D_3$, into inactive metabolite $1,24,25(OH)_3D_3$ [13, 39]. Regulation of the expression of this enzyme is one of the main mechanisms by which target cells respond to calcitriol and change their mode of functioning. VDR is known to respond to calcitriol exposure by increasing CYP24A1 gene expression to metabolize $25D_3$ and $1,25D_3$ to biologically inert water-soluble inactive forms (calcitroic acid and lactone, which are excreted in bile and urine). It is known that calcitriol suppresses its own synthesis and stimulates its own destruction through the induction of the CYP24A1 by a negative feedback mechanism [39]. In this study, we showed that

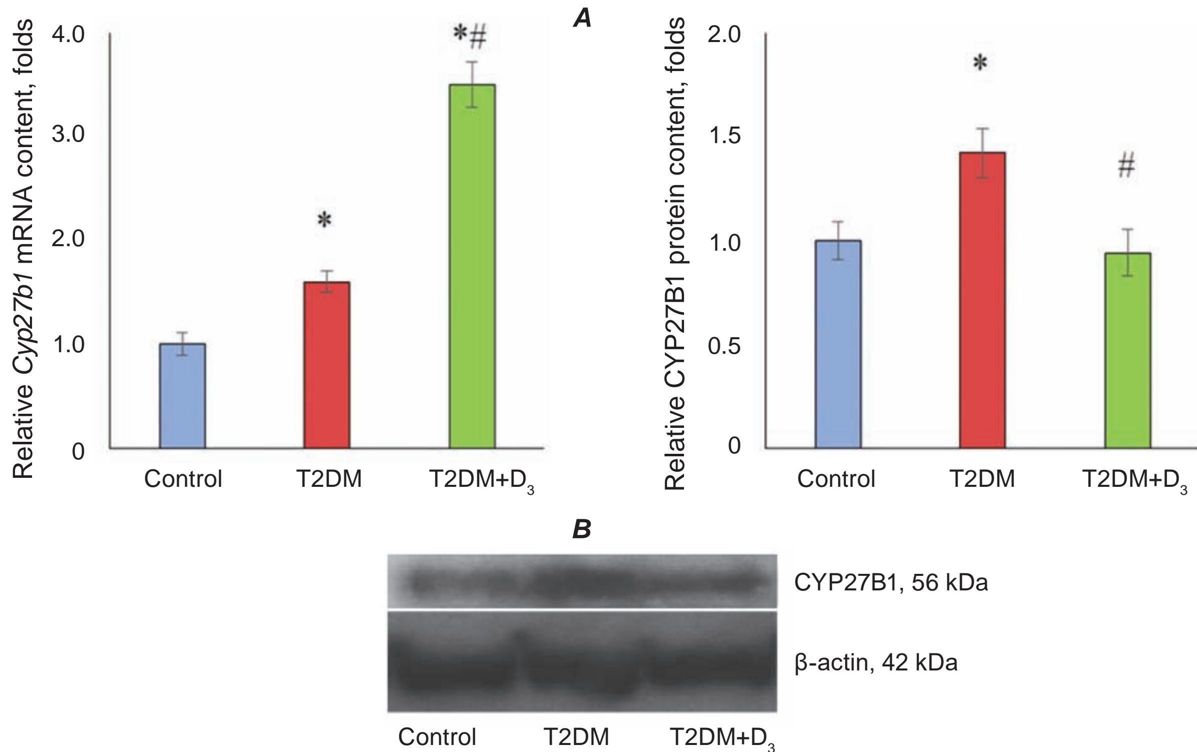

*Fig. 4. Relative content of CYP27B1 mRNA (**A**) and protein (**B**) in the brain of rats with type 2 diabetes and after supplementation of vitamin D_3. All data are presented as mean ± SEM, n = 6; *P < 0.05 denotes significance compared with control, #P < 0.05 denotes significance compared with T2DM*

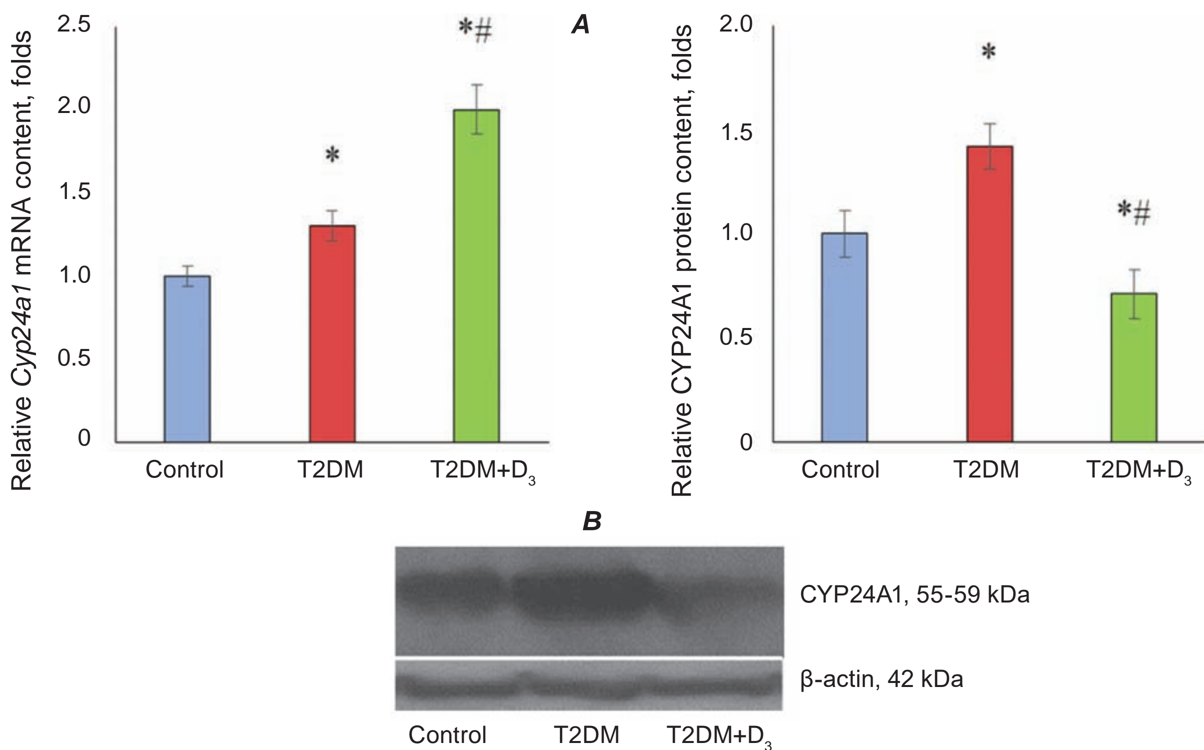

*Fig. 5. Relative content of CYP24A1 mRNA (**A**) and protein (**B**) in the brain of rats with type 2 diabetes and after supplementation of vitamin D_3. All data are presented as mean ± SEM, n = 6; *P < 0.05 denotes significance compared with control, #P < 0.05 denotes significance compared with T2DM*

in diabetic animals there is an increase in CYP24A1 mRNA level by 1.4 times, and its protein level by 1.5 times compared to the control (Fig. 5, *A*, *B*). Such a change may be a response to the increased expression of CYP27B1, which ensures the synthesis of calcitriol, as well as to the augmented content of $25D_3$ in the brain tissue. Administration of D_3 further stimulated the synthesis of CYP24A1 mRNA to a level that was two times higher than that of the intact control group. Instead, the content of CYP24A1 protein unexpectedly decreased to the values below control animals.

As part of the scientific discussion of the findings obtained, it should be emphasized that in this study, using quantitative real-time PCR and/or Western blotting, we carried out for the first time a comprehensive comparative assessment of the levels of proteins associated with the metabolism and $1{,}25D_3$ signaling in the brain of rats with experimental T2DM. Our data showed that when VDBP levels are reduced and D_3 deficiency occurs, there is a concomitant significant upregulation of mRNA and/or protein of VDR, CYP27B1 and CYP24A1 in diabetic brain compared with controls. We observed approximately 1.35-fold elevation of CYP24A1 mRNA expression, which can be explained by negative feedback through VDR signaling as a result of increased expression of CYP27B1 gene. The effectiveness of a compensatory increase in the expression of VDR protein is apparently limited by diabetes-induced insufficient level of prohormone ($25D_3$) in the nervous tissue, which is converted into a hormonally active form under the influence of CYP27B1. Most likely, it is precisely because of overcoming this deficiency that the supplementation of cholecalciferol and the complete restoration of $25D_3$ levels leads to a further increase in the level of cytochrome CYP27B1 and CYP24A1 gene expression to values even much higher than in control animals. Nevertheless, this claim requires further investigation in future experiments. Surprisingly, overexpression of CYP27B1 and CYP24A1 did not correlate with the protein levels of the corresponding genes, which were significantly reduced after cholecalciferol treatment.

Overall, we can speculate that our findings may, at least partially, be a consequence of T2DM-induced brain changes associated with a compensatory mechanism promoting antioxidant defense against oxidative/nitrosative stress as well as alleviating neuroinflammation and enhancing neurotrophic support, etc. The hormonally active form of D_3 modulates numerous factors acting in these pathways. Aberrant redox regulation is a hallmark of T2DM-induced neuropathology. The imbalance in the production of reactive oxygen and nitrogen species along with deficiency in enzymatic and nonenzymatic antioxidants can result in oxidative/nitrosative stress [8, 9]. Reduced glutathione (GSH) is thought to be one of the most pivotal cellular antioxidants. It is currently postulated that $1{,}25D_3$ enhances the expression of the γ-glutamyl transpeptidase GGT gene and increases the activation of GSH reductase, GSH peroxidase and the catalytic subunit of glutamyl cysteine ligase, promoting the synthesis and conversion of GSH in nerve cells [40]. Of interest, GSH has the capability to regulate glutamatergic and GABAergic neurotransmitter systems. GSH deficiency as a result of redox dysregulation can induce their hypofunction [41]. $1{,}25D_3$ also acts as a neuroprotective agent by reducing NF-κB-mediated synthesis of inducible nitric oxide synthase, which is a key enzyme producing nitric oxide (NO) [42]. NO and its reactive metabolites cause damage to neurons, glial cells and oligodendrocytes when generated in large quantities, further contributing to nitrosative stress. More recently, enhancement of genomic D_3 signaling by overexpression of VDR in human neural progenitor cells ACS-5003 was found to protect against hyperglycemia-induced oxidative stress and inflammation by activating the redox-sensitive transcription factor Nrf2 and its target genes, including SOD2 and HMOX1, and accordingly, knockdown of the VDR gene counteracts these effects [43]. There is also evidence suggesting a significant role for D_3 in immunomodulation and neuroinflammation in the CNS. It is reported that $1{,}25D_3$, by recruiting the genomic pathway, suppresses the transcription of the cyclooxygenase 2 (a rate-limiting enzyme in prostaglandin biosynthesis) gene, which may lead to subsequent inhibition of proinflammatory cytokines synthesis and secretion [44]. At the same time, $1{,}25D_3$ exerts neurotrophic support through upregulation of glial cell line-derived neurotrophic factor, neurotrophin-3 and suppression of neurotrophin-4 [20]. In addition, a lack of D_3 affects the expression of genes that are important for mitochondrial function, cytoskeletal maintenance and neurotransmission [21-23].

Collectively, our findings suggest that increased mRNA and/or protein expression of VDR, CYP27B1 and CYP24A1 can be involved in the modulation of mentioned pathways through the genomic mechanism in order to ameliorate the T2DM-assosiated

brain disturbances. However, further trials are needed to verify this suggestion and determine the exact regulatory mechanisms of D_3 action.

The main limitation of this study is that we performed all measurements on the whole brain, which makes it difficult to further analyze a multi-directional response from different cell types and brain regions. It would also be valuable, in addition to studying the level of RNA transcripts and proteins of target genes, to determine the activity of D_3 metabolizing enzymes as well as the transcriptional activity of VDR. In turn, the strength of our research lies in a comprehensive study characterizing the state of the D_3 auto-/paracrine system of the brain in the same animal model of T2DM in connection with D_3-deficient status and after $25D_3$ repletion. So far, most studies evaluating D_3 system-related genes in T2DM have mainly focused on polymorphisms of these genes or assessing serum D_3 levels in diabetic patients. Future studies should be designed to explore the expression levels of VDR and related enzymes simultaneously with the expression of other molecular players involved in oxidative/nitrosative stress, neuroinflammation, mitochondrial dysfunction and neurotrophic support in T2DM.

Conclusions. We demonstrated the relationship between the pool of circulating $25D_3$ and its level in the brain tissue of rats using an experimental model of type 2 diabetes. A significant decrease in the content of $25D_3$ in both blood serum and brain tissue was established. Profound abnormalities in the expression of key components of the D_3 auto-/paracrine system indicate marked changes in the D_3 status of diabetic animals: an increase in the level of mRNA transcripts and/or proteins of VDR, CYP27B1 and CYP24A1 against the background of a reduced level of VDPB protein. We found the systemic corrective effect of D_3 on the concentration of circulating $25D_3$, its bioavailability in the CNS and the expression level of CYP27B1 and CYP24A1 in the brain of T2DM rats. Overall, the established pattern of impaired expression of the key enzymes of D_3 conversion in brain tissue may indicate the compensatory intensification of calcitriol metabolism, presumably contributing to the maintenance of its physiological levels and effective VDR expression under pathological conditions of T2DM.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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СТАН ВІТАМІН D_3 АУТО-/ПАРАКРИННОЇ СИСТЕМИ ГОЛОВНОГО МОЗКУ ЩУРІВ В ЗАЛЕЖНОСТІ ВІД ЗАБЕЗПЕЧЕНОСТІ ОРГАНІЗМУ ВІТАМІНОМ D_3 ЗА ЕКСПЕРИМЕНТАЛЬНОГО ЦУКРОВОГО ДІАБЕТУ 2 ТИПУ

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Гормонально активна форма вітаміну D_3 ($1,25(OH)_2D_3$) є пара- і аутокринним нейростероїдним регулятором, який відіграє важливу роль у функціонуванні нервової системи. На експериментальній моделі цукрового діабету 2 типу (ЦД2) було досліджено стан забезпеченості тваринного організму вітаміном D_3 (за рівнем $25OHD_3$), а також залежність між пулом циркулюючого $25OHD_3$ та його рівнем у тканині головного мозку щурів. Виявлено суттєве зниження вмісту $25OHD_3$ у сироватці крові, що корелювало зі зниженням вмісту цього метаболіту у нервовій тканині. Порушення D_3-вітамінного статусу діабетичних тварин супроводжувалось змінами експресії ключових компонентів вітамін D_3 ауто-/паракринної системи. Показано підвищення рівня мРНК та протеїну 25OHD lα-гідроксилази (CYP27B1) під час зниження експресії вітамін

D_3-24-гідроксилази (CYP24A1), що відповідно забезпечують локальне утворення та деградацію у нервовій тканині гормонально активної форми вітаміну – $1,25(OH)_2D_3$. Крім того, рівень експресії рецептора вітаміну D_3 (VDR), через який реалізуються ауто-/паракринні ефекти $1,25(OH)_2D_3$ у головному мозку, істотно знижувався за ЦД2. Продемонстровано коригувальну дію вітаміну D_3 на забезпеченість організму тварин вітаміном D_3, його біодоступність у ЦНС та рівень експресії CYP27BI, CYP24A1 та VDR у головному мозку щурів за ЦД2.

Ключові слова: вітамін D_3, 25-гідроксивітамін D_3, ауто-/паракринна система вітаміну D_3, мозок, цукровий діабет 2 типу.

References

1. Eizirik DL, Pasquali L, Cnop M. Pancreatic β-cells in type 1 and type 2 diabetes mellitus: different pathways to failure. *Nat Rev Endocrinol.* 2020; 16(7): 349-362.
2. Sanaye MM, Kavishwar SA. Diabetic Neuropathy: Review on Molecular Mechanisms. *Curr Mol Med.* 2023; 23(2): 97-110.
3. Pickering J, Wong R, Al-Salami H, Lam V, Takechi R. Cognitive Deficits in Type-1 Diabetes: Aspects of Glucose, Cerebrovascular and Amyloid Involvement. *Pharm Res.* 2021; 38(9): 1477-1484.
4. Hamzé R, Delangre E, Tolu S, Moreau M, Janel N, Bailbé D, Movassat J. Type 2 Diabetes Mellitus and Alzheimer's Disease: Shared Molecular Mechanisms and Potential Common Therapeutic Targets. *Int J Mol Sci.* 2022; 23(23): 15287.
5. An Y, Xu BT, Wan SR, Ma XM, Long Y, Xu Y, Jiang ZZ. The role of oxidative stress in diabetes mellitus-induced vascular endothelial dysfunction. *Cardiovasc Diabetol.* 2023; 22(1): 237.
6. Batista TM, Haider N, Kahn CR. Defining the underlying defect in insulin action in type 2 diabetes. *Diabetologia.* 2021; 64(5): 994-1006.
7. Gholamhosseinian A, Abbasalipourkabir R, Ziamajidi N, Sayadi M, Sayadi K. The anti-inflammatory effect of omega-3 polyunsaturated fatty acids dramatically decreases by iron in the hippocampus of diabetic rats. *Life Sci.* 2020; 245: 117393.
8. Khaleghi-Mehr M, Delshad AA, Shafie-Damavandi S, Roghani M. Metformin mitigates amyloid β1-40-induced cognitive decline via attenuation of oxidative/nitrosative stress and neuroinflammation. *Metab Brain Dis.* 2023; 38(4): 1127-1142.
9. Muriach M, Flores-Bellver M, Romero FJ, Barcia JM. Diabetes and the brain: oxidative stress, inflammation, and autophagy. *Oxid Med Cell Longev.* 2014; 2014: 102158.
10. Luo JS, Ning JQ, Chen ZY, Li WJ, Zhou RL, Yan RY, Chen MJ, Ding LL. The role of mitochondrial quality control in cognitive dysfunction in diabetes. *Neurochem Res.* 2022; 47(8): 2158-2172.
11. Saha D, Paul S, Gaharwar U, Priya A, Neog A, Singh A, Bk B. Cdk5-mediated brain unfolded protein response upregulation associated with cognitive impairments in type 2 diabetes and ameliorative action of NAC. *ACS Chem Neurosci.* 2023; 14(15): 2761-2774.
12. Anirudhan A, Ahmad SF, Emran TB, Angulo-Bejarano PI, Sharma A, Ahmed SSSJ. Comparative Efficacy of Metformin and Glimepiride in Modulating Pharmacological Network to Increase BDNF Levels and Benefit Type 2 Diabetes-Related Cognitive Impairment. *Biomedicines.* 2023; 11(11): 2939.
13. Saponaro F, Saba A, Zucchi R. An Update on Vitamin D Metabolism. *Int J Mol Sci.* 2020; 21(18): 6573.
14. Shymanskyi IO, Lisakovska OO, Mazanova AO, Veliky MM. Vitamin D in transcriptional regulation of immune response and inflammation. *Adv Med Biol.* 2021; 183: 1-83.
15. Mesinovic J, Mousa A, Wilson K, Scragg R, Plebanski M, de Courten M, Scott D, Naderpoor N, de Courten B. Effect of 16-weeks vitamin D replacement on calcium-phosphate homeostasis in overweight and obese adults. *J Steroid Biochem Mol Biol.* 2019; 186: 169-175.
16. Martens PJ, Gysemans C, Verstuyf A, Mathieu AC. Vitamin D's effect on immune function. *Nutrients.* 2020; 12(5): 1248.
17. Christakos S, Dhawan P, Verstuyf A, Verlinden L, Carmeliet G. Vitamin D: Metabolism, Molecular Mechanism of Action, and Pleiotropic Effects. *Physiol Rev.* 2016; 96(1): 365-408.
18. Plantone D, Primiano G, Manco C, Locci S, Servidei S, De Stefano N. Vitamin D in neurological diseases. *Int J Mol Sci.* 2022; 24(1): 87.
19. Pertile RAN, Brigden R, Raman V, Cui X, Du Z, Eyles D. Vitamin D: A potent regulator

of dopaminergic neuron differentiation and function. *J Neurochem.* 2023; 166(5): 779-789.
20. Alqudah M, Khanfar M, Alfaqih MA, Al-Shboul O, Ghazi Al-U'Datt D, Al-Dwairi A, Allouh M. Correlation between vitamin D and serum brain derived neurotropic factor levels in type 2 diabetes mellitus patients. *Biomed Rep.* 2022; 16(6): 54.
21. Moretti R, Morelli ME, Caruso P. Vitamin D in neurological diseases: a rationale for a pathogenic impact. *Int J Mol Sci.* 2018; 19(8): 2245.
22. Peeyush KT, Savitha B, Sherin A, Anju TR, Jes P, Paulose CS. Cholinergic, dopaminergic and insulin receptors gene expression in the cerebellum of streptozotocin-induced diabetic rats: functional regulation with Vitamin D3 supplementation. *Pharmacol Biochem Behav.* 2010; 95(2): 216-222.
23. Cui C, Cui J, Jin F, Cui Y, Li R, Jiang X, Tian Y, Wang K, Jiang P, Gao J. Induction of the Vitamin D Receptor Attenuates Autophagy Dysfunction-Mediated Cell Death Following Traumatic Brain Injury. *Cell Physiol Biochem.* 2017; 42(5): 1888-1896.
24. Farhangi MA, Mesgari-Abbasi M, Nameni G, Hajiluian G, Shahabi P. The effects of vitamin D administration on brain inflammatory markers in high fat diet induced obese rats. *BMC Neurosci.* 2017; 18(1): 81.
25. Gooch H, Cui X, Anggono V, Trzaskowski M, Tan MC, Eyles DW, Burne THJ, Jang SE, Mattheisen M, Hougaard DM, Pedersen BN, Cohen A, Mortensen PB, Sah P, McGrath JJ. 1,25-Dihydroxyvitamin D modulates L-type voltage-gated calcium channels in a subset of neurons in the developing mouse prefrontal cortex. *Transl Psychiatry.* 2019; 9(1): 281.
26. Almeida Moreira Leal LK, Lima LA, Alexandre de Aquino PE, Costa de Sousa JA, Jataí Gadelha CV, Felício Calou IB, Pereira Lopes MJ, Viana Lima FA, Tavares Neves KR, Matos de Andrade G, Socorro de Barros Viana G. Vitamin D (VD3) antioxidative and anti-inflammatory activities: Peripheral and central effects. *Eur J Pharmacol.* 2020; 879: 173099.
27. Sui A, Xu Y, Pan B, Guo T, Wu J, Shen Y, Yang J, Guo X. Histone demethylase KDM6B regulates 1,25-dihydroxyvitamin D_3-induced senescence in glioma cells. *J Cell Physiol.* 2019; 234(10): 17990-17998.
28. Grygorieva N, Tronko M, Kovalenko V, Komisarenko S, Tatarchuk T, Dedukh N, Veliky M, Strafun S, Komisarenko Y, Kalashnikov A, Orlenko V, Pankiv V, Shvets O, Gogunska I, Regeda S. Ukrainian Consensus on Diagnosis and Management of Vitamin D Deficiency in Adults. *Nutrients.* 2024; 16(2): 270.
29. Mazanova A, Shymanskyi I, Lisakovska O, Hajiyeva L, Komisarenko Y, Veliky M. Effects of Cholecalciferol on Key Components of Vitamin D-Endo/Para/Autocrine System in Experimental Type 1 Diabetes. *Int J Endocrinol.* 2018; 2018: 2494016.
30. Shymanskyi I, Mazanova A, Lisakovska O, Labudzynskyi D, Makarova O, Komisarenko Yu, Veliky M. The role of vitamin D auto /paracrine system in the development of metabolic inflammation of liver tissue in experimental type 2 diabetes. *Endokrynologia.* 2021; 26(3): 271-280.
31. Natrus LV, Osadchuk YuS, Lisakovska OO, Labudzinskyi DO, Klys YuG, Chaikovsky YuB. Effect of Propionic Acid on Diabetes-Induced Impairment of Unfolded Protein Response Signaling and Astrocyte/Microglia Crosstalk in Rat Ventromedial Nucleus of the Hypothalamus. *Neural Plast.* 2022; 2022: 6404964.
32. Mazanova AO, Shymanskyy IO, Veliky MM. Development and validation of immunoenzyme test-system for determination of 25-hydroxyvitamin D in blood serum. *Biotechnol Acta.* 2016; 9(2): 28-36.
33. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−ΔΔC(T)) method. *Methods.* 2001; 25(4): 402-408.
34. Bivona G, Gambino CM, Lo Sasso B, Scazzone C, Giglio RV, Agnello L, Ciaccio M. Serum Vitamin D as a Biomarker in Autoimmune, Psychiatric and Neurodegenerative Diseases. *Diagnostics (Basel).* 2022; 12(1): 130.
35. Pop TL, Sîrbe C, Benţa G, Mititelu A, Grama A. The Role of Vitamin D and Vitamin D Binding Protein in Chronic Liver Diseases. *Int J Mol Sci.* 2022; 23(18): 10705.
36. Langer-Gould A, Lucas RM, Xiang AH, Wu J, Chen LH, Gonzales E, Haraszti S, Smith JB, Quach H, Barcellos LF. Vitamin D-Binding Protein Polymorphisms, 25-Hydroxyvitamin D, Sunshine and Multiple Sclerosis. *Nutrients.* 2018; 10(2): 184.
37. Bikle DD, Schwartz J. Vitamin D Binding Protein, Total and Free Vitamin D Levels in

Different Physiological and Pathophysiological Conditions. *Front Endocrinol (Lausanne).* 2019; 10: 317.

38. Nykjaer A, Fyfe JC, Kozyraki R, Leheste JR. Jacobsen C, Nielsen MS, Verroust PJ, Aminoff M, de la Chapelle A, Moestrup SK, Ray R, Gliemann J, Willnow TE, Christensen EI. Cubilin dysfunction causes abnormal metabolism of the steroid hormone 25(OH) vitamin D(3). *Proc Natl Acad Sci USA.* 2001; 98(24): 13895-13900.
39. Meyer MB, Pike JW. Mechanistic homeostasis of vitamin D metabolism in the kidney through reciprocal modulation of Cyp27b1 and Cyp24a1 expression. *J Steroid Biochem Mol Biol.* 2020; 196: 105500.
40. Garcion E, Sindji L, Leblondel G, Brachet P, Darcy F. 1,25-dihydroxyvitamin D_3 regulates the synthesis of gamma-glutamyl transpeptidase and glutathione levels in rat primary astrocytes. *J Neurochem.* 1999; 73(2): 859-866.
41. Beeraka NM, Avila-Rodriguez MF, Aliev G. Recent Reports on Redox Stress-Induced Mitochondrial DNA Variations, Neuroglial Interactions, and NMDA Receptor System in Pathophysiology of Schizophrenia. *Mol Neurobiol.* 2022; 59(4): 2472-2496.
42. Said MA. Vitamin D attenuates endothelial dysfunction in streptozotocin induced diabetic rats by reducing oxidative stress. *Arch Physiol Biochem.* 2022; 128(4): 959-963.
43. Liang Y, Yu H, Ke X, Eyles D, Sun R, Wang Z, Huang S, Lin L, McGrath JJ, Lu J, Guo X, Yao P. Vitamin D deficiency worsens maternal diabetes induced neurodevelopmental disorder by potentiating hyperglycemia-mediated epigenetic changes. *Ann N Y Acad Sci.* 2021; 1491(1): 74-88.
44. Wang Q, He Y, Shen Y, Zhang Q, Chen D, Zuo C, Qin J, Wang H, Wang J, Yu Y. Vitamin D inhibits COX-2 expression and inflammatory response by targeting thioesterase superfamily member 4. *J Biol Chem.* 2014; 289(17): 11681-11694.