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The Brain Adenylyl Cyclase Signaling System and Cognitive Functions in Rats with Neonatal Diabetes under the Influence of Intranasal Serotonin

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Abstract

Type 2 diabetes mellitus is often associated with the neurodegenerative changes, and this is regarded as being the cause of cognitive deficit and other brain dysfunctions. The data obtained by us and the other authors showed that alterations in the brain signaling systems regulated by different hormones and neurotransmitters contribute to triggering and development of neurodegenerative processes in diabetes. However, the activity of brain adenylyl cyclase system and the cognitive functions, their interrelation, and the influence of intranasal serotonin on them in type 2 diabetes are poorly understood yet. We investigated the hormonal sensitivity of adenylyl cyclase system in the brain of female rats with the neonatal model of type 2 diabetes and the influence of 8-weeks treatment with intranasal serotonin (20 μg/rat daily) on the brain adenylyl cyclase system and cognition in diabetes. It was shown that in the diabetic brain the regulatory effects of hormones (relaxin, adrenergic agonists, dopamine, serotonin) activating adenylyl cyclase via Gs proteins changed in a receptor-specific manner and were restored by intranasal serotonin. The effects of hormones inhibiting adenylyl cyclase via Gi proteins were significantly decreased, especially in the case of agonists of type 1 serotonin receptors. The intranasal serotonin treatment led to their partial or complete restoration. Using Morris water maze test we showed that intranasal serotonin improves diabetes-associated impaired learning and spatial memory. Summing up, in the brain of diabetic rats the functional activity of hormone-sensitive adenylyl cyclase signaling system was altered, most dramatically in the G_i-coupled cascades. The intranasal serotonin treatment improved both the signal transduction via the brain adenylyl cyclase system and cognitive functions in type 2 diabetes.

Abbreviations: AC: Adenylyl Cyclase; AR: Adrenergic Receptor; DAR: Dopamine Receptor; DM: Diabetes Mellitus; EMD-386088: 5-chloro-2-methyl-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-indole; G_s and G_i proteins: Heterotrimeric G proteins of the stimulating and inhibitory types, respectively; GppNHp, β,γ-imidoguanosine-5'-triphosphate; 5-HT, 5-hydroxytryptamine (serotonin); 5-HTR, 5-Hydroxytryptamine Receptor; IS: Intranasal Serotonin; 5-MeO-DMT: 5-Methoxy-N,N-Dimethyltryptamine; MWM test: Morris Water Maze Test; PACAP-38: Pituitary Adenylyl Cyclase-Activating Polypeptide-38; STZ: Streptozotocin; T1DM and T2DM: Types 1 and 2 Diabetes Mellitus.

Introduction

Diabetes mellitus (DM) is one of the most severe metabolic disorders in humans characterized by hyperglycemia due to a relative or an absolute lack of insulin or its action on the target tissue or both. Many neurodegenerative disorders, such as diabetic encephalopathy and Alzheimer's disease, are associated with the types 1 and 2 diabetes mellitus (T1DM and T2DM) [1]. Manifestation of these disorders in diabetic patients includes alterations in neurotransmission, electrophysiological abnormalities, structural changes and cognitive deficit [2]. In the case of T2DM it is generally accepted that the main factors responsible for the neurodegenerative changes are the resistance of the peripheral tissues to insulin action, which leads to moderate hyperglycemia, and the recurrent hypoglycemia induced by inadequate insulin therapy and glycemic control [3]. Recently, many evidences have been obtained to the effect that the alterations and abnormalities of hormonal signaling systems regulated by insulin, insulin-like growth factor-1, leptin, biogenic amines, and peptide hormones controlling fundamental processes in the neuronal and glial cells contribute to triggering and development of neurodegenerative changes in the diabetic brain [4,5]. In our view, the changes in the brain signaling systems, on the one hand, may be due to formation of a compensatory response to physiological and biochemical dysfunctions occurring in DM, and, on the other hand, may themselves be the cause of these dysfunctions.

Earlier we and the other authors showed that in human DM and animal models of this disease the functioning of hormone-sensitive adenylyl cyclase (AC) signaling system undergoes changes in tissue- and hormone-specific manner [6-11]. In both types of DM the most pronounced alterations were found in the regulation of AC activity by hormones inhibiting the enzyme via heterotrimeric G proteins of the inhibitory type (G_i) [6,7,10,11]. At the same time, the functional state of AC signaling system in the brain of rats with experimental models of T2DM and the regulation of AC activity by hormones interacting with G proteins of the stimulatory type (G_s) or G_i proteins, or activating simultaneously both G protein types, have not been investigated. The study of AC signaling in the diabetic brain may throw light on the molecular mechanisms of neurodegeneration in human T2DM; it may also be helpful in finding new approaches for the treatment of T2DM-induced cognitive deficit and neurological disorders.

Now insulin is commonly accepted as drug to be used in treatment of T1DM as well as T2DM, especially when β cell function falls [12]. Usually, insulin is injected subcutaneously, but recently the other ways of delivery of hormone and its analogues, such as oral and intranasal,

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have been used. Intranasal insulin allows successful glycemic control, improves learning and memory and prevents cognitive decline in diabetic patients [13]. This is confirmed by our findings that the therapy of rats with T2DM by intranasal insulin normalizes the functioning of insulin signaling system, restores cAMP-dependent signaling cascades and improves their cognitive functions [11,14]. However, in the recent years, along with insulin in the treatment of T2DM and pre-diabetic state and, first of all, to correct DM-associated neurodegenerative diseases, have been widely used bromocriptine, a selective agonist of type 2 dopamine receptor (DA_2R) [15], and the selective serotonin (5-HT) reuptake inhibitors [16]. It was found that both bromocriptine and 5-HT reuptake inhibitors increase the brain serotonin level markedly decreased in DM [15,17]. The data is available suggesting that abnormalities in the serotonergic system in the diabetic brain provoke disturbances in neuronal processing, induce alteration of the plasticity of neurotransmission and, thus, play an important role in DM-induced behavioral abnormalities, therefore there are all reasons to expect that the increased level of brain serotonin will lead to restoration of the serotonin signaling in the brain and improve the impaired cognitive functions [17]. With this in mind, we made an attempt to examine the influence of intranasal route of serotonin delivery on the functioning of the brain in rats with T2DM.

The aim of this work was to study the functional state and hormonal sensitivity of AC signaling system in the brain of female rats with the neonatal model of T2DM and to investigate the influence of 8-weeks intranasal serotonin (IS) on this system and cognitive functions in diabetic animals. To determine the specificity of alterations in brain AC system, we studied AC and β,γ-imidoguanosine-5'-triphosphate (GppNHp) binding effects of hormones and their analogues activating receptors of the serpentine type coupled with G_s or G_i proteins. To do this, were chosen the hormones activating G_s-coupled receptors, such as isoproterenol, agonist of β-adrenergic receptors (β-AR), $5\text{-}HT_6R$-agonist 5-chloro-2-methyl-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-indole (EMD-386088), pituitary AC-activating polypeptide-38 (PACAP-38) and relaxin-2, and the hormones activating G_i-coupled receptors, such as DA_2R-agonist bromocriptine, $5\text{-}HT_{1B/1D}R$-agonist 5-nonyloxytryptamine, $5\text{-}HT_{1/2}R$-agonist 5-methoxy-N,N-

dimethyltryptamine (5-MeO-DMT) and somatostatin-14, as well as the hormones activating both G_s- and G_i-coupled receptors, i.e. serotonin, norepinephrine and dopamine (Figure 1). Being involved in the regulation of synaptic plasticity and metabolic and growth processes in neuronal and glial cells, these hormones are very important for the functioning of the central nervous system (CNS). They are also responsible for cellular processes in the peripheral organs and tissues involved in the insulin synthesis and secretion, and thus controlling the glucose homeostasis in the brain and in the whole organism. To detect cognitive deficit in DM and the influence of IS on formation of the long-term spatial memory and learning, a spatial version of the Morris water maze (MWM) test was used.

Materials and Methods

Animals

The female Wistar rats were housed in plastic sawdust-covered cages with a normal light–dark cycle and free access to food and water. The experiments were carried out under the Institutional Guidelines (the Bioethics Committee, December 23, 2010) and "Guidelines for the treatment of animals in behavior research and teaching" [18]. All efforts were made to minimize animal suffering and reduce the number of animals used.

Four groups of animals were investigated: control animals (n = 9, Group C), IS-treated control animals (n = 8, Group C-S), animals with the neonatal model of T2DM (n = 7, Group D), and IS-treated diabetic animals (n = 7, Group D-S).

Neonatal model of T2DM

Neonatal DM was provoked by intraperitoneal administration of streptozotocin (STZ) dissolved in citrate-acidified solution containing 0.9% NaCl, pH 4.5, at the dose of 80 mg/kg of body weight in newborn (5 days) rats using 26 gauge (5/8 in. long) needle [19,20]. The injection site was the dorsal midpoint between pelvis and ribs close to the right side of the spine. Control animals received citrate-acidified physiological solution injected in a similar manner. Typically treatment with STZ induces destruction of β-cells and the insulin production is almost
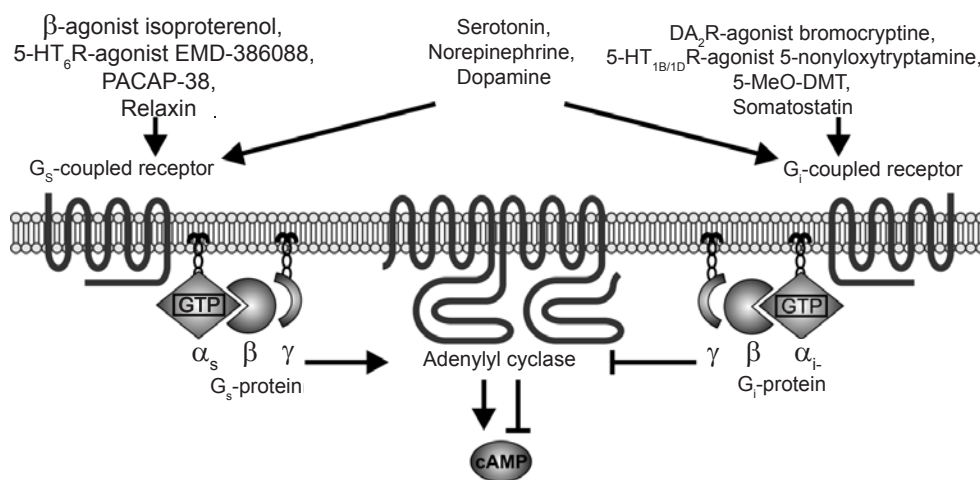
Figure 1: Transduction of hormonal signals generated by biogenic amines, polypeptide hormones and their synthetic analogues via AC signaling system. Isoproterenol, EMD-386088, PACAP-38 and relaxin interact with Gs protein-coupled receptors, stimulate AC activity, increase the intracellular level of second messenger cAMP and trigger cAMP-dependent signaling cascades involved in the regulation of growth, differentiation and metabolism of the neuronal and glial cells. Bromocriptine, 5-nonyloxytryptamine, 5-MeO-DMT and somatostatin interact with Gi protein-coupled receptors, inhibit AC activity and block cAMP-dependent pathways. Serotonin, norepinephrine and dopamine are capable of interacting with both Gs protein- and Gi protein-coupled receptors.

completely blocked. However, at the early stages of development of rats (the first week after birth), a partial restoration of insulin-producing function of β-cells can occur due to their regeneration [21,22]. As a rule, 50–70 % of STZ treatment infant rats show signs of T2DM on reaching the age of 2.5–3 months [23]. In our experiments 70 % of 3 month-old STZ-treated rats had glucose tolerance, as was assessed in the glucose tolerance test (GTT), and these animals were used for experiments (Groups D and D-S). Alongside, we observed moderate hyperglycemia (the fasting glucose concentration was no higher than 7 mM), moderate hypoinsulinemia and the increased plasma levels of triglycerides and HDL-cholesterol. This is in good agreement with the corresponding parameters of the rats with neonatal model of T2DM obtained by the other authors [19,20,23]. In our case impaired glucose tolerance can be associated with moderate hypoinsulinemia, which was due to damage of β-cells in the neonatal period of development and with insulin resistance, which was due to decrease of insulin signaling in the tissues of rats with STZ-induced neonatal DM [14,24]. Earlier, we showed that the insulin binding capacity of insulin receptors in the liver membranes of rats with neonatal model of T2DM was lower compared with healthy animals due to a smaller number of insulin receptors with high and low affinity [14].

Plasma glucose, insulin, triglycerides and HDL-cholesterol measurements

The glucose in the whole blood from the tail vein was measured using test strips One Touch Ultra (USA) and a glucometer (Life Scan Johnson & Johnson, Denmark). The insulin concentration in rat serum was determined using Rat Insulin ELISA (Mercodia AB, Sweden). Triglycerides was determined by Trinder-based GPO-PAP colorimetric end-point method and high density lipoproteins(HDL)-cholesterol by direct clearance method using kits of Randox Laboratories Ltd. (UK) and Sapphire 400 automated clinical chemistry analyzer (Niigata Mechatronics Co., Ltd., Japan). The whole blood was obtained from the tail vein under local (sc) anesthesia with 2 % Lidocaine per 2-4 mg/kg of body weight.

Intranasal delivery of serotonin

Intranasal delivery of serotonin to 6-months old healthy rats (Group C-S) and to diabetic rats of the same age with 3-months neonatal DM (Group D-S) was carried out according to Thorne and coworkers [25]. Serotonin at final concentration of 1 mg/ml was dissolved in saline, pH 4.5. Each rat was placed in a supine position and then an average of 20 µl of serotonin solution containing 20 µg of hormone was administrated by Eppendorf pipette as 5 µl drops in each nostril, in turn, every 1–2 min. Control animals were given the equal volume of saline, pH 4.5. All in all, the IS treatment covered 8 weeks (one week prior to physiological experiments and 7 weeks in the course of experiments).

Chemicals and radiochemicals

The chemicals used in the study were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Calbiochem (San Diego, CA, USA). STZ, GppNHp, somatostatin-14, PACAP-38, serotonin, norepinephrine, dopamine, isoproterenol, bromocriptine, and 5-MeO-DMT were purchased from Sigma-Aldrich (St. Louis, MO, USA). 5-Nonyloxytryptamine and EMD-386088 were purchased from Tocris Cookson Ltd. (United Kingdom). Human relaxin-2 was kindly provided by Prof. J. Wade (Howard Florey Institute, University of Melbourne, Australia). [α-32P]-ATP (4 Ci/mmol) was purchased from Isotope Company (St. Petersburg, Russia), β,γ-imido[8-³H]-guanosine-5'-triphosphate ([8-³H]-GppNHp) (5 Ci/mmol) was from Amersham

(UK); the type HA 0.45 µm nitrocellulose filters were from Sigma-Aldrich Chemie GmbH (Germany).

Synaptosomal membrane preparation

The 8-months old diabetic and non-diabetic animals were decapitated under anesthetics (a mixture of Ketamine and Xylazine at the doses of 90 and 10 mg/kg of body weight, respectively) 24 hours after the last intranasal administration of serotonin or saline, and the brain was rapidly removed and frozen to prepare synaptosomal membrane fractions. The preparation of the rat brain membranes was obtained as described earlier [26]. The brain tissues were dissected on ice and homogenized with a Polytron in 30 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM $MgCl_2$, 2 mM EGTA, 10% (w/v) sucrose and a cocktail of protease inhibitors (Buffer A). The obtained material underwent centrifugation at 4°C. The crude homogenate was centrifuged at $1000 \times g$ for 10 min; the resulting pellet was discarded and the supernatant was centrifuged at $9000 \times g$ for 20 min. The pellet was resuspended in Buffer A (without sucrose) and centrifuged at $35000 \times g$ for 10 min. The final pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4) to produce the membrane fraction with a protein concentration range 1–3 mg/ml and stored at -70°C. The protein concentration of each membrane preparation in all experiments was measured by the method of Lowry and colleagues, using BSA as a standard.

Adenylyl cyclase assay

The AC (EC 4.6.1.1) activity was measured as described earlier [26]. The reaction mixture (final volume 50 µl) contained 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM ATP, 1 µCi [α-³²P]-ATP, 0.1 mM cAMP, 20 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, and 15–45 µg of the membrane protein. Incubation was carried out at 37°C for 10 min. The reaction was initiated by the addition of membrane protein and terminated by the addition of 100 µl of 0.5 M HCl, followed by immersing the tubes with mixture in a boiling water bath for 6 min. 100 µl of 1.5 M imidazole was added to each tube. In these conditions the AC activity was linear. [³²P]-cAMP formed as a result of the enzyme reaction was separated using column chromatography. The samples were placed on neutral alumina columns and cAMP was eluted with 8 ml of 10 mM imidazole-HCl buffer (pH 7.4). The eluates were collected in scintillation vials and counted using a LS 6500 scintillation counter (Beckman Instruments Inc., USA). Each assay was carried out in triplicate at least three times, and the results were expressed as pmol cAMP/min per mg of membrane protein. The basal activity was measured in the absence of hormones, GppNHp and forskolin. To measure the AC inhibitory effect of hormone, the enzyme was pre-activated by diterpene forskolin (10^{-5} M).

GppNHp binding assay

[8-³H]-GppNHp binding to heterotrimeric G proteins was determined as described earlier [27]. The reaction mixture (final volume 50 µl) contained 25 mM HEPES-Na buffer (pH 7.4), 1 mM EDTA, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, 1 µM GppNHp, 0.1% BSA, 0.5–1 µCi [8-³H]-GppNHp. The reaction was started by the addition of 50–100µg of membrane protein and carried out at 30°C for 45 min. The reaction mixture was incubated and rapidly diluted with 100 µl of the washing buffer (20 mM K^+-Na^+ phosphate buffer, pH 8.0) containing 0.1% Lubrol-PX and the samples were filtered under vacuum through 0.45 µm nitrocellulose filters (type HA). In each case the filter was washed three times with 2 ml of washing buffer and dried. The filter-bound radioactivity was estimated in a toluene scintillator using a LKB 1209/1215 RackBeta scintillation counter. Unlabeled 10 mM

GppNHp was added to the reaction mixture to estimate non-specific binding. The specific GppNHp binding was assessed as the difference between a total and non-specific binding. Each assay was carried out in triplicate at least three times and the results were expressed as pmol [8-^{3}H]-GppNHp per mg of membrane protein.

Morris water-maze test

Throughout the behavioral experiments the groups of 6–8 months old diabetic and non-diabetic rats, IS-treated and untreated, were investigated. Prior to the experiments the animals were acclimated to handling over a week. A spatial training of rats was performed in MWM test [28]. The test efficiency was estimated by how long it took the animals swimming in a circular pool (120 cm in diameter and 30 cm in height filled with turbid water with chalk at 24 ± 1 °C) to find a 13 cm diameter hidden platform located 2 cm below the water level.

In the first series of experiments the animals swam daily for five days. Each rat had four attempts of 120 seconds for search of the hidden platform from four starting points. The animals were allowed to swim until either they located the platform, climbing upon it, or when 120 seconds elapsed. If the rats did not find the platform, they were compulsorily placed on it. Between the attempts the animals remained on the platform for 30 seconds rest. Post-testing, rats were placed under a heating lamp to get warm. The escape latency, the length of search path, and search speed during each trial were taken as indicators of successful formation and consolidation of spatial memory and learning.

A month later the testing was repeated (the second series) and the rats were given the trials similar to those of the first series. In addition, on the fifth day of the first and second series 40 minutes after the basic testing the rats were placed for 60 seconds in the pool where the hidden platform was removed and the number of annulus crossings over the previous platform location was recorded. This parameter provides replicates of the experiment for estimation of the strength and accuracy of the memory of the previous platform location. The swimming path and the escape latency were recorded by video camera for each rat during the trial. The obtained data were processed using Real Timer software 1.2 (Open Science Ltd., Russia) and Smart Junior (Panlab, S.L.U., Harvard Apparatus, USA).
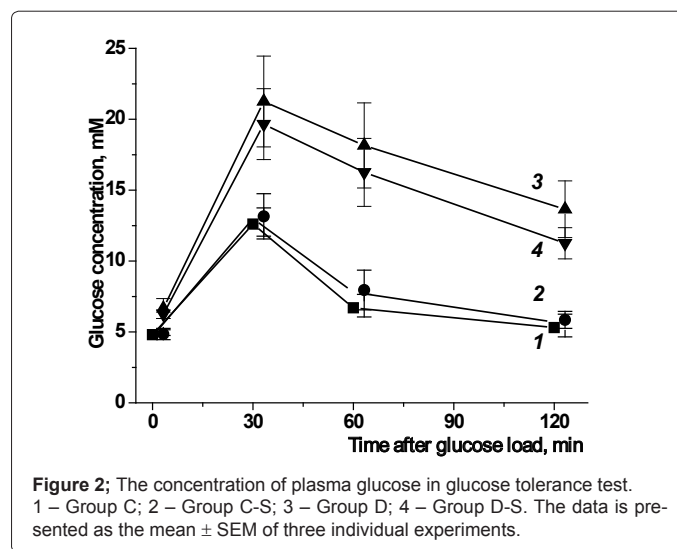
Statistical analysis

The data was presented as the mean ± SD. Testing the assumption of normality was performed by using Shapiro-Wilk test. The results of the test confirmed that the obtained data is a sample from a normal distribution at $\alpha = 0.05$. The difference in the basal activity of AC or the basal level of GppNHp binding in the tissue membrane fractions of control and diabetic animals as well as the difference in the AC activity or GppNHp binding in the membrane fractions treated by hormones and non-hormonal AC regulators in each case was statistically assessed using one-way analysis of variance (ANOVA) and considered significant at $P < 0.05$. The analysis of the data obtained in MWM test

was carried out using one-way analysis of variance (ANOVA) followed by Student's Newman-Keuls post hoc test or unpaired t-test as required, and the difference was considered significant at $P < 0.05$.

Results

Neonatal model of T2DM and IS treatment

In our biochemical and physiological experiments were used 6–8-months old diabetic rats with neonatal DM duration of 3–5 months. Prior to IS treatment the body weight of 6-months old diabetic rats was higher as compared with control animals of the same age and following 8-weeks treatment with IS the difference in the body weight between IS-treated diabetic (D-S) and healthy (C-S) animals decreased (Table 1). In diabetic rats the level of fasting blood glucose was no higher than 7 mM (moderate hyperglycemia) and the level of plasma insulin was slightly decreased (moderate hypoinsulinemia). The level of triglycerides in the blood of the diabetic rats was significantly increased compared with control animals (1.50 ± 0.19 vs. 0.88 ± 0.12 nM, $P < 0.001$), while the increase of the level of HDL-cholesterol in DM was not significant (0.55 ± 0.08 vs. 0.46 ± 0.07 mM). These data is in good agreement with characteristics of the rat neonatal DM obtained by the other authors [19,23]. In our case, the IS treatment had a weak influence on glucose, insulin, triglycerides and HDL-cholesterol concentrations both in diabetic and control rats. In glucose tolerance test 2 hours after glucose load the concentration of plasma glucose in control rats reached normal level, while in diabetic rats remained much higher compared with control (13.5 ± 2.6 vs. 5.3 ± 1.0 mM) (Figure 2). In the IS-treated diabetic rats the concentration of glucose in glucose tolerance test also did not decrease to normal level and was higher than in Group C-S (11.1 ± 2.0 vs. 5.7 ± 1.5 mM). At the same time,

Figure 2; The concentration of plasma glucose in glucose tolerance test. 1 – Group C; 2 – Group C-S; 3 – Group D; 4 – Group D-S. The data is presented as the mean ± SEM of three individual experiments.

	Body weight, g		Plasma glucose, mM		Plasma insulin, ng/mL	
	6 months	8 months	6 months	8 months	6 months	8 months
C (n = 9)	226 ± 19	259 ± 1[8&]	5.0 ± 0.7	4.7 ± 0.9[&]	2.4 ± 0.3	2.3 ± 0.3[&]
C-S (n = 8)	219 ± 17	256 ± 15[8&]	5.1 ± 0.6	4.5 ± 0.5[8&]	2.4 ± 0.4	2.1 ± 0.2[8&]
D (n = 7)	261 ± 22	296 ± 26[&]	6.9 ± 1.1*	6.5 ± 1.2**[&]	1.4 ± 0.3*	1.6 ± 0.4[&]
D-S (n = 7)	268 ± 12[++]	278 ± 16[8&]	6.9 ± 1.4[+]	6.0 ± 1.2[+8&]	1.5 ± 0.4[+]	1.8 ± 0.3[8&]

C – control animals, C-S – IS-treated healthy animals, D – diabetic animals; D-S – IS-treated diabetic animals. & - control and diabetic rats after 8-weeks intranasal administration of saline instead of serotonin; 8& - healthy and diabetic rats after 8-weeks IS treatment (20 μg/rat every day). Values are the mean ± SD of three individual experiments. *, ** - the statistically significant difference between Groups D and C at $P < 0.05$ and $P < 0.01$; +, ++ - the statistically significant difference between Groups D-S and C-S at $P < 0.05$ and $P < 0.01$, respectively.

Table 1: The body weight, fasting glucose and insulin levels in 6- and 8-months healthy and diabetic rats.
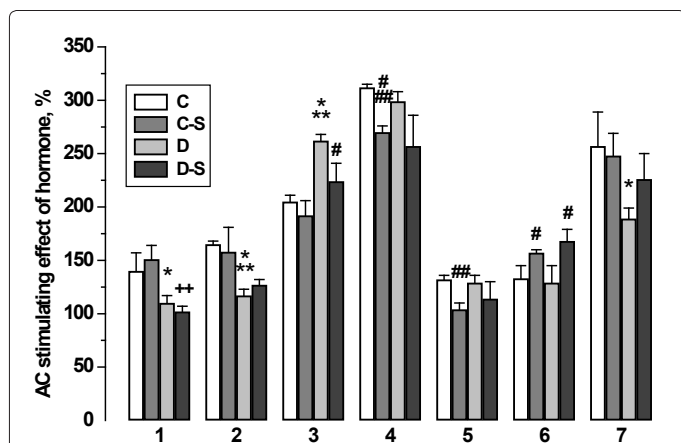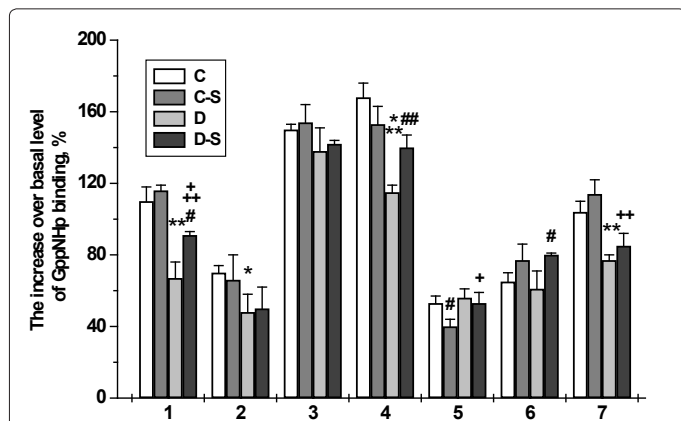
Figure 3: The stimulating effects of hormones on AC activity in the brain of untreated and IS-treated diabetic and control rats.
1 – norepinephrine; 2 – isoproterenol; 3 – dopamine; 4 – serotonin; 5 – EMD-386088 (10^{-5} M each); 6 – PACAP-38, 10^{-7} M; 7 – relaxin, 10^{-8} M.
C – control animals, C-S – IS-treated healthy animals, D – diabetic animals; D-S – IS-treated diabetic animals. Values are the mean $\pm$ SD of three individual experiments, each performed in triplicate.
*, *** - the statistically significant difference between Groups D and C at $P <$ 0.05 and $P < 0.001$; #, ##, ### - the statistically significant difference between Groups D-S and D or Groups C-S and C at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively; ++ - the statistically significant difference between Groups D-S and C-S at $P < 0.01$.

Figure 4: The hormone-induced increase of GppNHp binding in the brain of untreated and IS-treated diabetic and control rats.
1 – norepinephrine; 2 – isoproterenol; 3 – dopamine; 4 – serotonin; 5 – EMD-386088 (10^{-5} M each); 6 – PACAP-38, 10^{-7} M; 7 – relaxin, 10^{-8} M.
The basal level of GppNHp binding is taken as 100 %. Values are the mean $\pm$ SD of three individual experiments, each performed in triplicate.
*, **, *** - the statistically significant difference between Groups D and C at $P < 0.05$, $P < 0.01$ and $P < 0.001$; #, ## - the statistically significant difference between Groups D-S and D or Groups C-S and C at $P < 0.05$ and $P < 0.01$; +, ++, +++ - the statistically significant difference between Groups D-S and C-S at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

in Group D-S the plasma glucose level was 18 % lower compared with Group D, which indicates a rather moderate IS-induced improvement in sensitivity of the peripheral tissues to insulin.

The influence of IS on the basal and GppNHp- and forskolin-stimulated adenylyl cyclase activities and on GppNHp binding in the brain of diabetic and control rats

The basal AC activity in the brain of diabetic rats did not differ significantly from control (23.0 $\pm$1.7 vs. 24.4 $\pm$ 1.5 pmol cAMP/min per mg of membrane protein). Non-hydrolysable analog of GTP, GppNHp

(10^{-5} M), activator of G_s proteins, stimulated AC activity in control and diabetic rats by 188 and 197 %, and forskolin (10^{-5} M) acting directly on the catalytic site of the enzyme stimulated AC activity by 281 and 273 %, respectively. The basal AC activity in the brain of IS-treated diabetic and control rats was 21.3 $\pm$ 2.0 and 20.9 $\pm$ 1.3 pmol cAMP/min per mg of membrane protein, which did not differ significantly from the basal activity of the enzyme in the untreated animals. The IS treatment did not influence the stimulating effects of GppNHp and forskolin on AC activity.

The basal level of GppNHp binding in the brain of diabetic animals did not differ significantly from control (4.25 $\pm$ 0.28 vs. 4.51 $\pm$ 0.26 pmol [8-^{3}H]-GppNHp/mg of membrane protein), and 8-weeks IS treatment increased, but not significantly, it in Groups D-S and C-S, which was now 4.55 $\pm$ 0.19 and 4.73 $\pm$ 0.22 pmol [8-^{3}H]-GppNHp/mg of membrane protein, respectively. This prompts a suggestion that in the brain of rats with neonatal model of T2DM the functional activity of AC system in the basal state and/or under stimulation by non-hormonal agents does not undergo significant alterations, and IS did not significantly affect AC and G_s protein, the catalytic and the transducing components of this system.

The regulation of AC system by hormones acting via G_s-coupled receptors in DM under the influence of IS

In our experiments the biogenic amines and their analogues, such as norepinephrine, β-AR-agonist isoproterenol, dopamine, serotonin and 5-HT$_6$R-agonist EMD-386088, as well as the polypeptide hormones PACAP-38 and relaxin stimulated AC activity and GppNHp binding in the brain of both diabetic and control animals (Figures 3,4). In the diabetic brain AC stimulating effects of relaxin and AR-agonists isoproterenol and norepinephrine were decreased, the corresponding effect of dopamine was on the contrary increased, and AC effects of 5-HTR-agonists and PACAP-38 remained unchanged. The stimulating effects of relaxin, AR-agonists and serotonin on GppNHp binding were decreased in the diabetic brain, and the corresponding effects of the other hormones were close to control.

The IS treatment of control and diabetic rats led to a decrease of AC stimulating effects of serotonin and EMD-386088 and to an increase of the corresponding effect of PACAP-38. In the diabetic brain the stimulating effects of serotonin and PACAP-38 on GppNHp binding were increased, whereas the corresponding effect induced by EMD-386088 did not change significantly. IS led to partial restoration of both AC effect of relaxin decreased in the diabetic brain and the corresponding effect of dopamine increased in DM. IS did not significantly affect AC stimulating effects of AR ligands that in DM are decreased, but increased norepinephrine-induced stimulation of GppNHp binding (Figures 3,4). Thus, in the brain of rats with neonatal T2DM the functional activity of some signaling pathways mediating AC stimulation by hormones of different nature is changed. The changes we identified were hormone-specific, being as a rule associated with pathways regulated by AR ligands and relaxin. IS restored the transduction of AC stimulating signals in the diabetic brain and also affected AC stimulating effects of 5-HTR agonists and PACAP-38 in the healthy rats treated by IS.

The regulation of AC system by hormones acting via G_i-coupled receptors in DM under the influence of IS

The hormonal regulators acting via G_i-coupled receptors – the biogenic amines and their synthetic analogues, such as norepinephrine, dopamine, serotonin, selective 5-HT$_{1B/1D}$R agonist 5-nonyloxytryptamine, 5-HT$_{1/2}$R agonist 5-methoxy-
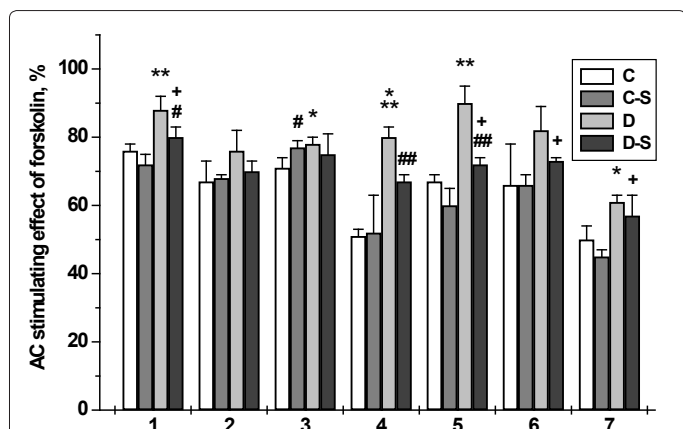
Figure 5: The inhibitory effects of hormones on forskolin-stimulated AC activity in the brain of untreated and IS-treated diabetic and control rats.
1 – norepinephrine; 2 – dopamine; 3 – bromocriptine; 4 – serotonin; 5 – 5-nonyloxytryptamine; 6 – 5-MeO-DMT (10^{-5} M each); 7 – somatostatin, 10^{-6} M.
The stimulating effect of forskolin (10^{-5} M) on the basal AC activity is taken as 100 %. Values are the mean ± SD of three individual experiments, each performed in triplicate.
*, **, *** - the statistically significant difference between Groups D and C at $P < 0.05$, $P < 0.01$ and $P < 0.001$; #, ## - the statistically significant difference between Groups D-S and D or Groups C-S and C at $P < 0.05$ and $P < 0.01$, respectively; + - the statistically significant difference between Groups D-S and C-S at $P < 0.05$.
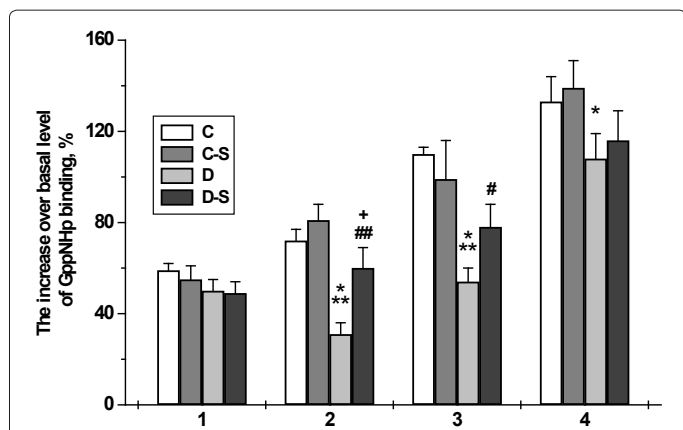
Figure 6: The increase of basal GppNHp binding by hormones activating preferably G_i proteins.
1 – bromocriptine; 2 – 5-nonyloxytryptamine; 3 – 5-MeO-DMT (10^{-5} M each); 4 – somatostatin, 10^{-6} M.
The basal level of GppNHp binding is taken as 100 %. Values are the mean ± SD of three individual experiments, each performed in triplicate.
*, *** - the statistically significant difference between Groups D and C at $P < 0.05$ and $P < 0.001$, respectively; #, ## - the statistically significant difference between Groups D-S and D or Groups C-S and C at $P < 0.05$ and $P < 0.01$, respectively; + - the statistically significant difference between Groups D-S and C-S at $P < 0.05$.

N,N-dimethyltryptamine, DA_2R agonist bromocriptine, and the neuropeptide somatostatin – all decreased forskolin-stimulated AC activity in the synaptosomal membranes in control rats (Figure 5). Besides, these hormones increased basal GppNHp binding (Figures 4,6). In Group D the inhibiting effects of hormones on AC activity and their stimulating effects on GppNHp binding were markedly decreased, especially in the case of serotonin and $5-HT_1R$ agonists. In the diabetic brain the stimulating effects of norepinephrine, serotonin, 5-nonyloxytryptamine, 5-methoxy-N,N-dimethyltryptamine and somatostatin on GppNHp binding reached 61, 68, 43, 49 and 81 % of

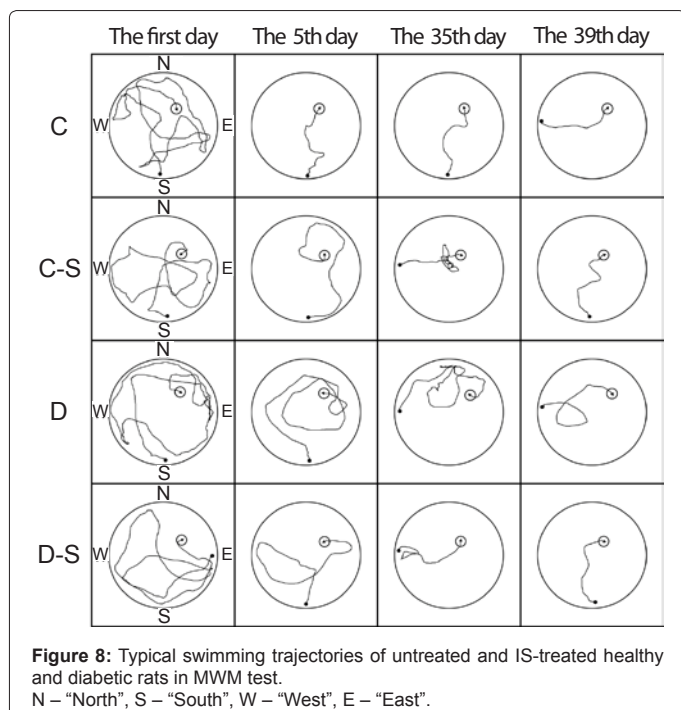those in Group C. The corresponding effects of dopamine and DA_2R-agonist bromocriptine changed a little.

IS weakly influenced the regulatory effects of hormones inhibiting AC via Gi proteins in the brain of animals in Group C-S. In Group D-S the IS treatment led to a significant restoration of both AC inhibitory effects of hormones and their stimulation of GTP-binding (Figures 4,5,6). The stimulating effects of norepinephrine, serotonin, 5-nonyloxytryptamine, 5-MeO-DMT and somatostatin on GppNHp binding in Group D-S covered 78, 92, 74, 79 and 83 % of those in Group C-S. These values are higher compared with those of untreated diabetic animals, especialy in the case of 5-HTR agonists. According to the obtained data, IS gives a significant impact to the stimulation of GppNHp binding of G_i proteins by hormones inhibiting AC activity in the diabetic brain, and this effect is most pronounced in serotonin-regulated G_i-coupled AC pathway.

The spatial memory and learning of diabetic and control rats under the influence of IS

The learning process was estimated according to the duration and dynamics of the latent period for finding the hidden platform. The latent period in Group D in the first series was 3.5 times and in the second series 4–4.5 times longer compared with Group C and the dynamics of time reduction in locating the platform was less pronounced (Figure 7). IS did not change the duration and dynamics of the latent period in control animals, but significantly improved the learning process in diabetic rats. On the 4th day of the first series the escape latency reached the plateau and the information about the location of platform was maintained throughout the experiment. In Group D-S the latent period in both series was lower compared with Group D and the difference was significant ($P < 0.05$). The maximal effect of IS was 4 times, at most, the reduction of the latent period on the first day of the second series (35th day of MWM experiment). In both series there was a significant difference in the trajectories of swimming to the remote platform in Groups D and C, especially in the beginning of the second series (Figure 8). IS did not influence the swimming tracking in Group C-S,
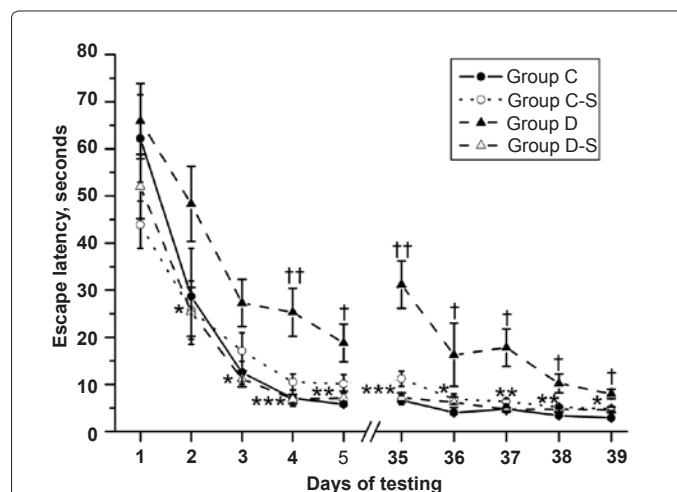
Figure 7: The duration and dynamics of latent period to find the platform by untreated and IS-treated diabetic and control rats in MWM test.
1 – Group C; 2 – Group C-S; 3 – Group D; 4 – Group D-S. Abscissa – the days of testing; ordinate – the escape latency, seconds. The first series covers days 1-5; the second days 35–39.
The data is presented as the mean ± SEM. †, †† - denotes the statistically significant difference between Groups D and C at $P < 0.05$ and $P < 0.01$; *, **, *** - denotes the statistically significant difference between Groups D-S and D at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

Figure 8: Typical swimming trajectories of untreated and IS-treated healthy and diabetic rats in MWM test.
N – "North", S – "South", W – "West", E – "East".

Rats	The first series, 5th day	The second series, 39th day
Group C (n = 9)	5.8 ± 2.0	7.0 ± 1.0
Group C-S (n = 8)	6.8 ± 1.5	6.6 ± 2.8
Group D (n = 7)	3.1 ± 2.5	$4.6 \pm 1.8**$
Group D-S (n = 7)	$5.4 \pm 1.6^{\#+}$	$7.9 \pm 2.3^{\#\#}$

The number of annulus crossings over the platform location was recorded on the fifth day of the first and second series when 40 minutes after the basic testing the animals were placed for 60 seconds in the pool where the hidden platform was removed.
C – control animals, C-S – IS-treated healthy animals, D – diabetic animals; D-S – IS-treated diabetic animals. Values are the mean ± SD of three individual experiments. ** - the statistically significant difference between Groups D and C at $P < 0.01$; #, ## - the statistically significant difference between Groups D-S and D at $P < 0.05$ and $P < 0.01$, respectively; + - the statistically significant difference between Groups D-S and C-S at $P < 0.05$.

Table 2: The number of annulus crossings in the experiments with IS-treated and untreated control and diabetic rats in Morris water-maze test.

but in Group D-S the trajectories were much shorter compared with Group D and similar to those of control rats.

On the last day of testing in the first and the second series there was no difference in the number of annulus crossings between Groups C and C-S (Table 2). However, in diabetic rats there was a 45 % decrease in the first series and a 40 % decrease in the second, compared with Group C and the treatment with IS significantly increased these parameters in both series. In the first and the second series the number of annulus crossings in Group D-S, on average, exceeded 1.7 times that of Group D. Paradoxically, in the second series the number of annulus crossings in Group D-S was larger than in Groups C and C-S (Table 2). This furnishes grounds for us to say that IS improves the long-term spatial memory in rats with neonatal model of T2DM, making it similar to healthy animals. A decrease of time to search the platform and an increase of the number of annulus crossings in Group D-S shows the serotonin-induced improvement of cognitive functions in diabetic rats.

Discussion

Cognitive deficit with impaired abstract reasoning and complex psychomotor functioning are likely to occur on the late stages of T2DM due to the triggering of neurodegenerative processes in the brain of diabetic patients [29]. However, the molecular mechanisms responsible for these processes in the diabetic brain are poorly understood, which hampers the development of effective approaches for the management and prevention of DM-induced CNS complications. Currently, it is commonly accepted that in DM especially disturbed is the signaling network regulated by insulin, insulin-like growth factor-1 and leptin, the principal hormones to be affected in diabetes; this leads to the disturbances of the growth, differentiation, metabolic and apoptotic processes in neuronal and glial cells. However, in addition to these hormones, there are a large number of other hormones and growth factors, as well as the signaling cascades regulated by them which are also involved in the etiology and pathogenesis of DM and its complications [5].

In this work we showed that in the brain of female rats with neonatal T2DM the brain signaling cascades regulated by biogenic amines and polypeptide hormones do indeed undergo a significant change and the most pronounced alterations are manifested in G_i protein-coupled cascades. The changes were found to occur in the AC system sensitive to AR agonists, serotonin, dopamine, and polypeptide hormones, which agrees very well with the data on the alterations and abnormalities in the adrenergic, serotonergic, dopaminergic and peptidergic neurotransmitter systems in the diabetic brain and on their role in the development of neurodegeneration [5].

We showed that in the diabetic brain the stimulating effects of norepinephrine and isoproterenol on AC activity and GppNHp binding mediated via G_s-coupled β-AR are reduced, so is the AC inhibitory effect of norepinephrine mediated via G_i-coupled $α_2$-AR. The abnormalities in adrenergic signaling in DM are likely, on the one hand, to have a negative influence on the synaptic transmission provoked by impairment of the mechanism involving increase of intracellular cAMP concentration and protein synthesis *de novo*, which leads to memory impairment and cognitive deficit [30], and on the other, to be responsible for DM-associated disturbances in the cerebral microvessels whose functions are controlled via different signaling systems, the adrenergic in particular [31]. It was shown earlier that in the hypothalamus of 4–16-weeks old *db/db* mice the number of β-ARs was reduced, which demonstrates a marked modification of the adrenergic signaling [32]. In the brainstem of rats with STZ-induced T1DM the affinity of $α_2$-ARs to agonists was significantly reduced and restored by insulin treatment [33]. The number of β-ARs in cerebral microvessels of fatty (*fa/fa*) and STZ rats is markedly decreased, and isoproterenol-stimulated AC activity in the microvessels of diabetic rats is much lower than that in control, giving evidence for the weakening of β-AR-mediated regulation of cAMP-dependent signaling [31].

In our study pronounced alterations of G_i-coupled serotonin signaling in the brain of diabetic rats are likely to be one of the direct causes of DM-induced behavioral abnormalities, so much so as the brain serotonergic system regulates many physiological functions, and the changes in serotonergic transmission in the diabetic brain provoke disturbances of neuronal processing and the altered plasticity of neurotransmission. We showed that the regulatory effects of 5-HT$_{1B/1D}$R-agonist 5-nonyloxytryptamine and 5-HT$_{1/2}$R-agonist 5-MeO-DMT were markedly reduced, which indicates a weakening of serotonin signaling mediated via 5-HT$_1$R in the brain of rats with neonatal T2DM . The data is available that the flat body posture of STZ diabetic and non-diabetic rats induced by 5-HT$_{1A}$R agonist 8-hydroxy-2-(dipropylamino)tetralin hydrobromide (8-OH-DPAT) changed, but not alike, which means that STZ-induced T1DM profoundly affects the sensitivity to drugs acting

via 5-HT$_{1A}$R [34]. One-week insulin treatment restored 8-OH-DPAT-induced behavioral effects. We showed earlier that the sensitivity of G$_i$-coupled AC system to agonists of 5-HT$_1$R was significantly decreased in the brain of STZ rats with T1DM [35], but there was no alteration of AC sensitivity to agonists of G$_s$ protein-coupled 5-HTR. This is consistent with the data obtained in the present study, i.e. that the efficiency of transduction of AC stimulating serotonin signal in the brain of rats with neonatal T2DM is not altered as compared with control. It seems quite possible that the decrease of efficiency of G$_i$-coupled serotonin signaling pathways may well be associated with reduced expression and with decreased functional activity of G$_i$ proteins, which might indeed be the case as in the brain of rats with neonatal DM was also detected a decrease of activity of the G$_i$-coupled cascades which undergo regulation by norepinephrine and somatostatin.

However, studying dopamine signaling in the diabetic brain, we showed that regulatory effects of dopamine and DA$_2$R-agonist bromocriptine on AC system mediated via G$_i$-coupled DA$_2$R did not change significantly, while stimulating effects of dopamine on this system mediated via G$_s$-coupled DA$_1$R increased. In our view, here a decreased expression of G$_i$ proteins was fully compensated by an increased expression of DA$_2$R or by the changes of their binding characteristics. This suggestion finds confirmation in the recent investigations showing that B$_{max}$ of a total DAR binding and the expression of DA$_2$R in the cerebral cortex of STZ rats with T1DM are increased significantly [36]. In the diabetic brain of T1DM rats the expression of DA$_1$R was also increased, which explains the increase of stimulating influence of dopamine on AC, as in the case of rats with neonatal DM in our investigation.

As far as the role of polypeptide hormones somatostatin, PACAP-38 and relaxin in the functioning of CNS is concerned, they are shown to be involved in the regulation of neuronal differentiation, synaptic plasticity, cognitive functions [37-39]. However, the data on functional activity of the brain signaling systems regulated by these hormones in DM are scarce. The information is available now that the intracerebral level of somatostatin and the expression of some types of brain somatostatin receptors are reduced in animal models of T1DM [40]. We showed that in the brain of rats with neonatal T2DM the regulatory action of somatostatin and relaxin on AC activity is decreased. This allowed a suggestion that somatostatin- and relaxin-sensitive AC system is involved in pathogenesis of T2DM and is a candidate to serve as a marker of this disease. At the same time, we found no significant changes in the response of AC in the diabetic brain to PACAP-38. It seems likely that the maintenance of AC stimulating effect of PACAP-38 is due to its important role in the protection of the brain from damage and neurodegenerative changes induced by DM [41].

The well-coordinated regulation of AC system in the normal brain is disrupted in T2DM, and the synergistic effect of alterations in this system leads to neurodegenerative changes in different brain areas. This is confirmed by our data on the impairment of the cognitive functions in rats with neonatal T2DM in MWM test. The diabetic rats demonstrated the problems in intellectual development and learning. The deterioration of the time allocated to search the platform and the increase of swimming trajectory in the second series indicates the impairment of spatial memory in neonatal T2DM. A number of reports are available describing a cognitive deficit associated with abnormalities in the hippocampus activity in obese rats [42]. Female KKAy mice with hyperinsulinemia and impaired glucose tolerance showed a significant impairment of cognitive functions evaluated by the shuttle avoidance and MWM tests [43]. The rats with T2DM induced by high fat diet and low-dose STZ treatment had deterioration of stroke-induced brain damage and cognitive deficit, as was illustrated by the impairment of the performance of rats in the MWM test [44].

One of the usual approaches used to restore the neuronal network in T2DM is the treatment of diabetic patients with glucose-lowering drugs and, at the late stages of disease, with insulin. Another approach is to treat patients with neurohormones and drugs controlling their synthesis, secretion and uptake, these substances can be used alone or in combination with insulin therapy. The best studied among them are the selective 5-HT reuptake inhibitors having antidepressant activity and DA$_2$R agonist bromocriptine widely used in the treatment of hyperprolactinemia, galactorrhoea and Parkinson disease [16,17]. The 5-HT reuptake inhibitors contribute to lowering the level of hyperglycemia, improve metabolic control through their positive effect on weight loss, thereby improving insulin resistance, and restore cognitive functions impaired in DM. The treatment of 60 patients with depression associated with T1DM and T2DM by fluoxetine, selective 5-HT reuptake inhibitor, reduced depressive symptoms and increased the sensitivity of the brain and the peripheral tissues to insulin [16]. Consequently, the approach leading to an increase of the brain 5-HT level is a successful strategy to treat DM [17]. Proceeding from this data, we applied IS for restoration of activity of the brain AC system and improvement of cognitive deficit of rats with neonatal T2DM. The intranasal route of serotonin administration has been chosen so that the hormone could be delivered to the brain areas, the targets of this action, more effectively. This method is successfully used to treat migraine by 5-HT$_{1B/1D}$R-agonist sumatriptan and its analogues [45]. In the diabetic brain IS restores hormonal sensitivity of AC system, and its positive effect was observed in the case of hormones, especially of serotonin and 5-HT$_1$R agonists, acting via G$_i$ proteins. A considerable restoration of 5-HT$_1$R-mediated signaling in the brain of IS-treated diabetic rats should be ascribed to an increase of the activity of serotonin-sensitive AC system as a result of normalization of the expression of components of this system, and to an increase of the efficiency of interaction between serotonin-regulated and the other signaling systems due to restoration of integrative signaling network in the neuronal and the glial cells impaired in DM. The restoration of the brain AC signaling regulated by neurotransmitters and polypeptide neurohormones leads to the improvement of cognitive functions. The evidence for this is considerable improvement of spatial memory and learning in IS-treated diabetic rats in MWM test. The cognitive functions we evaluated in this test did not differ from those of healthy rats treated as well as untreated with IS.

Summing up, in the brain of diabetic rats with neonatal T2DM the functional activity of hormone-sensitive AC signaling system is subject to changes, the most significant alterations being observed in G$_i$-coupled cascades that mediate the inhibition of AC activity. These alterations were associated with cognitive deficit and manifested as impaired learning and spatial memory. The intranasal delivery of serotonin improves both the brain AC system and cognitive functions of diabetic rats, which opens up a new avenue to correct and prevent the alterations and disturbances in the brain signaling and to improve cognitive functions in T2DM.

Acknowledgment

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