Hormonal Sensitivity of Adenylyl Cyclase in the Myocardium, Brain and Testes of 18-month-old Non-Diabetic and Diabetic Rats

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ABSTRACT

The alterations and abnormalities in hormone-sensitive adenylyl cyclase (AC) system occur at early stages of the type 2 diabetes mellitus (T2DM) and at later stages of the disease they are, according to our view, one of the factors causing T2DM complications. To study the changes in AC system likely to be involved in the development of these complications one needs a very long model of T2DM.

Purpose: The aim of this work was the study of AC system in the myocardium, brain and testes of three-, eight- and 18-month-old male rats with experimental T2DM compared with control animals of the same age.

Methodology: Neonatal model of T2DM was induced by the treatment of five-day pups with streptozotocin (80 mg/kg), at the age of three months streptozotocin-treated rats had glucose tolerance. The determination of the basal AC activity and its regulation by hormones was carried out in the plasma membranes from the tissues of diabetic and control rats, using [α-^{32}P]-ATP as substrate.

Results: With increasing age, three to 18 months, in the myocardium and testes of diabetic and control rats the AC stimulating effects of β-agonists and relaxin (myocardium), and gonadotropin and PACAP-38 (testes) decreased, in diabetic rats to a much greater extent. In the brain the influence of age on AC stimulation by hormones was less pronounced, and only AC effect of PACAP-38 drastically decreased in 18-month-old diabetic and control rats. AC inhibitory effects of somatostatin (all investigated tissues), 5-nonyloxytryptamine (brain) and noradrenaline (myocardium) reduced in eight- and 18-month-old diabetic rats, indicating the weakening of AC inhibiting pathways in a rat model of T2DM.

Conclusion: The hormonal regulation of AC system in the brain, myocardium and testes of 18-month-old control and diabetic rats significantly weakened, and in very long T2DM these abnormalities induced by the combined influence of DM and aging are expressed to a large extent.

Keywords: Adenylyl cyclase; adrenergic agonist; aging; brain; diabetes mellitus; myocardium; somatostatin; testes.

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1. INTRODUCTION

The type 2 diabetes mellitus (T2DM) leads to severe complications that include cardiovascular dysfunctions, neurodegenerative diseases, nephropathy, retinopathy, and neuropathy. The dysfunctions in the male reproductive system are also common in T2DM and cause male infertility and psychological and psychosocial problems. These complications are the result of the impairment of lipid and carbohydrate metabolism, endothelial dysfunction, as well as the alterations in the hormonal signaling systems of the brain and peripheral tissues [**1,2**]. However, in the case of type 1 DM, the complications occur rather rapidly as a result of the combined influence of absolute or relative insulin deficiency, pronounced hyperglycemia and hypoglycemic episodes caused by inadequate insulin therapy, while in T2DM, which is characterized by moderate hyperglycemia and insulin resistance, the complications develop much slower and are clearly detected only at later stages of the disease. Therefore, to study the etiology and pathogenesis of T2DM complications it is necessary to have the prolonged models of the disease. We developed very long-term, 18-months, neonatal model of DM in male rats that is closer to human T2DM compared with many other animal models of the disease. In rats with neonatal DM the insulin resistance occurs only at the age of three months, when the organism is completely formed, making this model close to human type 2 DM [**3,4**].

Earlier we and the other authors showed that the alterations and abnormalities in hormonal signaling systems, hormone-sensitive adenylyl cyclase (AC) signaling system in particular, is one of the main causes of the impairment in the nervous system and peripheral tissues in the types 1 and 2 DM, and the severity of these changes was correlated with the severity and duration of the disease [**5-15**]. Therefore, the study of the changes in hormonal signaling that occur in the tissues and organs of diabetic animals, even at the early stages of DM, makes it possible to monitor the functioning of the nervous and peripheral systems in DM. However, these studies were carried out using mostly the models of early DM, and the information on the changes in hormonal signaling in long-term models of the disease and their role in the etiology and pathogenesis of diabetic complications, T2DM in particular, is very scarce. There are no works on the hormonal signaling in the brain, myocardium, and reproductive tissues of the aging rats with T2DM. Only few works are available describing some physiological processes in the nervous and cardiovascular systems of rats with prolonged T2DM, but the functional activity of signaling systems in this case did not receive enough attention [**16-18**].

The aim of our investigation was to study the age-associated and DM-induced alterations and abnormalities in the basal and hormone-stimulated AC activity in the brain, myocardium and testes of three-, eight- and 18-month-old rats with experimental T2DM and to compare these changes with those in healthy rats of the same age. The choice of the tissues, such as the brain, myocardium, and testes, for the study was dictated by the following reasons. Functional abnormalities of the hormonal signaling in the diabetic brain lead to neurodegenerative diseases and cognitive deficit [**2,19**], and also induce a lot of dysfunctions in the peripheral organs and tissues, including the heart and testes [**20-23**]. It should be noted that the decline of reproductive functions in diabetes is a result of the disturbances in the hypothalamo-pituitary-gonadal axis, both at the central (hypothalamus) and at the peripheral (gonads) level [**20,21,24,25**]. The alterations in the cardiovascular and reproductive systems are one of the most frequent complications of T2DM, especially in the case of long-term T2DM and its inadequate therapy. Clinical studies show that diabetic cardiomyopathy is a leading cause of death of diabetic patients [**26,27**]. The close link between male infertility and T2DM-induced hypogonadotrophic hypogonadism and spermatogenic failure was also found [**22,28,29**]. On the other hand, many reports are available now on the hormonal signaling in healthy animals of different age, senescent and aged rats in particular [**30-34**], with attention focused, as a rule, on the nervous and cardiovascular systems, while age-associated changes in hormonal signaling of the reproductive organs and tissues remain poorly understood. To study the hormonal sensitivity of AC we turned to hormones stimulating AC activity via G_s-proteins or inhibiting the enzyme

via G_i-proteins, which are important regulators of the nervous, cardiovascular and reproductive systems.

2. MATERIALS AND METHODS

2.1. Animals

For experiments adult male Wistar rats housed in plastic sawdust-covered cages with a normal light–dark cycle and free access to food and water were obtained. Six groups of animals were investigated: three-month-old control rats (Group C3, *n* = 8), eight-month-old control rats (C8, *n* = 10), 18-month-old control rats (C18, *n* = 7), three-month-old diabetic rats (Group D3, *n* = 6), eight-month-old diabetic rats (D8, *n* = 10), and 18-month-old rats (D18, *n* = 7) (**Table 1**).

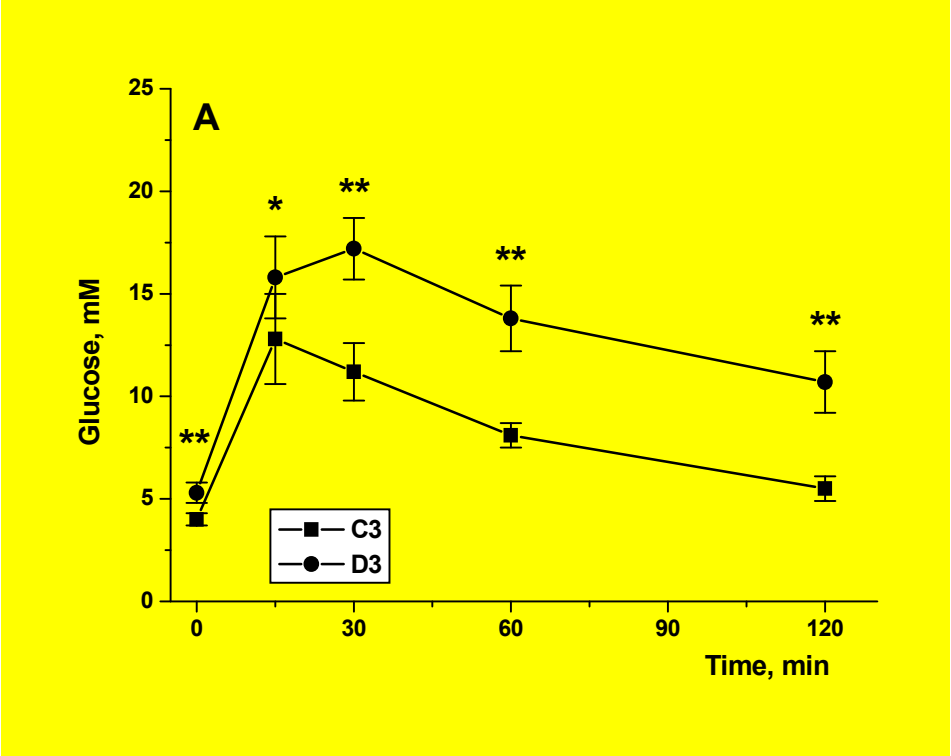
2.2. Neonatal model of type 2 diabetes mellitus

T2DM was induced by intraperitoneal administration of a single dose (80 mg/kg) of streptozotocin (STZ) freshly dissolved in citrate-acidified 0.9 % NaCl, pH 4.5 to newborn (5 day-old) rats. Pups from control group were injected by saline. Typically STZ treatment leads to destruction of β-cells followed by almost complete blocking of insulin production. In rats, however, at the early stages of development, the first week after birth, can occur partial restoration of insulin-producing function of β-cells due to their regeneration [**4**]. As a rule, 50–70 % of STZ treatment infant rats show signs of T2DM on reaching the age of 2.5–3 months [**3**]. In our experiments about 70 % of three-month-old rats treated with STZ had glucose tolerance, as was assessed in the glucose tolerance test, using intraperitoneal glucose loading (2 g/kg of body weight). This test showed that 2 hours after glucose loading the average levels of plasma glucose in Groups D3, D8, and D18 were 10.7 ± 1.5, 11.6 ± 1.9, and 10.5 ± 1.9, which is significantly higher than in the corresponding controls (**Fig. 1**).

Table 1. The body weight, fasting glucose and insulin levels in three-, eight- and 18-month-old diabetic and control rats

Animals group	Body weight, g	Glucose, mM	Insulin, ng/mL
C3, *n* = 8	206 ± 17*	4.4 ± 0.4*	ND
C8, *n* = 10	266 ± 15	4.7 ± 0.5	ND
C18, *n* = 7	326 ± 17	4.4 ± 0.4	1.8 ± 0.3*
D3, *n* = 6	214 ± 17	5.2 ± 0.5	ND
D8, *n* = 10	338 ± 25	6.9 ± 0.7*	ND
D18, *n* = 7	391± 22	6.5 ± 0.6	2.1 ± 0.4

** Values are expressed as the mean ± SD.*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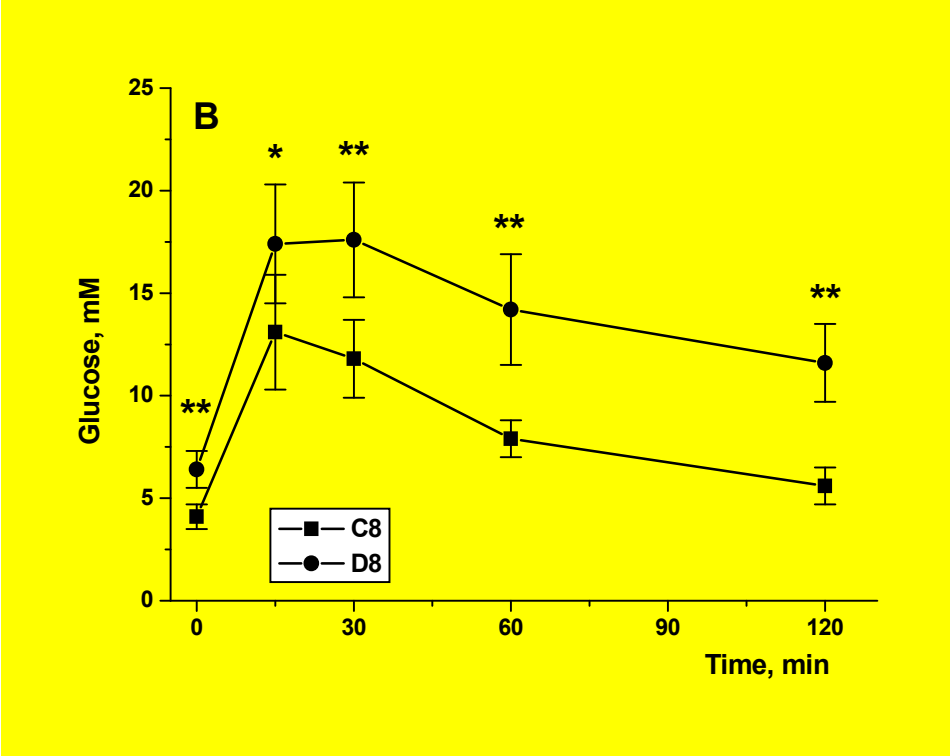
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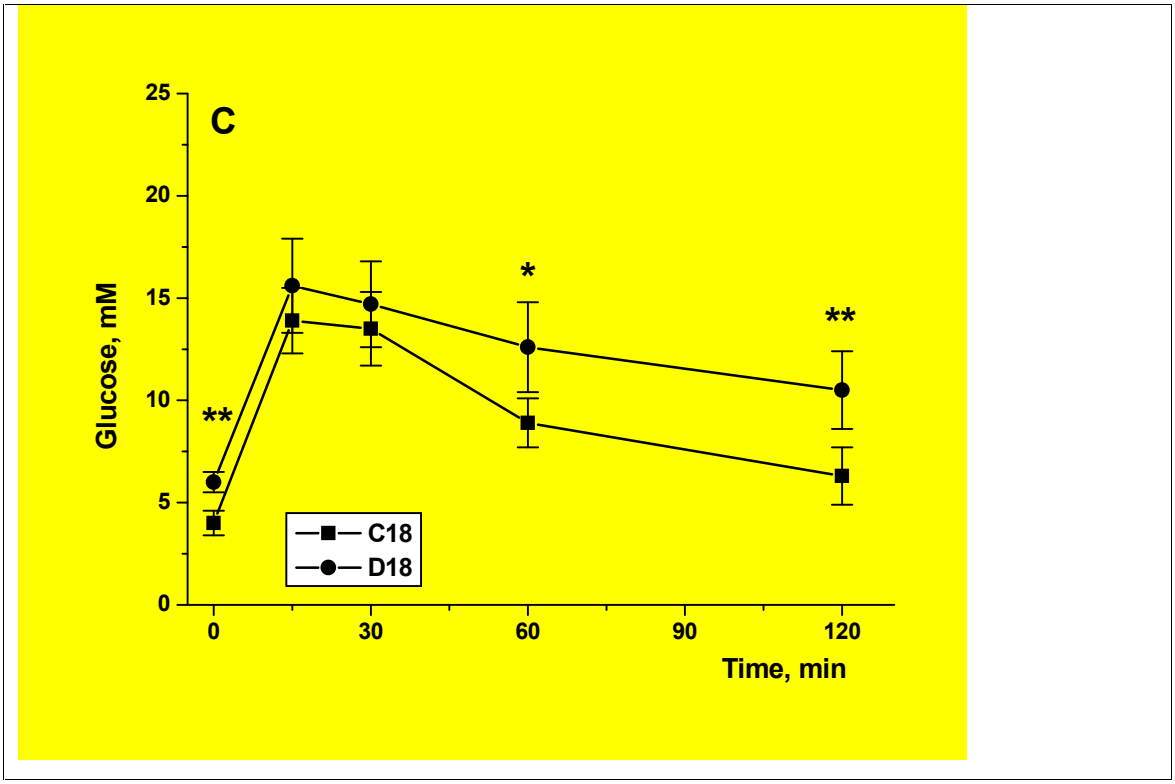


Fig. 1. The concentration of plasma glucose in glucose tolerance test in three- (A), eight- (B) and 18-months-old (C) diabetic and control rats

*Vertical axis – the concentrations of plasma glucose (mM); horizontal axis – time (minutes). Values are expressed as the mean ± SD. * and ** denote the statistically significant difference between control and diabetic rats of the same age at P = .05 and .001, respectively.*

2.3. Glucose and insulin assays

The whole blood was obtained from the tail vein under local anesthesia with 2 % Lidocaine per 2-4 mg/kg of body weight. The glucose measurement in the blood was performed 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).

2.4. Chemicals and radiochemicals

The chemicals used in the study were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Calbiochem (San Diego, CA, USA). Streptozotocin, β,γ-imidoguanosine-5'-triphosphate (GppNHp), forskolin, human chorionic gonadotropin (hCG), somatostatin-14, pituitary adenylyl cyclase-activating polypeptide-38 (PACAP-38), serotonin, noradrenaline and isoproterenol were purchased from Sigma-Aldrich (St. Louis, MO, USA), 5-nonyloxytryptamine (5-NOT) and 5-chloro-2-methyl-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1*H*-indole (EMD-386088) were purchased from Tocris Cookson Ltd. (United Kingdom). Human relaxin was kindly provided by Prof. J. Wade (Howard Florey Institute, University of Melbourne, Australia). [α-^{32}P]-ATP (4 Ci/mmol) was purchased from Isotope Company (St. Petersburg, Russia).

2.5. Plasma membrane preparation

The preparation of synaptosomal membranes from the rat brain was performed as described earlier [35]. 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 500 μM *O*-fenantrolin, 2 μM pepstatin and 1 mM phenylmethylsulphonyl fluoride (Buffer A). The obtained material underwent centrifuge procedures, each performed at 4°C. The crude homogenate was centrifuged at 1000 × *g* for 10 min; the resulting pellet was discarded and the supernatant was centrifuged at 9 000 × *g* for 20 min. The pellet was resuspended in Buffer A (without sucrose) and centrifuged at 35 000 × *g* for 10 min.

The preparation of ventricle membranes from the rat myocardium was performed according to Baker and Potter [36], with some modifications [37]. The dissected hearts were placed in ice-cold 0.9% NaCl and the atria, fat and valves were removed. The tissues were cut into small pieces, homogenized with a Polytron in 20 volumes of ice-cold 40 mM Tris-HCl buffer (pH 7.4) containing 5 mM $MgCl_2$, 320 mM sucrose and a cocktail of protease inhibitors (Buffer B) and centrifuged at 480 × *g* for 10 min at 4°C. The pellet was discarded and the supernatant was centrifuged at 27 500 × *g* for 20 min at 4°C. The pellet was suspended in Buffer B (without sucrose) and then centrifuged at 27 500 × *g* for 20 min.

The isolation of plasma membranes from the testes was carried out as described earlier [38]. The testes were placed in ice-cold Buffer B and homogenized with a Polytron. The homogenate was centrifuged at 1500 × *g* for 10 min at 4°C. The supernatant was centrifuged at 20 000 × *g* for 30 min at 4°C. The resulting pellet was washed in 10 volumes of Buffer B (without sucrose) and centrifuged again at 20 000 × *g* for 30 min.

The final pellet was resuspended in the 50 mM Tris-HCl buffer (pH 7.4) to obtain 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.

2.6. Adenylyl cyclase assay

Adenylyl cyclase (EC 4.6.1.1) activity was measured as described in [36]. 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-^{32}P]-ATP, 0.1 mM cAMP, 20 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, and 15–45 μg of 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. [^{32}P]-cAMP formed as a result of the enzyme reactions was separated using alumina for column chromatography. The samples were placed on neutral alumina columns and cAMP was eluted with 10 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 AC inhibiting effects of hormones, the enzyme was activated by forskolin (10^{-5} M), five minutes before the addition of hormone.

2.7. Statistical analysis

The data are presented as the weighted mean ± weighted standard deviation (SD). The difference in AC activity in control and hormone-, GppNHp- and forskolin-treated membranes in each case was statistically assessed using one-way analysis of variance (ANOVA) and considered significant at $P = .05$.

3. RESULTS

In all tissues of 18-month-old control rats used in investigation the basal AC activity was significantly deceased compared with the three- and eight-month-old control animals (**Table 2**). In the brain, myocardium and testes of 18-month-old diabetic rats the basal AC activity was also much lower than in Groups D3 and D8. In the testes of Groups D8 and D18 the enzyme activity was only 70 and 45 % of that in three-month-old diabetic animals that, as a rule, do not have pronounced manifestations of the disease. The basal AC activity in diabetic and control rats of the same age were compared and showed significant differences in the myocardium of eight-month-old animals (the basal AC activity was higher in DM), in the testes of eight- and 18-month-old animals and in the brain of 18-month-old rats (the basal AC activity was lower in DM) (**Table 2**). As is seen, at the age from three to 18 months the basal AC activity in the brain, myocardium, and testes of both control and diabetic rats had a tendency to decrease.

Table 2. The basal AC activity in the plasma membranes isolated from the brain, myocardium, and testes of three-, eight- and 18-month-old diabetic and control rats of the same age

Animals group	Brain	Myocardium	Testes
	AC activity, pmol cAMP/min per mg of protein		
C3	23.2 ± 1.7	26.7 ± 3.8	19.0 ± 2.5
C8	22.8 ± 2.2	25.2 ± 2.8	18.5 ± 2.1
C18	17.7 ± 2.5$^{\#, \#\#}$	20.5 ± 2.5$^{\#, \#\#}$	15.6 ± 2.2$^{\#, \#\#}$
D3	22.5 ± 2.1	26.0 ± 1.9	19.5 ± 2.5
D8	21.7 ± 2.0	28.1 ± 2.2*	13.6 ± 1.2$^{*,\&}$
D18	15.3 ± 1.6$^{*,\&,\&\&}$	18.6 ± 2.5$^{\&,\&\&}$	8.8 ± 1.0$^{*,\&,\&\&}$

** denotes the statistically significant difference between control and diabetic rats of the same age at P = .05; # the same between Groups C3 and C8 and between Groups C3 and C18 at P = .05; ## the same between Groups C3 and C18 at P = .05; & the same between Groups D3 and D8 and between Groups D3 and D18 at P = .05; and && the same between Groups D8 and D18 at P = .05. Values are expressed as the mean ± SD.*

Studying the influence of non-hormonal AC activators, such as diterpene forskolin directly interacting with the catalytic site of the enzyme, and GppNHp, a non-hydrolyzable analog of GTP, activating G_s-protein coupled to AC in a stimulating manner, the following results were obtained. The AC stimulating effect of forskolin (10^{-5} M) weakly changed with increasing age of animals, and did not differ significantly in the control and diabetic rats (data not shown), except the effect of forskolin in the testes. This effect was decreased in 18-month-old rats compared with three- and eight-month-old animals. E. g., in Group C18 compared with Groups C3 and C8 the forskolin-induced increase over the basal AC activity was 72 ± 3 (C18) *vs*. 88 ± 4 (C3) and 90 ± 5 (C8) pmol cAMP/min per mg of membrane protein, and its increase in Group D18 compared with Groups D3 and D8 was 64 ± 2 (D18) *vs*. 85 ± 2 (D3)

and 79 ± 4 (D8) pmol cAMP/min per mg of membrane protein. Thus, compared to the other tissues, with increasing age the catalytic potency of AC changed considerably only in the testes.

The AC stimulating effect of GppNHp (10^{-5} M) was significantly reduced in all tissues of 18-month-old control and diabetic rats compared with younger animals, and a weakening of this effect in the diabetic tissues was expressed much better then in their respective controls (**Table 3**). In the myocardium and testes of eight- and 18-month-old diabetic rats AC effects of GppNHp was decreased in comparison with Group D3 and with the respective groups of control rats (*P* = .001). No significant difference in AC stimulating effect of GppNHp in the brain of diabetic and control rats was found. Our data give evidence for a considerable influence of age and DM on the functional activity of G_s-proteins in the myocardium, testes and, to a much lesser extent, in the brain.

Table 3. The increase over the basal AC activity induced by treatment with 10^{-5} M GppNHp (pmol cAMP/min per mg of membrane protein) in the tissues of diabetic and control rats

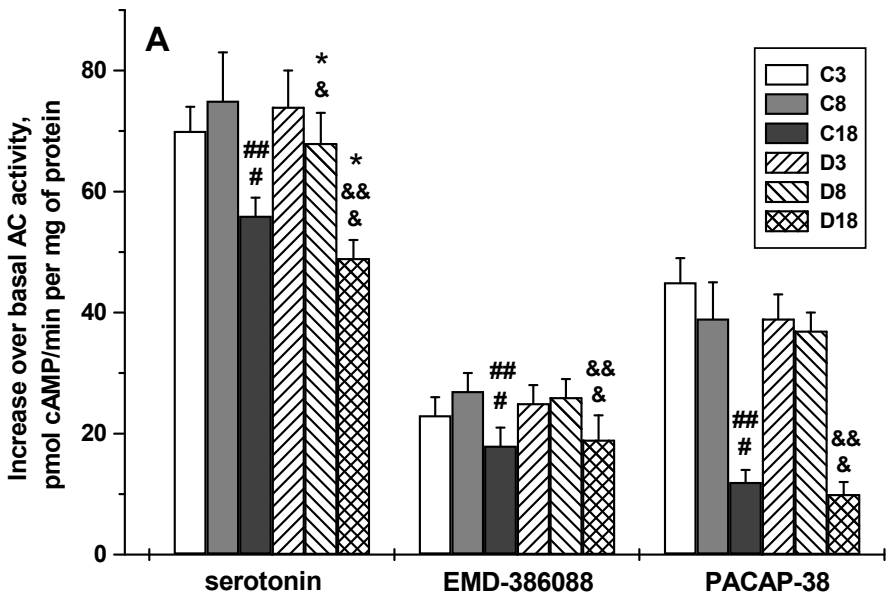
Animals group	Brain	Myocardium	Testes
	Over the basal AC activity, pmol cAMP/min per mg of protein		
C3	47 ± 5	69 ± 4	21 ± 3
C8	48 ± 4	71 ± 5	22 ± 3
C18	$43 \pm 5^{\#\#}$	$59 \pm 4^{\#,\#\#}$	$17 \pm 3^{\#,\#\#}$
D3	45 ± 3	63 ± 6*	19 ± 2
D8	48 ± 5	$48 \pm 4^{*,\&}$	$16 \pm 2^{*,\&}$
D18	$41 \pm 5^{\&,\&\&}$	$42 \pm 5^{*,\&,\&\&}$	$10 \pm 2^{*,\&,\&\&}$

** denotes the statistically significant difference between control and diabetic rats of the same age at P = .05; # the same between Groups C3 and C8 and between Groups C3 and C18 at P = .05; ## the same between Groups C3 and C18 at P = .05; & the same between Groups D3 and D8 and between Groups D3 and D18 at P = .05; and && the same between Groups D8 and D18 at P = .05. Values are expressed as the mean ± SD.*

Then the changes of AC stimulating effects of hormones acting via G_s-proteins that were induced by neonatal DM and increasing age were studied. In the brain of 18-month-old control and diabetic rats the AC stimulating effect of PACAP-38, a hormone regulating the growth and differentiation of neuronal cells, was significantly reduced, and AC effects of serotonin and EMD-386088, the latter being a selective agonist of the type 6 5-hydroxytryptamine receptor (5-HT_6R) involved in the control of cognitive functions, were also attenuated, but to a lesser degree (**Fig. 2,A**). In the brain of eight- and 18-month-old diabetic animals the AC stimulating effect of serotonin was decreased a little in comparison with that in control, while the corresponding effects of PACAP-38 and EMD-386088 were not.

In the myocardium of eight-month-old diabetic rats the AC effect of isoproterenol, an agonist of β-adrenergic receptor (β-AR), was increased, and in Group D18, on the contrary, reduced compared with the respective control (**Fig. 2,B**). This effect was decreased by 29 % as the age of control rats increased from eight to 18 months. AC effect of noradrenaline, a nonselective α/β-AR-agonist, was significantly attenuated in Groups C18 and D18 compared with the three- and eight-month-old animals, and in DM was by 34 % lower than in control. AC effect of relaxin, an insulin-like growth factor peptide, in the myocardium of diabetic rats was strongly reduced; in Group D18 it did not exceed 46 % of that in Group C18. These findings suggest significant age-induced changes in AC system sensitive to β-AR-agonists and relaxin in the myocardium, and the abnormalities occurring in this system in very long-term neonatal DM.

In the testes of 18-month-old control and diabetic rats, there was a significant weakening of AC effect of hCG, a structural and functional analog of luteinizing hormone (LH) regulating testosterone synthesis in Leydig cells, being most pronounced in DM. AC effect of PACAP-38 involved in the regulation of growth and regeneration of testicular tissue was strongly reduced in the testes in Group D18, but in the other groups of animals was altered to a lesser extent or did not change (**Fig. 2,C**). The decrease of sensitivity of the testicular AC system of rats with prolonged DM to gonadotropin and PACAP-38 in our view may well be regarded as one of causes of the weakening of the male reproductive system in T2DM.



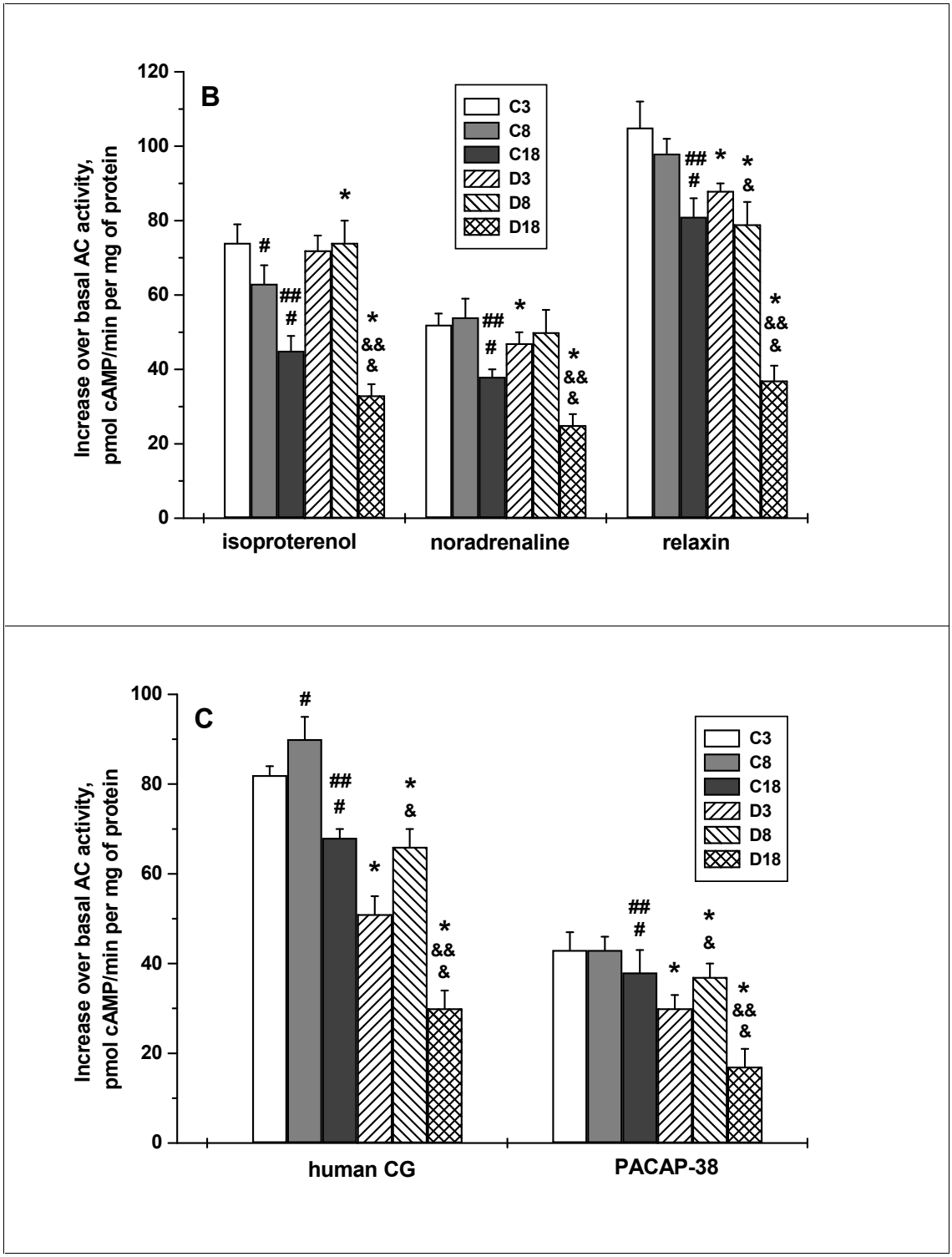


Fig. 2. The stimulation of AC activity by hormones in the brain (A), myocardium (B), and testes (C) of diabetic and control rats

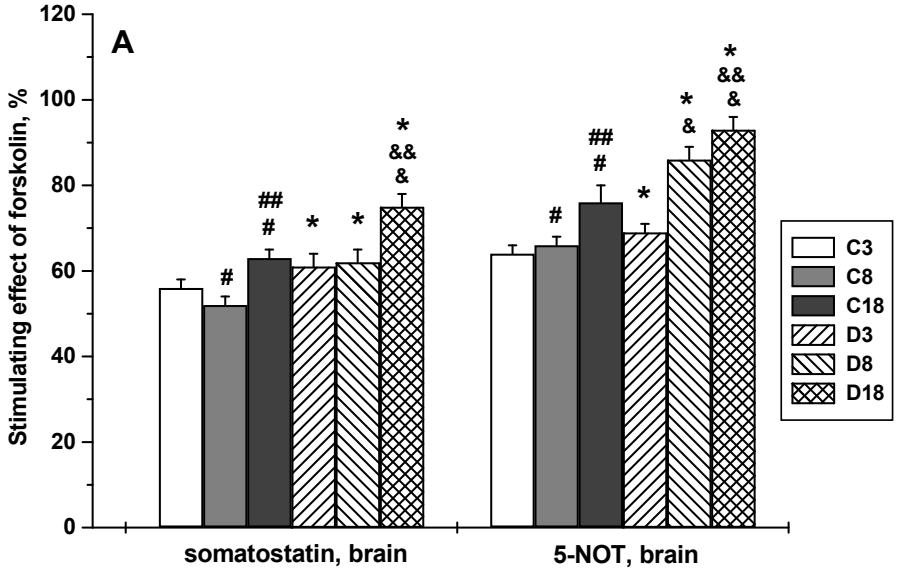
Vertical axis – the increase over basal AC activity, pmol cAMP/min per mg of membrane protein. The concentrations of serotonin, EMD-386088, isoproterenol and noradrenaline were 10^{-5} M, relaxin and hCG – 10^{-8} M, and PACAP-38 – 10^{-6} M.

*Values are expressed as the mean ± SD. * denotes the statistically significant difference between control and diabetic rats of the same age at P = .05; # the same between Groups C3 and C8 and*

between the Groups C3 and C18 at P = .05; ## the same between Groups C3 and C18 at P = .05; & the same between Groups D3 and D8 and between the Groups D3 and D18 at P = .05; and && the same between Groups D8 and D18 at P = .05.

The AC inhibitory effects of hormones acting via G_i-proteins were evaluated by their influence on forskolin-stimulated AC activity. The inhibitory effects of peptide hormone somatostatin that regulates the endocrine system functioning and affects neurotransmission and cell proliferation were studied in all tissues, the corresponding effects of 5-NOT, a selective agonist of 5-HT_{1B}R, in the brain, and of α/β-AR-agonist noradrenaline in the myocardium (**Fig. 3**).

In the brain of diabetic rats the AC inhibitory effect of 5-NOT was significantly reduced, in Group D8 by 59% and in Group D18 by 71% compared with the respective controls. The effect of 5-NOT in Group C18 was found to be decreased compared with Groups C3 and C8, but to a lesser degree (**Fig. 3,A**). In the myocardium of eight- and 18-month-old diabetic rats there was a significant decrease of AC inhibitory effect of noradrenaline in comparison with Group D3 and the respective controls. At the same time, in control rats with the increase of age the AC inhibitory effect of noradrenaline, on the contrary, increased, especially in Group C18 (**Fig. 3,B**). As a result, three-month-old control and diabetic rats had similar AC inhibitory effects of noradrenaline, while in Group D18 this effect was only 46% of that in Group C18.



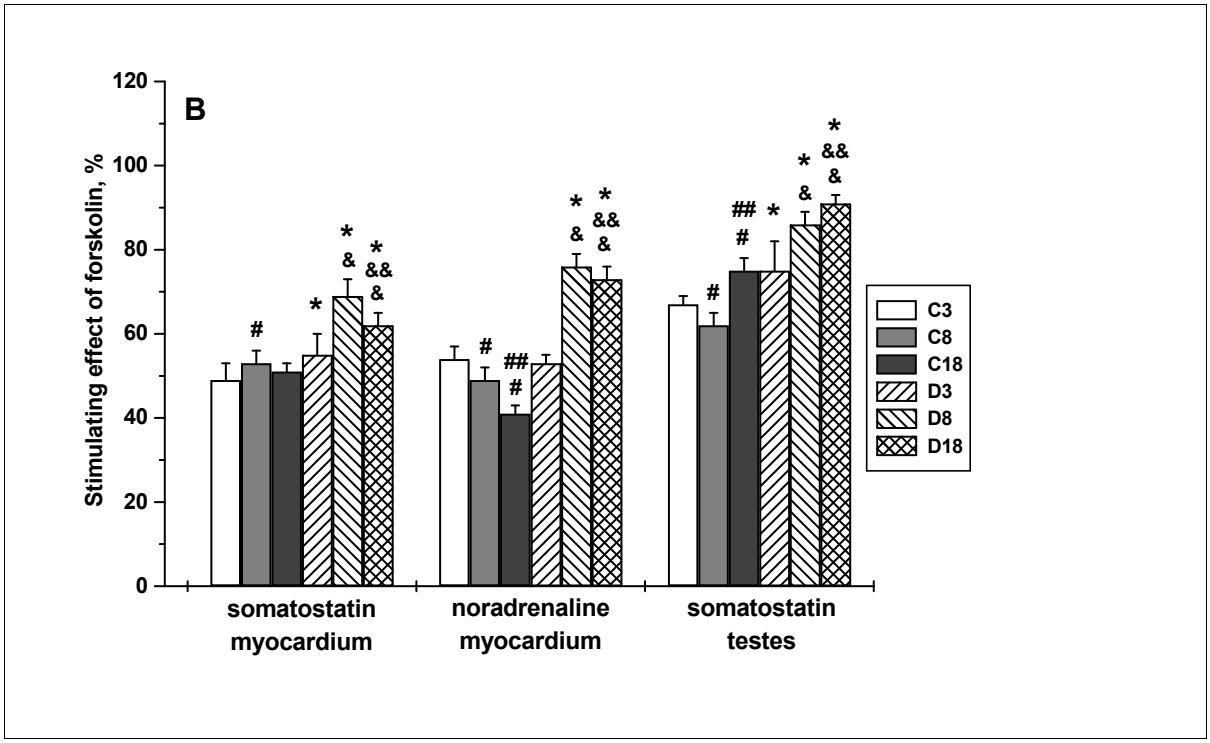


Fig. 3. The inhibition of forskolin-stimulated AC activity by hormones in the brain (A), myocardium and testes (B) of diabetic and control rats

Vertical axis – AC stimulating effect of forskolin (10^{-5} M) is taken as 100 %. The concentrations of 5-NOT and noradrenaline were 10^{-5} M, and somatostatin – 10^{-6} M. The other designations are the same as in Fig. 1.

*Values are expressed as the mean ± SD. * denotes the statistically significant difference between control and diabetic rats of the same age at P = .05; # the same between Groups C3 and C8 and between Groups C3 and C18 at P = .05; ## the same between Groups C3 and C18 at P = .05; & the same between Groups D3 and D8 and between Groups D3 and D18 at P = .05; and && the same between Groups D8 and D18 at P = .05.*

The AC inhibitory effect of somatostatin in all tissues under study was significantly reduced in Groups D8 and D18 compared with that in the corresponding controls (**Fig. 3**). In the brain, myocardium and testes of animals in Group D18 it was 68, 78 and 36%, respectively, of that in Group C18. At the same time, age-associated changes of the enzyme sensitivity to somatostatin in control rats were rather vague (brain, testes), if at all, like in the myocardium. This allows the conclusion that in the brain, myocardium and testes of rats with prolonged neonatal DM the G_i-coupled signaling pathways regulated by various hormones (somatostatin, noradrenaline, and 5-NOT) were strongly attenuated.

4. DISCUSSION

As has been said above, in the last few years a close relationship was established between the abnormalities and alterations in hormone-sensitive signaling systems and the pathogenesis of the types 1 and 2 DM and its complications [**5-15**], but no information is available on the functioning of these systems, AC system in particular, in long-term experimental T2DM. Therefore, our study of the functional state of AC system in rats with very long neonatal DM is the first investigation devoted to hormonal signaling in prolonged T2DM that is associated with aging. It should be mentioned that the changes in AC system of the brain, myocardium, and testes were studied in the dynamics of DM, using three-month-

old rats with the initial stages of the disease and aging 18-month-old animals with simultaneous influence of prolonged DM and age, and the obtained data were compared with control rats of the same age.

In all investigated tissues pronounced age-associated changes in the functioning of AC system were identified in 18-month-old control and diabetic rats. In the rat myocardium of 18-month-old control rats the basal and GppNHp-stimulated AC activity and AC stimulating effects of β-AR-agonists and relaxin were decreased, the difference between Groups C3 and C8 being, however, not significant. It follows that in the myocardium the alterations of G_s-coupled AC system occur only in old rats of 18 months of age. Earlier some authors showed a significant decrease of the number and functional activity of β-AR in the myocardium of aging rats, which is a consequence of age-associated increase of catecholamine level and also induces the impairment of chronotropic and ionotropic effects of β-AR-agonists and change the contractility of the heart muscle [**39-51**]. Along with a decrease in the density of β-AR and reduction of AC activation by AR agonists, forskolin and sodium fluoride [**39**], in the myocardium of aging rats the expression of G_i-proteins was increased [**40**]. It was shown that in the left ventricle of old Fisher 344 rats the expression of $G\alpha_{i2}$-subunit was significantly increased. As G_i-proteins at high concentrations are capable of interacting with G_s-coupled receptors, the increase of G_i-proteins content leads to attenuation of AC stimulating effect of β-AR-agonists and to impairment of vasodilatation [**40**]. The increased activity of G_i-proteins in the myocardium also leads to an increase of inhibitory effect of agonists of G_i-coupled muscarinic acetylcholine receptors on AC stimulation by β_1-AR- and β_2-AR-agonists, as well as to a decrease of the basal AC activity [**40**], which was also observed by us in the myocardium of 18-month-old control rats. It was shown that the increased expression and activity of G_i-proteins are linked directly to the age-associated disorders of the cardiovascular system [**42**]. Our results on increase of the AC inhibitory effect of noradrenaline and preservation of this effect of somatostatin, which act via G_i-coupled receptors, in the myocardium of 18-month-old control rats also point to the increased functional activity of G_i-proteins and G_i-mediated signaling pathways in the heart of aged animals. Carrying out investigation, we revealed a decrease of AC effect of relaxin, an important regulator of vasodilatation and angiogenesis, in the myocardium of rats of Group C18 that can be another cause of age-associated alterations in the cardiovascular system. It was shown that aging attenuates the physiological endothelium-dependent and -independent vasodilator response to relaxin, which is due to the weakening of the regulatory action of hormone on NO-dependent signaling pathways [**43**].

In the myocardium of eight-month-old diabetic rats the basal AC activity increased over that in Group C8, but in Group D18 it was strongly reduced, even below the respective control. In Groups D8 and D18 the AC stimulating effect of GppNHp was significantly decreased, while the same effect of forskolin changed, but a little. This suggests alteration of G_s-proteins activity and preservation of AC catalytic functions in the myocardium of diabetic rats. Of the same opinion are the other authors who showed the decrease of AC stimulating effect of GppNHp, that is mediated via G_s-proteins activation, in the myocardium of diabetic rats [**44,45**]. Their results are in good agreement with our data on the reduction of G_s-mediated AC stimulating effects of β-AR-agonists and relaxin in the myocardium of 18-month-old diabetic animals.

It seems quite likely that instability of the number and pattern of receptors, β_1- and β_2-AR in particular, as well as changes in the expression and activity of G_i-proteins can also be the factors that lead to the myocardium abnormalities. However, a majority of those who studied human T2DM and animal models of this disease did not find any significant changes in the number and proportion of β-AR despite a significant decrease of sensitivity of the diabetic myocardium to β-AR-agonists [**44,46,47**]. It should be pointed out in this connection that in the type 1 DM significant changes in β-AR density and in the ratio between β_1-, β_2- and β_3-AR were identified, which is considered to be a key cause of cardiovascular dysfunctions in this

type of DM [**8**]. The attenuation of AC inhibitory effects of noradrenaline and somatostatin acting via G_i-coupled receptors in Groups D8 and D18 indicates a decrease in G_i-proteins activity in the myocardium in prolonged neonatal DM. This is the main difference between aging control and diabetic rats. Earlier we and the other authors revealed a reduction of functioning of G_i-proteins in the myocardium of rats with different models of DM, which was associated with a decrease in their expression and functional impairment in G_i-protein coupling to receptors and the downstream effector proteins [**6,48,49**].

It was shown in our investigation that in the brain of 18-month-old control rats the basal AC activity and both stimulating and inhibiting effects of hormones on the enzyme activity were reduced, and AC stimulating effect of PACAP-38 decreased to a large extent. The data is available showing that in neurons of the suprachiasmatic nucleus of aging rats the number of G_s-coupled receptors for PACAP was strongly reduced [**50**], this may well account for the decrease of AC sensitivity to this neuropeptide. In the striatum of 40-months-old rats there was a decrease of the number of G_s-coupled D_1-dopamine receptors and their affinity for selective D_1-agonists, which led to a 57 % reduction of AC stimulating effect of dopamine [**51**]. In the brain of rats of Group C18 we showed a significant impairment of AC inhibitory effects of somatostatin and 5-NOT, a selective 5-HT_{1B}R-agonist, likely due to the weakening of G_i-protein functions. This is in good agreement with the data on significant decrease of activity of G_i-proteins and intracellular cascades coupled to them, D_2-dopamine signaling in particular, in the brain of senescent and old rats [**52,53**]. At the same time it is not to be excluded that the decrease of the number of receptors might provoke the reduction of AC inhibitory effects of hormones, as in the case of α_2-AR in the brain stem and hypothalamus of 34-month-old rats [**54**].

In the brain of diabetic rats the AC stimulating effects of hormones were similar to those in the respective controls; they also decreased with increasing age, indicating a crucial role of the age factors in attenuation of hormone-regulated AC stimulating pathways. Meanwhile, in Groups D8 and D18 the AC inhibitory effects of 5-NOT and somatostatin in the brain were diminished, to a great extent, compared with controls of the same age. This indicates a decrease of the expression and functional activity of G_i-proteins in neonatal DM, but not excluding, however, the influence of the changes in the number of G_i-coupled receptors. It was shown that in the hypothalamus of rat with the type 1 DM the expression of G_i-coupled somatostatin receptors of the types 1, 2, 3 and 5 was reduced by 50-80 % [**55**]. Along with the alterations of the initial stages of hormonal signaling, including the activation of receptors and G-proteins, in the brain of aged rats, both healthy and diabetic, the activity of some downstream effector proteins changed, which may be due to the influence of oxidative stress on the functioning of neuronal cells [**56**].

The best expressed alterations in AC system were found in the testes. The differences between the basal AC activity and its regulation by GppNHp and hormones in the testes of rats in Groups D8 and D18 and in the respective controls were significant, unambiguously indicating a negative impact of neonatal DM on AC system functioning in this tissue. There is a view that in Leydig cells of the elderly rats the number of receptors for LH mediating AC stimulating effect of hCG is reduced, this accounts for a decreased secretion of testosterone in response to gonadotropin treatment [**57,58**]. So far, there have been no reports on the influence of very long T2DM on the functioning of the testicular AC system. As for the short-term type 1 DM and neonatal DM up to six months, there is a decrease of the expression and activity of G_s- and G_i-proteins, and attenuation of the regulatory effects of gonadotropins and PACAP-38 [**12,59-61**]. In our view the changes of the testicular AC system we identified in very long neonatal DM, which increase significantly with the disease duration, are one of the factors that cause the dysfunctions of the male reproductive system in T2DM. It should be noted that the brain hormonal signaling also plays an important role in control of the male reproductive functions [**20,62**]. Thus, the alterations of hormone-sensitive AC system identified by us in the brain of rats with long-term neonatal T2DM can also be regarded as

causal factors leading to deterioration in the functioning of the male reproductive system in diabetes.

5. CONCLUSION

Thereby, in the brain, myocardium and testes of 18-month-old control and diabetic rats the functional activity of hormone-sensitive AC system undergoes significant changes, and in diabetic animals these changes are the result of aging as well as influence of metabolic abnormalities induced by neonatal DM. With age in the myocardium and testes of diabetic rats the AC stimulatory effects of guanine nucleotide and hormones were reduced, although the degree and dynamics of this decrease changed considerably. In the myocardium and testes of eight- and 18-month-old diabetic rats the influence of age on the regulatory effects of GppNHp and hormones was more pronounced than in the respective control groups. In the investigated tissues of control rats with increasing age the AC inhibitory effects of hormones changed a little while in diabetic tissues they were strongly disturbed, indicating the dysregulation of G_i-coupled AC system in very prolonged experimental T2DM. This data suggests that in prolonged experimental T2DM the alterations and abnormalities of AC signaling are due to the combined influence of two factors, i.e. aging and DM. This should be taken into account in deciphering the pathogenetic mechanisms of complications induced by long-term T2DM as well as in choosing the optimal strategies for their prevention and treatment.

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COMPETING INTERESTS

The author reports no conflict of interest in this research.

AUTHORS' CONTRUBUTIONS

Alexander O. Shpakov has carried out experiments on the preparation of plasma membrane fractions and the determination of AC activity, managed the literature searches, and wrote the first draft of the manuscript. Kira V. Derkach has carried out experiments on the determination of AC activity, participated in the control and monitoring of the diabetic model, and wrote the protocol. Irina V. Moyseyuk has carried out experiments on the preparation of plasma membrane fractions, performed the statistical analysis, and wrote the protocol. Oxana V. Chistyakova has carried out experiments on the determination of AC activity and participated in the control and monitoring of the diabetic model. Vera M. Bondareva participated in the control and monitoring of the diabetic model and managed the literature searches. All authors read and approved the final manuscript.

ETHICAL APPROVAL

The experiments were carried out under the Bioethics Committee of Sechenov Institute of Evolutionary Physiology and Biochemistry, St. Petersburg, Russia (Institutional Guidelines, December 23, 2010) and "Guidelines for the treatment of animals in behavior research and teaching". All efforts were made to minimize animal suffering and reduce the number of animals used.

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ABBREVIATIONS

AC, adenylyl cyclase; β-AR, β-adrenergic receptor; EMD-386088, 5-chloro-2-methyl-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-indole; GppNHp, β,γ-imidoguanosine-5'-triphosphate; hCG, human chorionic gonadotropin; 5-HT_{1B}R, 5-HT_6R –5-hydroxytryptamine receptor of the subtype 1B and type 6, respectively; LH, luteinizing hormone; 5-NOT, 5-nonyloxytryptamine; PACAP-38, pituitary adenylyl cyclase-activating polypeptide-38, STZ, streptozotocin; T2DM, diabetes mellitus of the type 2.