Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 22 (2011) 418–425
Journal of Nutritional Biochemistry

Vitamin D_3 restores altered cholinergic and insulin receptor expression in the cerebral cortex and muscarinic M3 receptor expression in pancreatic islets of streptozotocin induced diabetic rats

Peeyush T. Kumar, Sherin Antony, Mohan S. Nandhu, Jayanarayanan Sadanandan, George Naijil, Chiramadathikudiyil S. Paulose*

Molecular Neurobiology and Cell Biology Unit, Centre for Neuroscience, Cochin University of Science and Technology, Cochin, Kerala 682 022, India

Received 11 November 2009; received in revised form 6 March 2010; accepted 12 March 2010

Abstract

Nutritional therapy is a challenging but necessary dimension in the management of diabetes and neurodegenerative changes associated with it. The study evaluates the effect of vitamin D_3 in preventing the altered function of cholinergic, insulin receptors and GLUT3 in the cerebral cortex of diabetic rats. Muscarinic M3 acetylcholine receptors in pancreas control insulin secretion. Vitamin D_3 treatment in M3 receptor regulation in the pancreatic islets was also studied. Radioreceptor binding assays and gene expression was done in the cerebral cortex of male Wistar rats. Immunocytochemistry of muscarinic M3 receptor was studied in the pancreatic islets using specific antibodies. Y-maze was used to evaluate the exploratory and spatial memory. Diabetes induced a decrease in muscarinic M1, insulin and vitamin D receptor expression and an increase in muscarinic M3, α7 nicotinic acetylcholine receptor, acetylcholine esterase and GLUT3 expression. Vitamin D_3 and insulin treatment reversed diabetes-induced alterations to near control. Diabetic rats showed a decreased Y-maze performance while vitamin D_3 supplementation improved the behavioural deficit. In conclusion, vitamin D_3 shows a potential therapeutic effect in normalizing diabetes-induced alterations in cholinergic, insulin and vitamin D receptor and maintains a normal glucose transport and utilisation in the cortex. In addition vitamin D_3 modulated muscarinic M3 receptors activity in pancreas and plays a pivotal role in controlling insulin secretion. Hence our findings proved, vitamin D_3 supplementation as a potential nutritional therapy in ameliorating diabetes mediated cortical dysfunctions and suggest an interaction between vitamin D_3 and muscarinic M3 receptors in regulating insulin secretion from pancreas.

Keywords: Diabetes; Cerebral cortex; Insulin; Vitamin D_3; Cholinergic receptor pancreas and muscarinic M3

1. Introduction

Diabetes mellitus is a common metabolic disorder characterized by hyperglycemia due to an absolute or relative insulin deficiency. Diabetes mellitus is known to be associated with neurological complications in both peripheral nervous system and the central nervous system (CNS) [1]. Brain cells are particularly vulnerable to oxidative stress. Controlling blood glucose is essential for avoiding long-term complications of diabetes like learning and memory deficit.

Vitamin D_3 is either synthesised in the epidermis from 7-dehydrocholesterol by the absorption of ultraviolet light, or obtained from the diet in a limited number of foods such as eggs, fish oils and fortified milk. The biological actions of vitamin D_3 are mediated through binding to the vitamin D receptor (VDR), a member of the nuclear steroid hormone receptor family. An increased prevalence of diabetes has been described in vitamin D-deficient individuals [2]. Insulin synthesis and secretion has been shown to be impaired in β cells in vitamin D-deficient animals. It was also of interest to determine whether changes in the expression of the muscarinic M3 receptors using vitamin D_3 supplementation in pancreas might account for the increased synthesis and secretion of insulin. Immunohistochemistry showed the presence of VDR in human pituitary gland [3], suggesting a possible role of vitamin D in regulation of the brain endocrine system. It is of particular importance that VDR and catalytic enzymes are colocalized in the brain, supporting an autocrine/paracrine function for vitamin D. These findings support a functional role for vitamin D in the human brain [4].

Diabetes is also found to be associated with changes in somatic sensations which involve the cerebellum, cerebral cortex and thalamus. The cholinergic innervation of the cerebral cortex has been extensively investigated because of its role in arousal, learning and memory [5]. Alterations in glucose utilization are known to occur in the important regions of brain connected with learning and memory [6]. The brain glucose uptake is ultimately dependent on facilitative glucose transporters, the modulation of brain glucose transporters intrinsic activity. GLUT3 is the main neuronal glucose transporter [7] abundant in the brain.

* Corresponding author. Tel.: +91 484 2576267, 257588; fax: +91 484 2575588, +91 484 2576699.
E-mail addresses: cspaulose@cusat.ac.in, paulosecs@yahoo.co.in, cspaulose@gmail.com (C.S. Paulose).

0955-2863/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2010.03.010

Glucose-dependent insulin secretion is regulated by several neurotransmitters released from peripheral autonomic nerves. Acetylcholine, a major neurotransmitter of the peripheral parasympathetic nervous system helps to facilitate the release of insulin in a glucose-dependent mode. Hence this activity has been shown to be mediated by the activation of muscarinic acetylcholine receptors located on the pancreatic β cells [8].

The role of vitamin D_3 in the functional regulation of cholinergic and insulin receptors in cerebral cortex and muscarinic M3 receptor modulation with vitamin D_3 in pancreas has not been studied. The current experiments were designed to analyse the neuroprotective effect of vitamin D_3 on the cholinergic, insulin receptors and GLUT3 in the cerebral cortex of streptozotocin (STZ)-induced diabetic rats and interaction between vitamin D_3 and pancreatic M3 receptors, thereby evaluating the therapeutic role of vitamin D_3 in insulin release and diabetes associated cortical dysfunctions. Our present study on vitamin D_3 supplementation and its restorative effect on neurotransmitter receptors will definitely enlighten novel therapeutic possibilities for complications associated with diabetes.

2. Materials and methods

Biochemicals used in the present study were purchased from Sigma Chemical, St. Louis, MO, USA. All other reagents of analytical grade were purchased locally. Quinuclidinyl benzilate, L-[Benzilic-4,4′-3H], ([^{3}H] QNB)(Sp. Activity 42 Ci/mmol) and 4-DAMP, [*N*-methyl-3H] (Sp. Activity 83 Ci/mmol) were purchased from NEN Life Sciences Products, Boston, MA, USA. Pirenzepine, 4-DAMP mustard, and cholecalciferol and Tri-reagent kit were purchased from Sigma Chemical Co., St Louis, MO, USA. Real-time PCR Taqman probe assays on demand were from Applied Biosystems, Foster City, CA, USA.

Male adult Wistar rats of 180–240 g body weight were used for all experiments. They were housed in separate cages under 12 hour light and 12 hour dark periods. Rats had free access to standard food and water ad libitum. All animal care and procedures were done in accordance with the Institutional and National Institute of Health guidelines. Diabetes was induced in rats by single intra femoral vein injection of STZ (55 mg/kg body weight) freshly dissolved in 0.1 M citrate buffer [9]. Animals were divided into the following groups: (i) control, (ii) diabetic, (iii) insulin-treated diabetic and (iv) vitamin D_3-treated diabetic rats. Each group consisted of six to eight animals. The insulin-treated diabetic group received subcutaneous injections (1 U/kg body weight) of Lente and Plain insulin (Boots India) daily during the entire period of the experiment. The last injection was given 24 h before sacrificing the rats. Vitamin D_3 treated groups received 12 μg/kg vitamin D_3 dissolved in 0.3 ml of coconut oil. The supplementation was administrated via gavage for a period of 2 weeks [10] for the entire period of the experiment. Rats were sacrificed on 15th day by decapitation. The cerebral cortex was dissected out quickly over ice according to the procedure of Glowinski and Iversen [11], and the tissues collected were stored at −80° C until assay.

2.1. Estimation of blood glucose

Blood glucose was estimated by the spectrophotometer method using glucose oxidase-peroxidase reactions. Blood samples were collected from the tail vein at 0 h (before the start of the experiment) and on the 3rd, 6th, 10th and 14th day, and the glucose levels were estimated subsequently. Along with this blood samples were collected 3hrs after the administration of morning dose of insulin and vitamin D_3. The results were expressed in terms of milligrams per decilitre of blood.

2.2. Y-maze test

Short-term spatial memory performance was assessed by Y-maze [12]. The Y-maze was made of grey wood, covered with black paper, and consisted of three arms with an angle of 120 degrees between each of the arms. Each arm was 8-cm width×30-cm length×15-cm height. The three identical arms were randomly designated: Start arm, in which the rat started to explore (always open); novel arm, which was blocked at the first trial but open at the second trial and the other arm (always open). The maze was placed in a separate room with enough light. The floor of the maze was covered with sawdust, which was mixed after each individual trial in order to eliminate olfactory stimuli. Visual cues were placed on the walls of the maze.

The Y-maze test consisted of two trials separated by an inter-trial interval (ITI). The first trial (training) was of 10-min duration and allowed the rat to explore only two arms (start arm and the other arm) of the maze, with the third arm (novel arm) blocked. After a 1-h ITI, the second trial (retention) was conducted, during which all three arms were accessible and novelty vs. familiarity was analyzed through comparing behavior in all three arms. For the second trial, the rat was placed back in the maze in the same starting arm, with free access to all three arms for 5 min. The time spent in each arm was analyzed. Data was expressed as percentage of performance in all three arms during the 5 min of test.

2.3. Total muscarinic, muscarinic M1 and M3 receptor binding studies in the cerebral cortex

Binding assay in cerebral cortex was done according to the modified procedure of Yamamura and Synder [13]. Total muscarinic, and muscarinic M1 receptor binding parameter assays were done using [^{3}H] QNB (0.1–2.5 nM) and M3 receptor using [^{3}H] DAMP (0.01–5 nM). The nonspecific binding was determined using 100 μM atropine for total muscarinic, pirenzepine for muscarinic M1 and 4-DAMP mustard for M3 receptor. Total incubation volume of 250 μl contains 200-250μg protein concentrations. Tubes were incubated at 22°C for 60 min and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 5.0 ml of ice cold 50 mM Tris–HCl buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The nonspecific binding determined showed 10% in all our experiments.

2.4. Receptor data analysis

The receptor binding parameters were determined using Scatchard analysis [14]. The specific binding was determined by subtracting non-specific binding from the total. The binding parameters, maximal binding (B_{max}) and equilibrium dissociation constant (K_d), were derived by linear regression analysis by plotting the specific binding of the radioligand on *x*-axis and bound/free on *y*-axis using Sigma plot software (version 2.0, Jandel, Erkrath, Germany). The maximal binding is a measure of the total number of receptors present in the tissue and the equilibrium dissociation constant is the measure of the affinity of the receptors for the radioligand. The K_d is inversely related to receptor affinity.

2.5. Analysis of gene expression by real-time polymerase chain reaction

RNA was isolated from the cerebral cortex of experimental rats using the Tri-reagent. Total cDNA synthesis was performed using ABI PRISM cDNA archive kit. The cDNA synthesis reactions were carried out at 25 °C for 10 min and 37°C for 2 h using an Eppendorf Personal Cycler. Real-time polymerase chain reaction (PCR) assays were performed in 96-well plates in ABI 7300 real-time PCR instrument (Applied Biosystems). The primers and probes were purchased from Applied Biosystems, Foster City, CA, USA. β-Actin was used as endogenous control. The following thermal cycling profile was used (40 cycles): 50°C for 2 min, 95°C for 10 min, 95°C for 15 s and 60°C for 1 min. The relative ratios of mRNA levels were calculated using the $^{\Delta}$CT method normalized with β-actin CT value as the internal control and control CT value as the calibrator [14].

2.6. Muscarinic M3 receptor expression studies in the pancreas of control and experimental rats using confocal microscope

Pancreatic islets were prepared from adult rats by standard collagenase digestion procedures using aseptic techniques [15]. The islets were seeded in culture wells and allowed to adhere to the plate. The islets were rinsed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.0, for 30 min on ice. After fixation, the islets were washed thrice with blocking buffer containing 0.1 M phosphate buffer, pH 7.0, 0.1% Triton X and 10% bovine serum albumin. Then the islets were incubated with primary antibody for muscarinic M3 (Sigma Aldrich, diluted in PBST at 1: 1000 dilution), prepared in blocking buffer with 1% serum and incubated overnight at 4°C. After the incubation, the islets were washed thrice with blocking buffer. Then the islets were incubated with secondary antibody tagged with fluorescein isothiocyanate (FITC) (No. AB7130F, Chemicon, diluted in PBST at 1: 1000 dilution) diluted in blocking buffer with 1% serum and incubated at room temperature in dark for two hours. After incubation the islets were rinsed with blocking buffer and were observed and photographed using confocal imaging system (Leica SP 5). The specificity of the immunocytochemical procedure is validated by negative controls (data not shown) to ensure that the labelling method accurately identifies the antibody bound to the specific muscarinic M3 receptors in the pancreatic islets. Expression of muscarinic M3 receptor was analysed using pixel intensity method. The given pixel value is the net value which is deducted from the negative control pixel value [16].

2.7. Statistics

Statistical evaluations were done by analysis of variance (ANOVA), expressed as mean±S.E.M using In Stat (Ver.2.04a) computer programme.

3. Results

Blood glucose level of all rats before STZ administration was within the normal range. STZ administration led to a significant increase (P<.001) in blood glucose level of diabetic rats compared to

Table 1
Blood glucose (mg/dl) level in experimental rats

Animal status	0 day (Before STZ injection)	3rd day (Initial)	6th day	10th day	14th day (final)
Control	82.3±1.6	86.5±1.6	89.6±1.2	92.3±1.4	90.7±1.21
Diabetic	80.3±1.3	255.1±0.8	317.3±1.4	306.8±0.7	313.3±1.4‡
D+I	84.2±0.8	256.8±0.5	303.6±0.7	190.9±1.5	137.0±1.3*†
D+V	86.3±1.5	257.4±1.4	310±0.8	195±1.5	170.4±1.5*†

Values are mean±S.E.M of four to six rats in each group. Each group consist of six to eight rats. ‡$P<.001$ when compared to control; *$P<.001$ when compared to diabetic group; †$P<.001$ when compared with initial reading. D+I, Insulin-treated diabetic rats; D+V, vitamin D3-treated diabetic rats.

control rats. Insulin and vitamin D_3 treatment were able to significantly reduce ($P<.001$) the increased blood glucose level to near the control value compared to diabetic group (Table 1).

3.1. Y-maze performance of control and experimental groups of rats

Number of visits and time spent in the novel arm decreased significantly ($P<.001$) in the diabetic rats compared to control. Both insulin treatment and vitamin D_3 treatment to diabetic rats significantly increase the number of visits and time spent in the novel arm (Fig. 1).

3.2. Total muscarinic receptor analysis

3.2.1. Scatchard analysis of [^{3}H] QNB binding against atropine in the cerebral cortex of control, diabetic, diabetic+insulin- and diabetic+vitamin D_3-treated diabetic rats

The Scatchard analysis showed that the B_{max} and K_d of the [^{3}H] QNB receptor binding decreased significantly ($P<.001$) in the cerebral cortex of diabetic rats compared to control group. In vitamin D_3 and insulin treated diabetic groups, B_{max} reversed to near control value. K_d of insulin treated and vitamin D_3 group reversed to near control (Table 2).

3.3. Muscarinic M1 receptor analysis

3.3.1. Scatchard analysis of [^{3}H]QNB binding against pirenzepine in the cerebral cortex of control, diabetic, diabetic+insulin- and diabetic+vitamin D_3-treated diabetic rats

The Scatchard analysis showed that the B_{max} and Kd of muscarinic M1 receptors of Cerebral cortex were decreased significantly ($P<.001$) in diabetic condition compared to control group. Insulin- and vitamin D_3-treated diabetic rats B_{max} and K_d were reversed to near control value compared to diabetic group (Table 3).

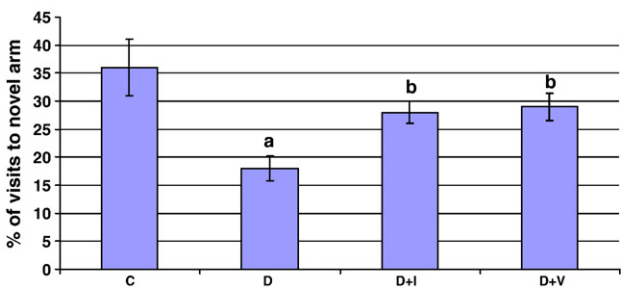

Fig. 1. Y-maze performance of control, diabetic, insulin-treated diabetic and vitamin D_3-treated diabetic rats. Values are mean±S.E.M. of four to six separate experiments (n=5–6 rats per group) ANOVA followed by Student-Newman-Keuls test. [a] $P<.01$ when compared to control. [b] $P<.01$ when compared to diabetic rats.

Table 2
Scatchard analysis of [^{3}H] QNB binding against atropine in the cerebral cortex of control, diabetic, insulin treated diabetic and vitamin D_3-treated diabetic group rats

Animal status	B_{max} (fmol/mg protein)	K_d (nM)
Control	316±8.5	0.21±0.02
Diabetic	131±6.2‡	0.17±0.02$
D+I	300±8.2*	0.3±0.01†
D+V	301±7.4*	0.3±0.03†

Values are mean±S.E.M of four to six separate experiments. Each group consist of six to eight rats. ‡$P<.001$ when compared to control; *$P<.001$ when compared to diabetic group; $$P<.05$ when compared to control group; †$P<.05$ when compared to diabetic group.

3.4. Muscarinic M3 receptor analysis

3.4.1. Scatchard analysis of [^{3}H] DAMP binding against 4-DAMP mustard in the cerebral cortex of control, diabetic, diabetic+insulin and diabetic+vitamin D_3 diabetic rats

The Scatchard analysis showed that the B_{max} and K_d of muscarinic M3 receptors of cerebral cortex were increased significantly ($P<.001$) in diabetic rats compared to control group. Insulin- and vitamin D_3-treated diabetic rats showed B_{max} and K_d were reversed to near control value compared to diabetic group (Table 4).

3.5. Real time-PCR analysis of muscarinic M1 receptor

Real-time PCR analysis showed that the muscarinic M1 receptor gene expression in the cerebral cortex was decreased significantly ($P<.001$) in diabetic condition and it reversed to near control value in insulin- and vitamin D_3-treated diabetic rats (Fig. 2).

3.6. Real time-PCR analysis of muscarinic M3 receptor

Real-time-PCR analysis showed that the muscarinic M3 receptor gene expression in the cerebral cortex was increased significantly ($P<.001$) in diabetic condition and it reversed to near control value in insulin- and vitamin D_3-treated diabetic rats (Fig. 3).

3.7. Real-time-PCR analysis of α7 nicotinic acetylcholine receptor

Real-time PCR analysis showed that α7 nicotinic acetylcholine receptor gene expression in the cerebral cortex was increased significantly ($P<.001$) in diabetic condition and it reversed to near control value in vitamin D_3-treated diabetic rats (Fig. 6). But insulin treatment did not show any significant change in α7 nicotinic acetylcholine receptor gene expression in the cerebral cortex when compared to diabetes (Fig. 4).

3.8. Real-time PCR analysis of acetylcholine esterase

Real-time PCR analysis showed that the acetylcholine esterase gene expression in the cerebral cortex was increased significantly ($P<.001$) in diabetic condition and it reversed to near control value in insulin- and vitamin D_3-treated diabetic rats (Fig. 5).

Table 3
Scatchard analysis of [^{3}H] QNB binding against pirenzepine in the cerebral cortex of control, diabetic, insulin treated diabetic and vitamin D_3-treated diabetic group rats

Animal status	B_{max} (fmol/mg protein)	K_d (nM)
Control	180±12.4	1.6±0.2
Diabetic	65±13.2‡	0.58±0.02‡
D + I	225±8.6*	1.8±0.01*
D + V	215±8.4*	2.1±0.03*

Values are mean±S.E.M of four to six separate experiments. Each group consist of six to eight rats. ‡$P<.001$ when compared to control; *$P<.001$ when compared to diabetic group.

Table 4
Scatchard analysis of [^{3}H] DAMP binding against 4-DAMP mustard in the cerebral cortex of control, diabetic, insulin-treated diabetic and vitamin D_3-treated diabetic group rats

Animal status	B_{max} (fmol/mg protein)	K_d (nM)
Control	56±1.4	0.20±0.02
Diabetic	202±2.2‡	0.49±0.02‡
D + I	52±0.5*	0.25±0.01$^{*,\$}$
D + V	49±0.4*	0.25±0.03$^{\$}$

Values are mean±S.E.M of four to six separate experiments. Each group consist of six to eight rats. $^{‡}P$<.001 when compared to control; $^{*}P$<.001 when compared to diabetic group; $^{\$}P$<.01 when compared to diabetic group.

3.9. Real-time PCR analysis of choline acetyl esterase

Real-time PCR analysis showed that the acetylcholine esterase gene expression in the cerebral cortex was decreased significantly (P<.001) in diabetic condition and it reversed to near control value in insulin- and vitamin D_3-treated diabetic rats (Fig. 6).

3.10. Real-time PCR analysis of insulin receptor

Real-time PCR analysis showed that the insulin receptor gene expression in the cerebral cortex was decreased significantly (P<.01) in diabetic condition and it reversed to near control value in insulin- and vitamin D_3-treated diabetic rats (Fig. 7).

3.11. Real time-PCR analysis of GLUT3 receptor

Real-time PCR analysis showed that the GLUT3 gene expression in the cerebral cortex was increased significantly (P<.001) in diabetic condition and it reversed to near control value in insulin-and vitamin D_3-treated diabetic rats (Fig. 8).

3.12. Real-time PCR analysis of VDR receptor

Real-time PCR analysis showed that the VDR receptor gene expression in the cerebral cortex was decreased significantly (P<.01) in diabetic condition and it reversed to near control value in insulin-and vitamin D_3-treated diabetic rats (Fig. 9).

3.13. Immunocytochemistry of muscarinic M3 receptor antibody staining in control and experimental groups of rats

The muscarinic M3 receptor antibody staining in the pancreas showed significant (P<.001) decrease in the receptor expression in diabetic compared to control. There was a significant increased expression of muscarinic M3 receptors in the pancreas of insulin (P<.01) and vitamin D_3 (P<.001) treated diabetic rats compared to diabetic group (Fig. 10, Table 5).

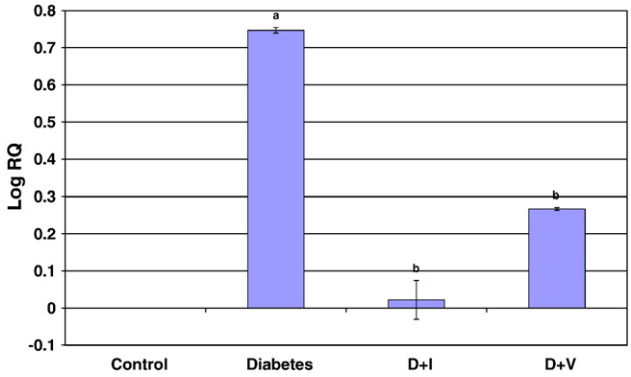

Fig. 3. Real-time amplification of muscarinic M3 receptor mRNA from the cerebral cortex of control, diabetic, insulin-treated diabetic and vitamin D_3-treated Diabetic rat. Values are mean±S.E.M. of four to six separate experiments. Each group consist of six to eight rats. ^{a}P<.001 when compared with control; ^{b}P<.001 when compared with diabetic group.

4. Discussion

Diabetes mellitus is a major global health problem currently affecting more than 180 million people worldwide. The disease is one of the most severe metabolic disorders in humans and it is characterised by hyperglycaemia as a result of a relative or an absolute lack of insulin or the action of insulin on its target tissue or both. The neurological consequences of diabetes mellitus in the CNS are now receiving greater attention. Manifestations of cerebral disorders in diabetic patients include alterations in neurotransmission, electrophysiological abnormalities, structural changes and cognitive deficits [17].

Our results showed that vitamin D_3 and insulin treatment showed restorative effect on blood glucose homeostasis and body weight of diabetic rats. There is evidence that vitamin D stimulates pancreatic insulin secretion directly. Vitamin D exerts its effects through nuclear vitamin D receptors [18], which are found in a wide variety of tissues, including T and B lymphocytes, skeletal muscle and the pancreatic islet β-cells [19]. In individuals with diabetes mellitus, vitamin D_3 treatment increases insulin secretion and improve glucose tolerance [20]. The facts of increased blood glucose level and decreased body

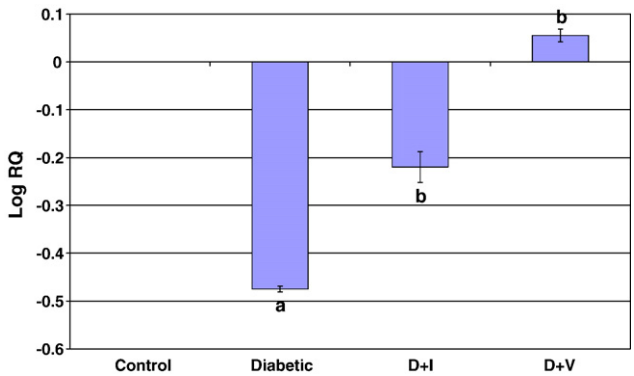

Fig. 2. Real-time amplification of muscarinic M1 receptor mRNA from the cerebral cortex of control, diabetic, insulin-treated diabetic and vitamin D_3-treated diabetic rat. Values are mean±S.E.M. of four to six separate experiments. Each group consist of six to eight rats. ^{a}P<.001 when compared with control; ^{b}P<.001 when compared with diabetic group.

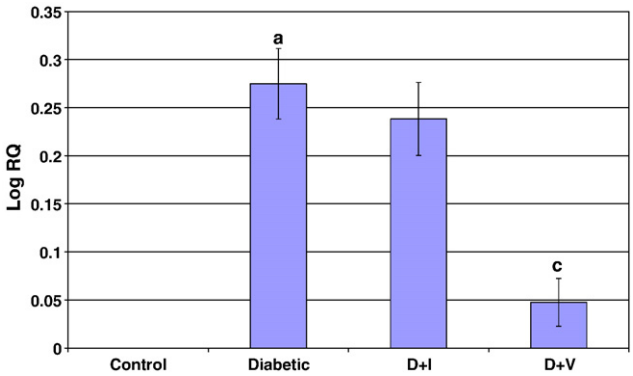

Fig. 4. Real-time amplification of α7 nicotinic acetylcholine receptor mRNA from the Cerebral cortex of Control, Diabetic, insulin treated Diabetic and vitamin D_3-treated Diabetic rat. Values are mean±S.E.M. of four to six separate experiments. Each group consist of six to eight rats. ^{a}P<.001 when compared with control; ^{b}P<.001 when compared with diabetic group; ^{c}P<.01 when compared with diabetic group.

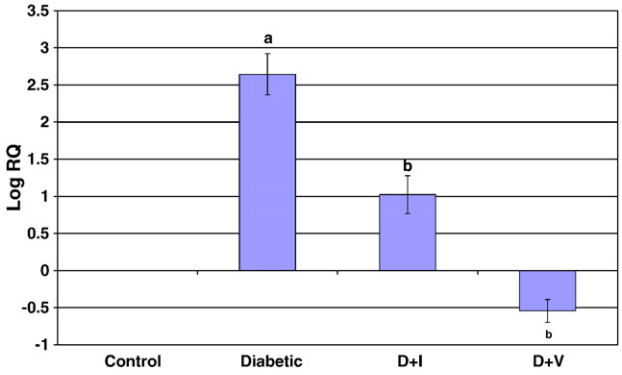

Fig. 5. Real-time amplification of Acetylcholine esterase mRNA from the cerebral cortex of control, diabetic, insulin-treated diabetic and vitamin D_3-treated diabetic rat. Values are mean±S.E.M. of four to six separate experiments. Each group consist of six to eight rats. [a]$P<.001$ when compared with control; [b]$P<.001$ when compared with diabetic group.

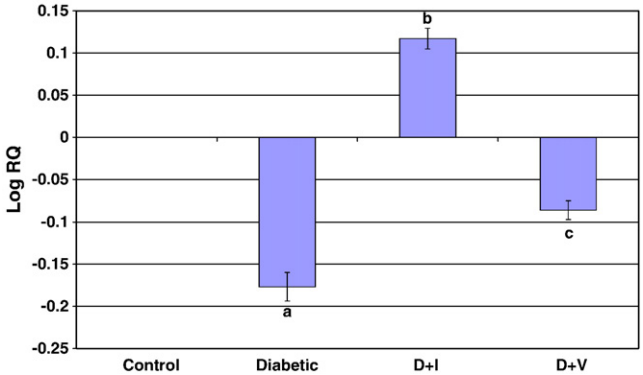

Fig. 7. Real-time amplification of Insulin receptor mRNA from the Cerebral cortex of Control, Diabetic, insulin treated Diabetic and vitamin D_3-treated Diabetic rat. Values are mean±S.E.M. of four to six separate experiments. Each group consist of six to eight rats. [a]$P<.001$ when compared with control; [b]$P<.001$ when compared with diabetic group; [c]$P<.01$ when compared with diabetic group.

weight, observed during diabetes, are similar with previous reports as a result of the marked destruction of insulin secreting pancreatic β-cells by STZ [9].

Y-maze performance showed that intensity of derangement in diabetic rats increased. In agreement with this finding, it has been found that the intensity of deficits of learning and memory has been found to increase in the course of diabetes which is associated with intensification of pathological processes within the cortical and other brain regions engaged in these processes [21]. Furthermore, spatial memory and exploratory activity have an influence on some behavioral tests including Y-maze performance. In this regard, the number of novel arm entries, and time spent was significantly lower in STZ-diabetic rats. There are also reports on the involvement of the cholinergic system abnormality in the impaired acquisition and/or retention of passive avoidance learning. In this respect, it has been postulated that the observed behavioural abnormalities consequent on an impairment of cerebral glucose metabolism may be suggestive of cholinergic dysfunction [22]. The vitamin D_3 and insulin-treated diabetic rats showed a significantly increased time spent and number of novel arm entry compared to STZ- induced diabetic rats. Our findings indicate that vitamin D_3 normalizes the cholinergic receptor dysfunction which assists in lowering their time for spatial recognition and thus improving the cognitive functions.

Functional abnormalities in muscarinic and nicotinic receptors are associated with many major diseases of the CNS. Current results showed the contribution of vitamin D_3 in modulating the muscarinic receptors of cerebral cortex thereby ameliorating cognitive performance. Earlier studies, from our laboratory have proved the functional regulation of the central neurotransmitter receptor subtypes during diabetes, pancreatic regeneration, cell proliferation and insulin secretion [14]. The result of this study demonstrate that mRNA level of muscarinic M1, M3 receptors, cholinergic enzyme, acetylcholine esterase and choline acetyl transferase in the cerebral cortex was substantially altered in the STZ-induced diabetic rats compared to control. Binding parameters of total muscarinic, muscarinic M1 showed a decreased receptor binding and M3 receptor showed an increase in diabetic rats. It is hypothesized that the cerebral cortex participates in the memory, attention, perceptual awareness, thought, language and consciousness which are necessary for the normal life style. Thus the current study reveals neuroprotective role of vitamin D_3 in cerebral cortex by normalising the altered cholinergic synaptic transmission.

It is well established that muscarinic receptors as well as nicotinic receptors are present mainly on pyramidal neurons in the human and rodent cerebral cortex [23]. The present research reveals a major increase in α7 nicotinic receptor gene expression in the cerebral cortex of STZ-induced diabetes rats. Thus, these receptors significantly influence the activity within the cortex circuitry and diabetes mellitus associated deregulation of this activity could contribute to disorders involving the cerebral cortex. As nicotine increases cerebral blood flow [24] glucose utilization, acetylcholine release [25] in the

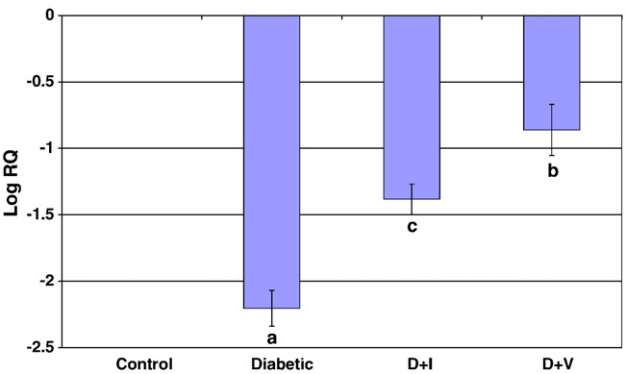

Fig. 6. Real-time amplification of Choline acetyl transferase mRNA from the Cerebral cortex of Control, Diabetic, insulin treated Diabetic and vitamin D_3-treated Diabetic rat. Values are mean±S.E.M. of four to six separate experiments. Each group consist of six to eight rats. [a]$P<.001$ when compared with control; [b]$P<.001$ when compared with diabetic group; [c]$P<.01$ when compared with diabetic group.

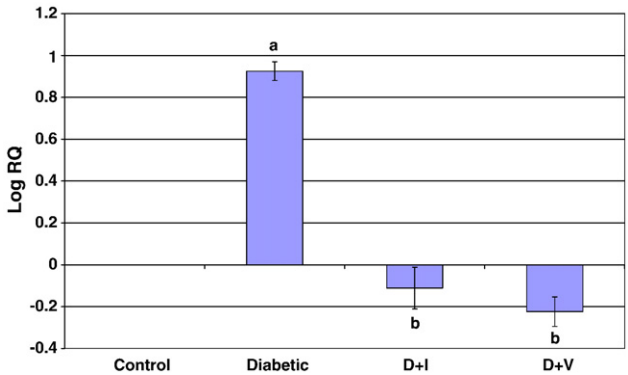

Fig. 8. Real-time amplification of Glut 3 receptor mRNA from the Cerebral cortex of Control, Diabetic, insulin treated Diabetic and vitamin D_3-treated Diabetic rat. Values are mean±S.E.M. of four to six separate experiments. Each group consist of six to eight rats. [a]$P<.001$ when compared with control; [b]$P<.001$ when compared with diabetic group.

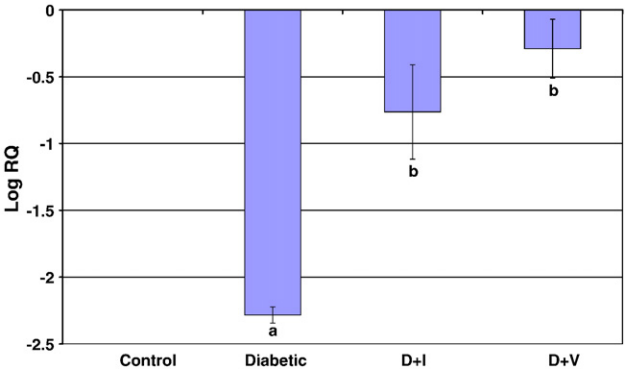

Fig. 9. Real-time amplification of VDR mRNA from the Cerebral cortex of Control, Diabetic, insulin treated Diabetic and vitamin D_3-treated Diabetic rat. Values are mean±S.E.M. of four to six separate experiments. Each group consist of six to eight rats. [a]$P<.001$ when compared with control; [b]$P<.001$ when compared with diabetic group; [c]$P<.01$ when compared with diabetic group.

brain, alterations in nAChR expression may be related to cognitive deficits in STZ-induced diabetes. Abnormalities of nicotinic acetylcholine receptor function in the hippocampus lead to cognitive and memory impairments [26]. Vitamin D_3 supplementation proved a beneficial effect in standardising the altered gene expression to near control stage. Also, insulin treatment showed no significant effect in the gene expression level of STZ induced diabetic rats.

Table 5
Muscarinic M3 Receptor Expression in the pancreatic islets of control and experimental Rats

Condition	Pixel Intensity
	Muscarinic M3
Control	40567±175
Diabetic	20156±151[a]
D + I	31542±128[b]
D + V	45986±160[c]

Values are mean±S.E.M. of three to four separate experiments. The given pixel value is the net value which is deducted from the negative control pixel value.
[a] $P<.001$ when compared to control.
[b] $P<.01$ when compared to diabetic.
[c] $P<.001$ when compared to diabetic.

The memory-improving effect of glucose was shown by Lapp [27]. Experiments have shown the ability of small doses of insulin (0.4–0.8 U/kg) to reverse the amnesia produced by a 2 mg/kg scopolamine injection [27] and intra-cerebro-ventricular injection of insulin facilitates memory [28]. The wide distribution of insulin and insulin receptors in the brain as well as the presence of insulin-dependent glucose transporters suggests that insulin in the brain participates in several cognitive functions, including learning and memory. An obvious problem that has impeded further research is that exogenous insulin injection can reduce blood glucose and lead to hypoglycaemia which is associated with impaired memory [29]. Cognitive impairments associated with diabetes mellitus caused by inadequate

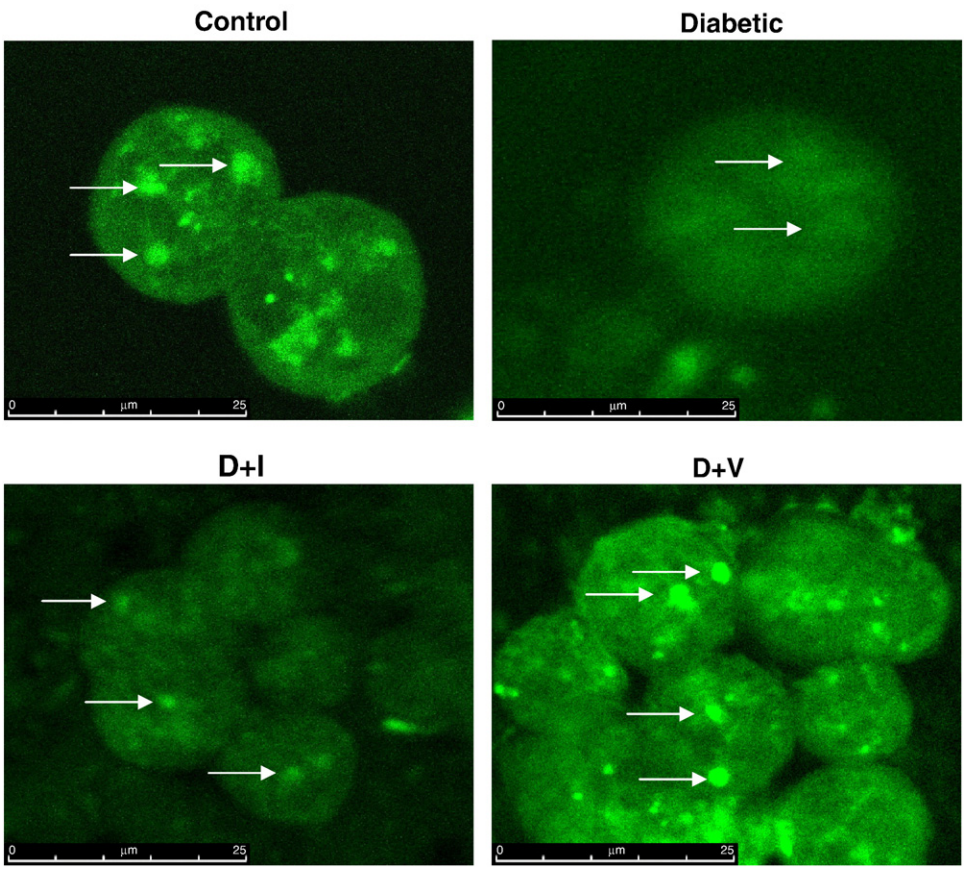

Fig. 10. Muscarinic M3 receptor Localization in Pancreatic islets of Control, Diabetic, insulin treated Diabetic and vitamin D_3-treated diabetic rat. Confocal immunofluorescent analysis of M3 receptors in the pancreatic islets of control, diabetic, D+I and D+V rats using immunofluorescent muscarinic M3 receptor specific primary antibody and FITC as secondary antibody. Pancreatic islets were excited with a 488 laser line and detected with a 510–600- band pass in PMT1. There was decreased expression of muscarinic M3 receptors in the pancreas of diabetic rats when compared to control rats. Insulin and vitamin D_3 treatment show an increased expression. (➞) shows muscarinic M3 receptors. The figures show representative image of three to four separate experiments (negative control is not shown). Scale bar=25 µm.

insulin/insulin receptor functions have also been documented. Our results provide a novel role of vitamin D_3 in reversing the disrupted expression of insulin receptor in the cerebral cortex of diabetic.

Glucose transport into the brain is critical for the maintenance of brain metabolism. Although under basal conditions the rate of glucose transport is not the rate-limiting step for glycolysis in the CNS, hypoglycaemia or hyperglycaemia is known to change the glucose transport system in the brain [30], suggesting that there should be glucose-regulatable mechanisms associated with the transport of glucose. Our study investigated the effect of learning-induced neuronal activation on cerebral cortex glucose utilization. Result confirms the alterations in GLUT3 expression, a major glucose transport in neurons of cerebral cortex with STZ-induced diabetes. Also, vitamin D_3 treatment improves the glucose transport system in cerebral cortex of diabetes rats by controlling the increased GLUT3 expression. Alterations in glucose utilization are known to occur in the important regions of brain connected with learning and memory [31]. Learning and memory processing are found to produce increases of glucose metabolism in the cortical brain regions that are functionally related to memory processing as well as to the sensorimotor task requirements [32].

VDR is expressed in most brain areas. Vitamin D_3, has been detected in the cerebrospinal fluid, and this hormone has been shown to cross the blood- brain barrier [33]. The presence of VDR in the limbic system, cortex, cerebellum of rodents and humans [34] supports a functional role for vitamin D_3 in the regulation of behavior and cognitive functions. Studies have shown that vitamin D_3 confers regulatory benefits in neuronal Ca++ homeostasis and protects neurons from excess calcium entry in the brain [35]. The present study shows a decreased expression of vitamin D receptor in the cortex of STZ-induced diabetic rats, which was standardized by the insulin and vitamin D_3 treatment.

The acetylcholine/vagus effects on pancreatic insulin release are mediated by activation of muscarinic acetylcholine receptors located on the pancreatic β cells. Receptor localization studies suggest that multiple muscarinic receptors (M1, M3, M4 and M5) are expressed in pancreatic islets/β-cells or β-cell derived tumor cell lines [36]. However, the M3 muscarinic receptor appears to be the predominant subtype expressed by pancreatic β-cells [8,37]. Earlier study demonstrated that muscarinic stimulation of pancreatic insulin and glucagon release is mediated by the M3 muscarinic receptor subtype [38]. Immunocytochemistry analysis in pancreas showed an increased expression of muscarinic M3 receptor in vitamin D_3-treated diabetic rats. Diabetes pancreas showed a decreased expression of muscarinic M3 receptors compared to control. An improvement in insulin secretion and response to an intravenous glucose tolerance test has also been seen with vitamin D_3 replacement in vitamin D-deficient rabbits [39]. In individuals with diabetes mellitus, vitamin D treatment may increase insulin secretion and improve glucose tolerance [40]. Our result showed that vitamin D_3 supplementation plays a pivotal role in regulating muscarinic M3 receptor expression through the VDR present in the pancreas and there by enhancing the insulin synthesis and secretion. Thus, our results demonstrate a possible mechanism of reducing the neuronal disorders in diabetes with vitamin D_3 supplementation through muscarinic M3 receptors in pancreas.

Treatment of diabetes mellitus is complex, requiring multifaceted lifestyle change and, for many, self-regulation of insulin levels in the blood. Uncontrolled hyperglycaemia, deficiencies of central insulin or both contributes to cortical dysfunction mediated with cholinergic neurons. Vitamin D_3 exhibited a potential effect in improving glucose homeostasis through modulation of pancreatic muscarinic M3 receptors and reversing the altered functional regulation of cholinergic, insulin and vitamin D receptors, acetylcholine esterase, choline acetyl transferase and GLUT3 activity in the cerebral cortex of STZ-induced diabetic rats. These results represent a novel possibility of vitamin D_3 as a therapeutic agent for the better management of neurological complications associated with diabetes.

References

[1] Greene DA, Stevens MJ, Feldman EL. Diabetic neuropathy: scope of the syndrome. Am J Med 1999;30:2S–8S.
[2] Chiu KC, Chu A, Go VL, Saad MF. Hypovitaminosis D is associated with insulin resistance and beta cell dysfunction. Am J Clin Nutr 2004;79:820–5.
[3] Perez-Fernandez R, Alonso M, Segura C, Munoz I, Garcia-Caballero T, Diguez C. Vitamin D receptor gene expression in human pituitary gland. Life Sci 1997;60: 35–42.
[4] McGrath J, Feron F, Eyles D, Mackay-Sim A. Vitamin D: the neglected neurosteroid? Trends Neurosci 2001;24:570–2.
[5] Olton D, Markowska A, Voytko ML, Givens B, Gorman L, Wenk G. Basal forebrain cholinergic system: a functional analysis. Advances in Experimental Medicine and Biology 1991;295:353–72.
[6] McNay EC, Fries TM, Gold PE. Decreases in rat extracellular hippocampal glucose concentration associated with cognitive demand during a spatial task. PNAS 2000;97:2881–5.
[7] Maher F, Vannucci SJ, Simpson IA. Glucose transporter isoforms in brain: absence of GLUT3 from the blood-brain barrier. J Cereb Blood Flow Metab 1993;13:342–5.
[8] Gilon P, Henquin JC. Mechanisms and physiological significance of the cholinergic control of pancreatic beta-cell function. Endocr Rev 2001;22:565–74.
[9] Junod A, Lambert AE, Staufferacher W, Renold AE. Diabetogenic action of streptozotocin relationship of dose to metabolic response. J Clin Invest 1969;48: 2129–39.
[10] Rosane de Souza Santos, Lucia Marques Vianna T. Effect of cholecalciferol supplementation on blood glucose in an experimental model of type 2 diabetes mellitus in spontaneously hypertensive rats and Wistar rats. Clinica Chimica Acta 2005;358:146–50.
[11] Glowinski J, Iversen LL. Regional studies of catecholamines in the rat brain, the disposition of [^{3}H] norepinephrine, [^{3}H]dopa in various regions of brain. J Neurochem 1966;13:655–69.
[12] Nitta A, Murai R, Suzuki N, Ito H, Nomoto H, Katoh G, et al. Diabetic neuropathies in brain are induced by deficiency of BDNF. Neurotoxicol Teratol 2002;24: 695–701.
[13] Yamamura HI, Synder G. Binding of [^{3}H] QNB in rat brain. Proc Natl Acad Sci USA 1981;71:1725–9.
[14] Gireesh G, Balarama Kaimal S, Peeyush Kumar T, Paulose CS. Decreased muscarinic M1 receptor gene expression in the hypothalamus, brainstem, and pancreatic islets of streptozotocin-induced diabetic rats. J Neurosci Res 2008;86: 947–53.
[15] Howell SL, Taylor KW. Potassium ions the secretion of insulin by islets of Langerhans incubated in vitro. Biochemical Journal 1968;108:17–24.
[16] Joseph A, Peeyush KT, Nandhu MS, Paulose CS. Enhanced NMDAR1, NMDA2B and mGlu5 receptors gene expression in the cerebellum of insulin induced hypoglycaemic and streptozotocin induced diabetic rats. Eur J Pharmacol 2010;630:61–8.
[17] Biessels GJ, Smale S, Duis SE, Kamal A, Gispen WH. The effect of gamma-linolenic acid-alpha-lipoic acid on functional deficits in the peripheral and central nervous system of streptozotocin-diabetic rats. J Neurol Sci 2001;182:99–106.
[18] Zeitz U, Weber K, Soegiarto DW, Wolf E, Balling R, Erben RG. Impaired insulin secretory capacity in mice lacking a functional vitamin D receptor. FASEB J 2003;17:509–11.
[19] Walters MR. Newly identified actions of the vitamin D endocrine system. Endocr Rev 1992;13:719–64.
[20] Borissova AM, Tankova T, Kirilov G, Dakovska L, Kovacheva R. The effect of vitamin D_3 on insulin secretion and peripheral insulin sensitivity in Type 2 diabetic patients. Int J Clin Pract 2003;57:258–61.
[21] Artola A, Kamal A, Ramakers GM, Biessels GJ, Gispen WH. Diabetes mellitus concomitantly facilitates the induction of long-term depression and inhibits that of long-term potentiation in hippocampus. Eur J Neurosci 2005;22:169–78.
[22] Jackson-Guilford J, Leander JD, Nisenbaum LK. The effect of streptozotocin-induced diabetes on cell proliferation in the rat dentate gyrus. Neurosci Lett 2000;293:91–4.
[23] Nakayama H, Shioda S, Okuda H, Nakashima T, Nakai Y. Immunocytochemical localization of nicotinic acetylcholine receptor in rat cerebral cortex. Mol. Brain Res 1995;32:321–8.
[24] Uchida S, Kagitani F, Nakayama H, Sato A. Effect of stimulation of nicotinic cholinergic receptors on cortical cerebral blood flow and changes in the effect during aging in anesthetized rats. Neurosci Lett 1997;228:203–6.
[25] Armitage AK, Hall GH, Sellers CM. Effects of nicotine on electrocortical activity and acetylcholine release from the cat cerebral cortex. Br J Pharmacol 1969;35: 152–60.
[26] Levin ED, Bradley A, Addy N, Sigurani N. Hippocampal alpha 7 and alpha 4 beta 2 nicotinic receptors and working memory. Neuroscience 2002;109:757–65.
[27] Lapp JE. Effects of glycemic alterations and noun imagery on the learning of paired associates. J Learn Disab 1981;14:35–8.
[28] Park CR, Crofford OB, Kono T. Mediated nonactive transport of glucose in mammalian cells and its regulation. J Gen Physiol 1968;52:S296–318.

[29] Santucci AC, Schroeder H, Riccio DC. Homeostatic disruption and memory effect of insulin administration in rats. Behav Neural Biol 1990;53:321–33.
[30] Devivo DC, Trifiletti RR, Jacobson RI, Rosen GM, Behmand RA, Harik SI. Defective glucose transport across the blood-brain barrier as a cause of persistent hypoglycorrhachia, seizures, and developmental delay. N Engl J Med 1991;325: 703–9.
[31] van der Zee EA, Streefland C, Strosberg AD, Schro der H, Luiten PGM. Visualization of cholinoceptive neurons in the rat neocortex: colocalization of muscarinic and nicotinic acetylcholine receptors. Mol Brain Res 1992;14:326–36.
[32] Friedman HR, Goldman-Rakic PS. Coactivation of prefrontal cortex and inferior parietal cortex in working memory tasks revealed by 2DG functional mapping in the rhesus monkey. J Neurosci 1994;14:2775–88.
[33] Gascon-Barre M, Huet PM. Apparent [3*H*]1,25-dihydroxyvitamin D_3 uptake by canine and rodent brain. Am J Physiol 1983;244:E266–271.
[34] Musiol IM, Stumpf WE, Bidmon HJ, Heiss C, Mayerhofer A, Bartke A. Vitamin D nuclear binding to neurons of the septal, substriatal and amygdaloid area in the Siberian hamster (*Phodopus sungorus*) brain. Neuroscience 1992;48:841–8.
[35] Brewer LD, Thibault V, Chen KC, Langub MC, Landfield PW, Porter NM. Vitamin D hormone confers neuroprotection in parallel with downregulation of L-type calcium channel expression in hippocampal neurons. J Neurosci 2001;21:98–108.
[36] Tang SH, Sharp GW. Identification of muscarinic receptor subtypes inRINm5F cells by means of polymerase chain reaction, subcloning, and DNA sequencing. Diabetes 1997;46:1419–23.
[37] Iismaa TP, Kerr EA, Wilson JR, Carpenter L, Sims N, Biden TJ. Quantitative and functional characterization of muscarinic receptor subtypes in insulin secreting cell lines and rat pancreatic islets. Diabetes 2000;49:392–8.
[38] Duttaroy A, Zimliki CL, Gautam D, Cui Y, Mears D. Muscarinic stimulation of pancreatic insulin and glucagon release is abolished in m3 muscarinic acetylcholine receptor-deficient mice. Diabetes 2004;53:1714–20.
[39] Nyomba BL, Bouillon R, De Moor P. Influence of vitamin D status on insulin secretion and glucose tolerance in the rabbit. Endocrinology 1984;115:191–7.
[40] Rudnicki PM, Molsted-Pedersen L. Effect of 1,25-dihydroxycholecalciferol on glucose metabolism in gestational diabetes mellitus. Diabetologia 1997;40: 40–4.