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Research Paper

Muscarinic M_1, M_3 receptor modulation in the corpus striatum of streptozotocin induced diabetic rats as a function of age

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Abstract

Objectives In this study we have investigated muscarinic M_1, M_3 receptor kinetics and the functional role of IP3 and cGMP in the corpus striatum of both young and old diabetic and insulin-treated diabetic rats.

Methods Radioreceptor binding assays was done in the corpus striatum using specific antagonists QNB and DAMP. IP3 and cGMP assay using [3H]IP3 and [3H]cGMP Biotrak assay system kits.

Key findings M_1 receptor increased and M_3 receptor decreased in control old rats when compared with young control rats. In young diabetic groups M_1 receptor increased and M_3 receptor decreased. Old diabetic groups showed reversed M_1 and M_3 receptors compared with their controls. IP3 and cGMP content increased in old control rats compared with young control rats. IP3 content increased in young diabetic rats and decreased in old diabetic rats. cGMP content was increased significantly in both young and old diabetic groups. Insulin treatment reversed these altered parameters near to control.

Conclusions Our studies showed that M_1 and M_3 receptors, IP3 and cGMP were functionally regulated during diabetes as function of age, which will have immense clinical significance.

Keywords ageing; corpus striatum; diabetic rats; muscarinic receptors; second messengers

Introduction

Muscarinic acetylcholine (ACh) receptors are members of a large family of plasma membrane receptors that transduce the intracellular signals via coupling to guanine nucleotide binding regulatory proteins and subserve numerous vital functions in both the brain and the autonomic nervous system.[1] These receptors are widely distributed throughout the body, predominantly expressed within the parasympathetic nervous system and exert both excitatory and inhibitory control over central and peripheral tissues. ACh has complex and clinically important actions in the striatum that are mediated predominantly by muscarinic receptors. Cholinergic terminals within the striatum contain presynaptic muscarinic receptors that inhibit neurotransmitter release. Various anatomical, electrophysiological, and pathological observations provide evidence that ACh plays a major role in the control of striatal function and in the regulation of motor control.[2] Striatal ACh is released from a population of large cholinergic interneurons that establish complex synaptic contacts with dopamine terminals, originating from the substantia nigra, and with several striatal neuronal populations. The structures within the basal ganglia observed to express muscarinic receptor mRNAs are the striatum, substantia nigra, pars compacta and subthalamic nucleus. The cholinergic innervation of the striatum is intrinsic and originates from a small number (<3%) of large neurons.[3] Streptozotocin (STZ)-induced rats were used as an experimental model for diabetes since they provide a relevant example of endogenous hyperglycaemia.[4] STZ is a toxic glucose analogue that preferentially accumulates in pancreatic beta cells via the GLUT2 glucose transporter and is used as a prominent diabetogenic chemical in diabetes research.[5]

Changes in muscarinic acetylcholine receptors (mAChRs) have been implicated in the pathophysiology of many major diseases of the CNS. Neurochemical studies have revealed age-related changes in neurotransmitter enzyme activity and receptor binding.[6] M_1

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mAChRs are abundantly expressed in all major forebrain areas, including cerebral cortex, hippocampus and striatum, where they constitute 40–50% of the total mAChRs.[7] These receptors have an important role in learning and memory processes. Receptor localization studies suggest the presence of multiple muscarinic receptors (M_1, M_3, M_4 and M_5) in pancreatic β-cells or β-cell-derived tumour cell lines.[8] The activation of mAChRs located on the pancreatic β-cells mediates ACh/vagus effects on pancreatic insulin release.[9] However, the muscarinic M_3 receptor appears to be the predominant cholinergic receptor subtype expressed by pancreatic β-cells and involved in pancreatic insulin and glucagon release. Studies from our laboratory have reported the regulatory role of mAChRs in insulin secretion.[10] M_1 receptors of diabetic rats were decreased in the hypothalamus, brainstem and pancreatic islets and insulin treatment reversed this altered expression near to control.[11] However, the functional role of striatal M_1 and M_3 receptors in regulating central cholinergic activity during diabetes, insulin treatment and on ageing has not been examined.

Muscarinic receptor stimulation by ACh leads to activation of phospholipase C, which in turn hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2) to produce D-myo-inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG).[12] IP3 is a second messenger that regulates intracellular Ca^{2+} release through IP3 receptors located in the endoplasmic reticulum and many prohypertrophic G protein-coupled receptor signalling events lead to IP3 liberation. In pancreatic β-cells, IP3 mobilizes Ca^{2+} from intracellular stores, resulting in an elevation of the intracellular concentration of Ca^{2+} and allowing activation of Ca^{2+}/calmodulin. DAG activates protein kinase C and accelerates exocytosis of insulin granules.[13] cGMP mediates physiological effects in the cardiovascular, endocrine and immunological systems as well as in the CNS. In the CNS, activation of the NMDA receptor induces Ca^{2+}-dependent nitric oxide synthase (NOS) and nitric oxide (NO) release, which then activates soluble guanylate cyclase for the synthesis of cGMP. These compounds are important mediators in long-term potentiation and long-term depression and play an important role in the mechanisms of learning and memory. Also, altered modulation of cGMP levels in the brain seems to be responsible for impairment of cognitive function.[14] However, the involvement of striatal IP3 and cGMP in mediating brain function during diabetes and ageing remains to be studied. In this study, we have investigated muscarinic M_1 and M_3 receptor kinetics in the corpus striatum of STZ-induced and insulin-treated diabetic rats as a function of age. Also, in-vivo studies were carried out in corpus striatum to elucidate the regulatory role of IP3 and cGMP during diabetes in both young and old rats.

Materials and Methods

Chemicals

All biochemicals used were of analytical grade. Quinuclidinyl benzilate, L-[benzilic-4,4′-^{3}H]-[4-^{3}H] (Sp. Activity 42 Ci/mmol) and 4-DAMP, [*N*-methyl-^{3}H] (Sp. Activity 83 Ci/mmol) were purchased from NEN life sciences products, Inc. (Boston, USA). [^{3}H]D-myo-inositol 1,4,5-triphosphate (IP3) Biotrak assay kit and [^{3}H]Cyclic GMP Biotrak assay kit was purchased from GE Healthcare UK Ltd (Buckinghamshire, London, UK). Pirenzepine and 4-DAMP mustard were purchased from Sigma Chemical Co. (St Louis, USA). Ethylenediaminetetraacetic acid, Tris-HCl and other chemicals of reagent grade for buffer solutions were obtained from SRL (Mumbai, India) and Merck (Mumbai, India).

Animals

Male Wistar weanling rats, 7–9 weeks old and 90–100 weeks old, purchased from Amrita Institute of Medical Sciences, Cochin, India and Kerala Agriculture University, Mannuthy, India, were used for all experiments. They were housed in separate cages under 12-h light–dark periods and allowed free access to standard food pellets and water. All animal care and procedures were in accordance with the CPCSEA and National Institute of Health guidelines and ethical committee approved procedures of the study.

Diabetes induction

Rats were divided into the following groups: (1) Control, (2) Diabetic, (3) Insulin-treated diabetic (D + I) rats, either 7 weeks old or 90 weeks old. Each group consisted of 4–6 rats. Diabetes was induced by a single intrafemoral dose (55 mg/kg body weight) of STZ (Sigma chemicals Co., St Louis, USA) freshly prepared in citrate buffer, pH 4.5 under anaesthesia.[4] The control rats were given the citrate buffer injection. Insulin treatment was started from the fourth day after STZ injection after confirming diabetes in rats by measuring the serum glucose and insulin level. The insulin-treated diabetic group received a daily dose (1 Unit/kg body weight) of Lente and Plain insulin (Abbott, Mumbai, India). The dose was increased daily according to the blood glucose level.[15] Blood glucose was estimated by the glucose-oxidase peroxidase method using a Glucose estimation kit (Merck, Mumbai, India).

Sacrifice and tissue preparation

On the 15th day of the experiment the rats were sacrificed by decapitation . The corpus striatum was dissected out quickly over ice according to the procedure of Glowinski and Iversen[16] and stored at −70°C until assay.

Muscarinic M_1 and M_3 receptor binding studies

Muscarinic M_1 and M_3 receptor binding assays were performed using specific antagonists [^{3}H]QNB and [^{3}H]DAMP in the corpus striatum of 7-week-old and 90-week-old rat groups, respectively.[17] [^{3}H]QNB has been widely used as a specific ligand for muscarinic ACh receptors in the CNS and in peripheral tissues.[17] Also, the DAMP biding assay is specific for muscarinic M_3 receptors. The QNB and DAMP act as competitive inhibitors of ACh at postsynaptic and postjunctional muscarinic receptor sites in the CNS.

The tissues were homogenised in a polytron homogeniser with 20 volumes of cold 50 mM Tris-HCl buffer, pH 7.4 containing 1 mM EDTA. The supernatant was then centrifuged at 30 000*g* for 30 min and the pellets were suspended in Tris-HCl-EDTA buffer. The muscarinic M_1 binding assay was performed using different concentrations (0.1–2.5 nM) of [^{3}H]QNB in the incubation buffer, pH 7.4, in a total incubation volume of 250 μl containing appropriate protein concen-

trations (200–250 μg). Competition studies were carried out with 1.0 nM [^{3}H]QNB in each tube with unlabelled ligand concentrations varying from 10^{-9} M to 10^{-4} M of pirenzepine. The muscarinic M_3 binding assay was carried out using different concentrations (0.01–5 nM) of [^{3}H]DAMP in the incubation buffer, pH 7.4, in a total incubation volume of 250 μl containing appropriate protein concentrations (200–250 μg). Competition studies were carried out with 0.5 nM [^{3}H]DAMP in each tube with unlabelled ligand concentrations varying from 10^{-9} M to 10^{-4} M of 4-DAMP mustard. Tubes were incubated at 22°C for 60 min and filtered rapidly through GF/C filters (Whatman Maidstone, UK). The filters were washed quickly by three successive washes 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. Specific binding was determined by subtracting non-specific binding from total binding.

Receptor data analysis

Protein was measured by the method of Lowry *et al*.,[18] using bovine serum albumin as standard. The receptor binding parameters were determined using Scatchard analysis.[19] The 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 GmbH, Erkrath, Germany). The displacement data were analysed by non-linear regression using Graphpad Prism software (GraphPad, Inc., San Diego, CA USA).

In-vivo studies

The corpus striatum of control and experimental rats was homogenised in a polytron homogeniser with 20 volumes of cold 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA. The homogenate was then centrifuged at 30 000*g* for 30 min and the supernatant was transferred to fresh tubes for IP3, cGMP assay using [^{3}H]IP3 and [^{3}H]cGMP Biotrak assay system kits.

Statistics

Statistical evaluations were performed by Student's *t*-test and analysis of variance by using Graphpad InStat (Ver. 2.04a; San Diego, USA) computer program. Linear regression Scatchard plots were made using SIGMA PLOT (Ver 2.03; Jandel GmbH, Erkrath, Germany). Competitive binding data were analysed using a non-linear regression curve-fitting procedure (GraphPad PRISM, San Diego, CA USA).

Results

The body weight was significantly decreased ($P < 0.05$) in 7-week-old and 90-week-old diabetic rats when compared with their respective controls. After insulin treatment for 14 days, the decrease in body weight was reversed to near the initial level in both groups (Table 1). STZ administration led to a significant increase ($P < 0.05$) in the blood glucose level of 7-week-old and 90-week-old diabetic rats when compared with controls. In both groups, insulin treatment significantly reduced ($P < 0.05$) the increased blood glucose level to near the control value when compared with the diabetic groups (Table 2).

Scatchard analysis of [^{3}H]QNB against pirenzepine in the corpus striatum showed a significant increase ($P < 0.05$) in the B_{max} of the 7-week-old diabetic rat group with a significant increase ($P < 0.05$) in the K_d compared with control. B_{max} decreased significantly ($P < 0.05$) in 90-week-old diabetic rats and there was a significant decrease ($P < 0.01$) in K_d compared with control. Insulin treatment significantly reversed ($P < 0.05$) the B_{max} to the control value with a significant increase ($P < 0.05$) in the affinity in 7-week-old rats

Table 1 Body weight of 7-week-old and 90-week-old experimental rats

Animal status	Initial weight (g)		Weight on 7th day (g)		Weight on 14th day (g)	
	7 weeks old	90 weeks old	7 weeks old	90 weeks old	7 weeks old	90 weeks old
Control	110 ± 10	320 ± 12	120 ± 12	325 ± 14	133 ± 15	330 ± 15
Diabetic	120 ± 6	360 ± 16	87 ± 5	280 ± 3	76 ± 4*$^{\phi}$	251 ± 2*$^{\phi}$
D + I	117 ± 3	350 ± 13	98 ± 2	301 ± 5	106 ± 5¶	310 ± 6¶

D + I, insulin treated diabetic rats. Values are means ± SD of 6 rats in each group. *$P < 0.05$ when compared with control. $^{\phi}P < 0.05$ when compared with initial weight. $^{¶}P < 0.05$ when compared with diabetic group.

Table 2 Blood glucose level in 7-week-old and 90-week-old experimental rats

Animal status	Blood glucose level on day 0 (before STZ injection) (mg/dl)		Blood glucose level on 3rd day (initial) (mg/dl)		Blood glucose level on 10th day (mg/dl)		Blood glucose level on 14th day (mg/dl)	
	7 weeks old	90 weeks old	7 weeks old	90 weeks old	7 weeks old	90 weeks old	7 weeks old	90 weeks old
Control	108.6 ± 1.7	110.1 ± 1.5	113.1 ± 0.8	120.2 ± 1.7	123.7 ± 0.6	127.9 ± 2.1	127.5 ± 0.8	128.3 ± 1.21
Diabetic	105.9 ± 0.7	115.7 ± 1.2	256.2 ± 0.4	248.9 ± 1.5	305.8 ± 0.7	265.7 ± 0.4	306.9 ± 1.3*	267.9 ± 1.2*
D + I	113.6 ± 1.2	119.2 ± 0.7	258.7 ± 0.3	254.8 ± 0.6	182.8 ± 1.3	193.8 ± 1.2	113.0 ± 1.1$^{¶\phi}$	164.6 ± 0.9$^{¶\phi}$

D + I, Insulin treated diabetic rats. Values are means ± SD of 6 rats in each group. *$P < 0.05$ when compared with control. $^{¶}P < 0.05$ when compared with diabetic group. $^{\phi}P < 0.05$ when compared with initial reading.

Table 3 Scatchard analysis of [^{3}H]QNB receptor binding against pirenzepine in the corpus striatum of control and experimental 7-week-old (young) and 90-week-old (old) rats

Animal status	B_{max} (fmol/mg protein)		K_d (nM)	
	7 weeks old	90 weeks old	7 weeks old	90 weeks old
Control	4200.0 ± 53.33	6600.0 ± 44.00$^{\Psi}$	1.31 ± 0.02	1.65 ± 0.04$^{\Psi}$
Diabetic	7400.0 ± 85.00*	4200.0 ± 28.30*†	1.48 ± 0.07*	1.31 ± 0.01*†
D + I	5200.0 ± 36.20¶	5800.0 ± 52.50$^{\P a}$	1.36 ± 0.01¶	1.61 ± 0.02$^{\P a}$

B_{max}, maximal binding; D + I, insulin treated diabetic rats; K_d, equilibrium dissociation constant. Values are means ± SD of 5 separate experiments. Each group consists of 8 rats. *$P < 0.05$, when compared with respective controls. $^{\P}P < 0.05$, when compared with diabetic rats. $^{\Psi}P < 0.05$, when compared with 7-week-old control. $^{\dagger}P < 0.05$, when compared with 7-week-old diabetic rats. $^{a}P < 0.05$ when compared with 7-week-old insulin-treated diabetic rats.

Table 4 Binding parameters of [^{3}H]QNB against pirenzepine in the corpus striatum of 7-week-old (young) control, diabetic and insulin-treated diabetic rats

Animal status	Log (EC50)-1	Log (EC50)-2	$K_{i(H)}$	$Ki_{(L)}$	Hill slope
Control	–9.323	–4.473	2.1×10^{-9}	3.36×10^{-5}	–0.540
Diabetic	–6.296	–4.690	5.05×10^{-7}*	2.04×10^{-5}	–0.218
D + I	–9.024	–4.570	$9.47 \times 10^{-10\P}$	2.65×10^{-5}	–0.269

D + I, insulin treated diabetic rats. EC50 is the concentration of the competitor that competes for half the specific binding. -1 and -2 indicates two affinity sites for the respective receptor. K_i is the affinity of the receptor for the competing drug. The affinity for the first and second site of the competing drug is designated as $K_{i(H)}$ (for high affinity) and $K_{i(L)}$ (for low affinity). Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, USA). Values are means ± SD of 5 separate experiments. Each group consists of 8 rats. *$P < 0.05$, when compared with respective control. $^{\P}P < 0.05$, when compared with diabetic rats.

whereas 90-week-old rats showed a significant decrease ($P < 0.01$) in the affinity when compared with the diabetic group. An increased B_{max} ($P < 0.05$) was observed in control old rats compared with the young rats, with a significant decrease ($P < 0.01$) in the affinity. B_{max} decreased significantly ($P < 0.05$) in 90-week-old diabetic rats with a significant decrease in K_d ($P < 0.01$) when compared with 7-week-old diabetic rats. The 90-week-old D + I group showed a significant increase in the B_{max} ($P < 0.01$) and K_d ($P < 0.01$) compared with the 7-week-old D + I group (Table 3). The competition curve for [^{3}H]QNB fitted for two-sited model in 7-week-old control, diabetic and D + I groups with Hill slope value away from unity. The competition curve fitted for one-sited model in 90-week-old control, diabetic and insulin-treated diabetic groups with unity as Hill slope value (Table 4).

Scatchard analysis of [^{3}H]DAMP against 4-DAMP mustard in the corpus striatum showed a significant decrease ($P < 0.05$) in the B_{max} of the 7-week-old diabetic rats whereas B_{max} increased significantly ($P < 0.05$) in the 90-week-old diabetic rats with a significant decrease in the affinity ($P < 0.01$) compared with control. Insulin treatment significantly reversed ($P < 0.05$) the B_{max} to the control when compared with the diabetic. B_{max} showed a significant decrease ($P < 0.05$) in control old rats compared with the young rats, with a significant increase ($P < 0.05$) in the affinity. B_{max} and K_d decreased significantly ($P < 0.001$, $P < 0.01$) in the 90-week-old diabetic and D + I groups when compared with the 7-week-old diabetic and D + I groups, respectively (Table 5). The competition curve for [^{3}H]DAMP fitted for one-sited model in 7-week-old control, diabetic and D + I groups with unity as Hill slope value. The competition curve

Table 5 Binding parameters of [^{3}H]QNB against pirenzepine in the corpus striatum of 90-week-old control, diabetic and insulin-treated diabetic rats

Animal status	Log (EC50)	K_i	Hill slope
Control	–5.251	5.60×10^{-6}	–0.990
Diabetic	–5.091	8.11×10^{-6}	–0.991
D + I	–5.209	6.18×10^{-6}	–0.970

D + I, insulin treated diabetic rats. EC50 is the concentration of the competitor that competes for half the specific binding. K_i is the affinity of the receptor for the competing drug. Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA).Values are means ± SD of 5 separate experiments. Each group consists of 8 rats.

fitted for one-sited model in 90-week-old rat groups with unity as Hill slope value (Tables 6 and 7).

IP3 content in the corpus striatum was increased significantly ($P < 0.05$) in 7-week-old diabetic groups whereas it decreased significantly ($P < 0.05$) in 90-week-old diabetic groups compared with respective controls. Insulin treatment significantly reversed ($P < 0.05$) the IP3 content near to control. In control old rats, the IP3 content increased significantly ($P < 0.05$) compared with the young rats. In 90-week-old diabetic group, we observed decreased IP3 content ($P < 0.05$) whereas the D + I group showed an increased IP3 content ($P < 0.05$) compared with 7-week-old diabetic and D + I groups, respectively (Table 8). The cGMP content in the corpus striatum was increased significantly ($P < 0.05$) in both 7-week-old and 90-week-old diabetic groups compared with control. Insulin treatment significantly reversed ($P < 0.05$) the increased cGMP content near to control. In control old rats,

Table 6 Scatchard analysis of [^{3}H] DAMP receptor binding against 4-DAMP mustard in the corpus striatum of control and experimental 7-week-old (young) and 90-week-old (old) rats

Animal status	B_{max} (fmol/mg protein)		K_d (nM)	
	7 weeks old	90 weeks old	7 week olds	90 weeks old
Control	6050.0 ± 28.05	1150.0 ± 31.79$^{\Psi}$	0.85 ± 0.01	0.52 ± 0.03$^{\Psi}$
Diabetic	4250.0 ± 18.33*	2100.0 ± 46.18*†	0.91 ± 0.07*	0.61 ± 0.05*†
D + I	5600.0 ± 46.00¶	1350.0 ± 23.53¶ ^{a}	0.87 ± 0.02	0.54 ± 0.02^{a}

B_{max}, maximal binding; D + I, insulin treated diabetic rats; K_d, equilibrium dissociation constant. Values are means ± SD of 5 separate experiments. Each group consists of 8 rats. *$P < 0.05$, when compared with respective controls. $^{\P}P < 0.05$ when compared with diabetic rats. $^{\Psi}P < 0.05$ when compared with 7-week-old controls. $^{\dagger}P < 0.05$, when compared with 7-week-old diabetic rats. $^{a}P < 0.05$ when compared with 7-week-old insulin-treated diabetic rats.

Table 7 Binding parameters of [^{3}H]DAMP against 4-DAMP mustard in the corpus striatum of 7-week-old (young) and 90-week-old (old) control, diabetic and insulin-treated diabetic rats

Animal status	Log (EC50)		K_i		Hill slope	
	7 weeks old	90 weeks old	7 weeks old	90 weeks old	7 weeks old	90 weeks old
Control	–5.251	–6.587	5.60×10^{-6}	2.58×10^{-7}	–0.990	–0.996
Diabetic	–5.091	–6.725	8.11×10^{-6}	1.83×10^{-7}	–0.991	–0.993
D + I	–5.209	–6.624	6.18×10^{-6}	2.37×10^{-7}	–0.970	–0.972

D + I, insulin treated diabetic rats; EC50, the concentration of the competitor that competes for half the specific binding; K*i*, the affinity of the receptor for the competing drug. Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, USA). Values are means ± SD of 5 separate experiments. Each group consists of 8 rats.

Table 8 IP3 and cGMP content in the corpus striatum of control and experimental 7-week-old (young) and 90-week-old (old) rats

Animal status	IP3 Concentration (pmol/g tissue)	cGMP Concentration (pmol/g tissue)
Control-young	200.0 ± 5.65	236.0 ± 15.50
Diabetic-young	356.0 ± 17.58*	376.0 ± 34.56*
D + I-young	252.0 ± 7.80¶	264.0 ± 10.25¶
Control-old	308.0 ± 15.25$^{\Psi}$	316.0 ± 8.40$^{\Psi}$
Diabetic-old	268.0 ± 8.50*†	432.0 ± 25.80*†
D + I-old	304.0 ± 12.50$^{\P a}$	356.0 ± 12.55$^{\P a}$

D + I, insulin-treated diabetic rats. Values are means ± SD of 5 separate experiments. Each group consists of 8 rats. *$P < 0.05$ when compared with respective control groups. $^{\P}P < 0.05$ when compared with respective diabetic groups. $^{\Psi}P < 0.05$ when compared with 7-week-old control. $^{\dagger}P < 0.05$ when compared with 7-week-old diabetic rats. $^{a}P < 0.05$ when compared with 7-week-old insulin-treated diabetic rats.

the cGMP content increased significantly ($P < 0.05$) compared with the young rats. Also, the 90-week-old diabetic and D + I groups showed increased cGMP content ($P < 0.05$) compared with the 7-week-old diabetic and D + I groups, respectively (Table 8).

Discussion

Neurotransmitters show significant alterations during hyperglycaemia and cause degenerative changes in neurons of the CNS.[20] Studies using STZ-induced diabetic rat models have shown similar results, which demonstrate morphological, behavioral and electrophysiological alterations in diabetes.[21] Earlier studies from our laboratory have established the functional regulation of central neurotransmitter receptor subtypes during diabetes, pancreatic regeneration, cell proliferation and insulin secretion.[22] During diabetes there is a decrease in body weight as a result of altered metabolic function. Hyperglycaemia occurs as a result of increased glycogenolysis, decreased glycogenesis, increased gluconeogenesis, impaired glucose transport across membranes and almost complete suppression of the conversion of glucose into fatty acids through acetyl-coenzyme A. The decreased body weight in the diabetic rats is due to excessive breakdown of tissue proteins. Treatment of diabetic rats with insulin improved body weight and decreased blood glucose level significantly, which indicates prevention of muscle tissue damage due to hyperglycaemia.

Muscarinic ACh receptors are widely distributed throughout the body and subserve numerous vital functions in both the brain and autonomic nervous system. CNS mAChRs regulate a large number of important central functions including cognitive, behavioural, sensory, motor and autonomic processes. The striatum, a neuronal nucleus intimately involved in motor behaviour, is one of the brain regions with the highest acetylcholine content.[23] Muscarinic receptor density was found to decrease significantly with ageing in certain brain regions, depending on the ligand used.[24] In our present study on muscarinic M_1 receptor binding using [^{3}H]QNB with pirenzepine, we found that M_1 receptor number increased significantly in the corpus striatum of control old rats compared with young rats, with an increase in K_d indicating a decrease in the affinity of receptors during ageing. Our analysis on the affinity states of these receptors by displacement studies showed that the

competition curve for [^{3}H]QNB fitted for a two-sited model in 7-week-old rat groups whereas it fitted for a one sited-model in 90-week-old rat groups with unity as Hill slope value. The 7-week-old diabetic rats showed an increased log (EC50) and K_i value, indicating a shift from a higher affinity state to a lower affinity state. There was no shift in affinity of the receptor in the old groups as indicated by unchanged K_i and log (EC50) values. Scatchard analysis of [^{3}H]DAMP against 4-DAMP mustard showed decreased B_{max} and K_d of control old rats compared with the young rats indicating increased affinity of muscarinic M_3 receptors on ageing. The competition curve fitted for a one-sited model in both 7-week-old and 90-week-old rat groups. There was no shift in affinity of the receptor as indicated by no change in K_i and log (EC50) in all these groups. The affinity change is thus confirmed by displacement analysis where we have observed the structural and functional alterations of muscarinic receptor subtypes during diabetes and ageing.

Age-related shrinkage of the midbrain has been observed previously[25] and the age-related decline in midbrain size could be accounted for in part by neuronal death or degeneration of nuclei or tracts of the midbrain. In STZ-induced diabetes, there are reported to be some structural alterations of mAChRs in the brain which in turn alter cholinergic nerve components, with a decrease in the Na^+, K^+-ATPase activity.[26] The studies of Latifpour and McNeill[27] on long-term STZ-induced diabetes demonstrated a large reduction in muscarinic receptor density as compared with age-matched controls. The M_1 receptor is present in more than 80% of striatal neurons, including cholinergic, substance P, enkephalin and somatostatin neurons. M_1 receptors of the corpus striatum were increased in young rats whereas old rats showed increased receptors during diabetes. However, M_3 receptors in the diabetic striatum showed reversed binding parameters in both age groups of rats when compared with striatal M_1 receptors. The functional regulation of both M1 and M3 receptors in young and old diabetic rats suggests a direct correlation between the diabetes-induced biochemical and functional alterations in muscarinic receptor properties on ageing. The variations in membrane structure and synaptic deficit of ACh concentration due to the ageing process could induce alteration in the mAChRs' conformation and this would modify the antagonist affinity. Also, altered muscarinic receptor numbers and axonal transport of receptor-bound opiate observed in STZ-induced hyperglycaemia suggests that impaired axonal transport of mAChRs is involved in the neurological disturbances which are seen in diabetic patients.[28] Insulin-treated diabetic rats showed an alteration in the muscarinic M_1 and M_3 receptors binding parameters in the corpus striatum to near control values. Studies by Iismaa *et al*.[8] highlighted cholinergic-mediated insulin secretion by muscarinic M_1 and M_3 receptor subtypes. Muscarinic receptor subtypes other than M_1 and M_3 may also be affected differentially in different age groups by the diabetic condition. Insulin has been reported to be mitogenic and to stimulate pancreatic β-cell proliferation *in vitro*. There have been previous reports from our laboratory regarding the regulatory role of mAChRs in glucose-induced insulin secretion. McEvoy and Herge[29] demonstrated that administration of insulin to diabetic rats implanted with fetal pancreas resulted in a three-fold increase in β-cell mass. Insulin favoured regeneration of β-cells by activating their neogenesis from precursor cells.[30] Rabinovitch *et al*.[31] have demonstrated that insulin can stimulate islet β-cell replication directly, possibly through a receptor for multiplication stimulating activity or insulin-like growth factor. Insulin signalling controls synaptic plasticity by modulating the activity of mAChRs and by triggering signal transduction cascades that are required for memory consolidation.[32]

The activation of muscarinic receptors results in hydrolysis of PIP2 and Ca^{2+} release from intracellular stores via the PLC-IP3 signalling pathway.[12] The resultant cytosolic Ca^{2+} transients serve numerous signalling functions in neurons, including modulation of membrane excitability, synaptic plasticity and gene expression.[33] The IP3 content in the corpus striatum was increased in young diabetic rats whereas it was decreased in old diabetic rats compared with control; insulin treatment significantly reversed the altered IP3 content near to control. Activation of endoplasmic reticulum membrane IP3 receptors produces excessive calcium release from the lumen of the endoplasmic reticulum to the cytoplasm.[34] This IP3-dependent increase in Ca^{2+} influx suggests a diabetes-induced modification of the nuclear membrane IP3 receptors. The IP3 content was increased in the corpus striatum of control old rats compared with the young rats. Increase in intranuclear Ca^{2+} leads to an altered transcription of apoptotic genes and activation of nuclear endonucleases resulting in programmed neuronal death.[35] cGMP has been implicated in the regulation of brain functions such as synaptic plasticity, phototransduction, olfaction and behavioural states.[36] The cGMP content in the corpus striatum was increased in both young and old diabetic rat groups compared with control. Insulin treatment significantly reversed the altered cGMP content to control. An increased cGMP content and NMDA receptor-NO (iNOS) activity was reported in striatum during ischaemic injury, indicating an important role of NMDA receptor-NO-cGMP in ischaemic injury of the striatal region.[37] Ageing coincided with a decrease in the basal level of cGMP as a consequence of a more active degradation of cGMP by phosphodiesterase. Moreover, a loss of NMDA receptor-stimulated enhancement of the cGMP level in the presence of cGMP-phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) was observed in aged rats. cGMP modulates cGMP-stimulated phosphodiesterase, which is widely expressed in the brain and regulates cAMP levels.[38] cGMP-dependent signal transduction becomes insufficient in the senescent brain and there are functional disturbances in learning and memory processes.[39] Thus our results revealed the significance of central muscarinic receptor and secondary messengers changes during diabetes in different age groups and the regulatory role of insulin on muscarinic receptors and secondary messengers.

Conclusion

From our results, it is suggested that acetylcholine differentially regulates striatal cholinergic function in the corpus striatum of both young and old rats through muscarinic M_1 and M_3 receptors. Also, this study elucidates the functional regulatory

role of IP3 and cGMP in striatal function during diabetes in both young and old rats. Uncontrolled hyperglycaemia or deficiencies of central insulin, or both, with age will contribute to corpus striatum dysfunction syndromes mediated through cholinergic neurons. Thus our results show the significance of managing cholinergic impairment, calcium release and functional deterioration of brain activity during diabetes as a function of age. This study suggests that drugs that can selectively activate muscarinic receptors may be of significant therapeutic benefit in diabetes management. This will have immense clinical significance in managing cholinergic impairment and functional deterioration of brain activity in striatum during diabetes and ageing.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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