MUSCARINIC M1 AND M3 RECEPTORS, IP3 AND cGMP FUNCTIONAL REGULATION IN STREPTOZOTOCIN INDUCED DIABETIC RATS: INSULIN AND SOMATOTROPIN INDUCED REJUVENATION AS A FUNCTION OF AGE

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CERTIFICATE

This is to certify that the thesis entitled "**MUSCARINIC M1 AND M3 RECEPTORS, IP3 AND cGMP FUNCTIONAL REGULATION IN STREPTOZOTOCIN INDUCED DIABETIC RATS: INSULIN AND SOMATOTROPIN INDUCED REJUVENATION AS A FUNCTION OF AGE**" is a bonafide record of the research work carried out by **Ms. Savitha Balakrishnan,** under my guidance and supervision in the Department of Biotechnology, Cochin University of Science and Technology and that no part thereof has been presented for the award of any other degree.

(C. S. Paulose)

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I hereby declare that the thesis entitled "**MUSCARINIC M1 AND M3 RECEPTORS, IP3 AND cGMP FUNCTIONAL REGULATION IN STREPTOZOTOCIN INDUCED DIABETIC RATS: INSULIN AND SOMATOTROPIN INDUCED REJUVENATION AS A FUNCTION OF AGE**" is based on the original research carried out by me at the Department of Biotechnology, Cochin University of Science and Technology, under the guidance of **Dr. C. S. Paulose,** Director, Centre for Neuroscience, Reader & Head, Department of Biotechnology and no part thereof has been presented for the award of any other degree, diploma, associateship or other similar titles or recognition.

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Savitha

Savitha Balakrishnan

Dedicated To My Beloved Parents ...

ABBREVIATIONS USED IN THE TEXT

5-HIAA	5-Hydroxyindole acetic acid
5-HT	5-Hydroxytryptamine
ACh	Acetylcholine
AChE	Acetylcholine esterase
AD	Alzheimer's disease
ADP	Adenosine diphosphate
AMPA	Amino-3-hydroxy-5-methyl-4isoxole propionic acid
APE	Arecaidine propargyl ester
ATP	Adenosine triphosphate
BBB	Blood Brain Barrier
BDNF	Brain Derived Neurotropic factor
B_{max}	Maximal binding
BS	Brainstem
BSA	Bovine Serum Albumin
BARC	Bhabha Atomic Research Centre
cAMP	Cyclic Adenosine mono phosphate
CAT	Choline acetyl transferase
CC	Cerebral cortex
CICR	Ca^{2+} induced calcium release
cDNA	Complementary deoxy ribonucleicacid
cGMP	Cyclic Guanosine monophosphate
CREB	cAMP responsive element binding protein
CNS	Central nervous system
cpm	Counts per minute
CS	Corpus striatum
CSF	Cerebrospinal fluid

D3	Iodothyronine deiodinase
DA	Dopamine
DAG	Diaceylglycerol
DOPAC	3,4-dihydroxyphenyl acetic acid
DAMP	4- deoxy acetyl methyl piperidine mustard
DEPC	Diethylpyrocarbonate
dNTP	Dinucleotide triphosphate
EAA	Excitatory amino acid
ECD	Electro chemical detector
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalogram
ER/SR	Reticulum/Sarcoplasmic Reticulum
GABA	γ- aminobutyric acid
GAD	Glutamic acid decarboxylase
GDP	Guanosine diphosphate
GH	Growth Hormone (Somatotropin, STH)
GHBP	Growth Hormone Binding Protein
GHR	Growth Hormone Receptor
GHRH	Growth Hormone Releasing Hormone
GLUT 2	Glucose transporter type 2
GLUT 3	Glucose transporter type 3
GLUT 4	Glucose transporter type 4
GOD	Glucose oxidase
GQPCR	GQ protein coupled receptors
GRP	Gastrin releasing peptide
GTP	Guanosine triphosphate
HBSS	Hank's balanced salt solution
HEPES	[n' (2-hydroxy ethyl)] piperazine-n'-[2-ethanesulfonic acid]

HIF	Hypoxia Inducible Factor
HPA	Hypothalamic- Pituitary -Adrenal
HPLC	High performance liquid chromatography
HYPO	Hypothalamus
IDDM	Insulin dependent diabetes mellitus
IGF	Insulin like growth factor
INS	Insulin
IP3	D, myo1, 4, 5 inositol triphosphate
IRS	Insulin Receptor Substrates
JAK-2	Janus kinase
K_i	Inhibitory coefficient
K_m	Michaelis constant
K_d	Dissociation constant
KRB	Krebs ringer bicarbonate
LSD	Lysergic acid diethylamide
LTP	Long term potentiation
LTD	Long term depression
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
MCI	Mild cognitive impairment
MIF	Macrophage migration inhibiting factor
MNCV	Motor nerve conduction velocity
mAChRs	Muscarinic acetylcholine receptors
mGlu-5	Metabotropic glutamate receptor 5
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
NE	Norepinephrine

NGF	Nerve Growth Factor
NIDDM	Non-insulin dependent diabetes mellitus
NMDAR1	N-methyl-D-aspartate receptor
NO	Nitric Oxide
NTS	Nucleus tractus solitarius
OD	Optical density
p	Level of significance
PACAP	Pituitary adenylate cyclase activating polypeptide
PEG	Polyethylene glycol
PIP_2	Phosphatidylinositol biphosphate
PKC	Protein kinase C
PLC-C	Phospho lipase C
PMCA	Plasmalemma calcium ATPase
PNS	Peripheral nervous system
PZ	Pirenzepine
QNB	Quinuclidinyl benzilate, L-benzilic-4,4'
RIA	Radioimmuno assay
RNA	Ridonucleic acid
ROI	Reactive oxygen intermediate
RPMI	Roswell park memorial Institute
RT-PCR	Reverse-transcription-polymerase chain reaction
S.E.M.	Standard error of mean
SERCA	Smooth endoplasmic reticulum calcium ATPase
SMOCC	Second messenger-operated calcium channels
SNc	Substantia nigra pars compacta
SNCV	Sensory nerve conduction velocity
STH	Somatotropin (Growth Hormone, GH)
STZ	Streptozotocin

T3	3,3', 5-tri-iodo-L-thyronine
T4	Thyroxine
TRH	Thyrotropin releasing hormone
TSH	Thyroid stimulating hormone
TZD	Thiazolidinediones
VIP	Vasoactive intestinal peptide
VMH	Ventro medial hypothalamus
V_{max}	Maximum velocity
VICC	Voltage intensive Ca^{2+} Channels
VOCC	Voltage sensitive Ca^{2+} channels
VTA	Ventral tegmental area

CONTENTS

MATERIALS AND METHODS

Introduction

Ageing is the biological process characterized by the progressive and irreversible loss of physiological function accompanied by increasing mortality with advancing age. It is a complex physiological phenomenon associated with a multitude of biological changes at the molecular level, which is eventually manifested at the tissue and organism level. Understanding the molecular mechanism behind the ageing process is therefore, key to the development of therapeutics which prolong human life span, and more importantly, to reduce morbidity among the elderly. The mechanisms involved in plasticity in the nervous system are thought to support cognition, and some of these processes are affected during normal ageing. Genes that are down-regulated over the age include GluR1 AMPA receptor subunit, NMDA R2A receptor subunit involved in learning, subunits of the $GABA_A$ receptor, genes involved in long-term potentiation like calmodulin 1 and CAM kinase IIα, calcium signalling genes, synaptic plasticity genes, synaptic vesicle release and recycling genes. Genes that are up regulated include genes associated with stress response, DNA repair and antioxidant defense. Several studies showed that the age-related loss of a number of functions is associated with an oxidative damage in the tissues mediating those functions. In the ageing brain, neurological deficits related to ageing have been suggested to be due to a breakdown of calcium (Ca^{2+}) homeostasis, and an increase in intracellular Ca^{2+}. It is possible that age-related changes in Ca^{2+} regulation cause some portion of the observed age-related plasticity deficits.

Ageing is characterized by progressive impairment of bodily activities. Normal human ageing is associated with a progressive impairment of glucose tolerance (Davidson 1979). Total glucose stimulated insulin secretion has been described as being unchanged (Draznin *et al.*, 1985), suppressed (Molina *et al.*, 1985)

or increased (Curry *et al.*, 1984) as an animal ages. Recently it was demonstrated in Wistar rats ageing is indeed associated with progressive decline in beta cell number, the pancreatic insulin content, amount of insulin secreted and insulin mRNA levels (Perfetti *et al.*, 1995). Impairment of insulin action as a function of age has also been reported. There is an impairment of insulin induced glucose disposal in old compared with young subjects (Haruo *et al.*, 1988). Few laboratories have attributed the alterations in glucose stimulated insulin secretion with age to changes in diet rather than ageing, *per se* (Hara *et al.*, 1992).

The central nervous system (CNS) neurotransmitters play an important role in the regulation of glucose homeostasis. During normal ageing prominent alterations occur in various neurotransmitter systems in the brain, related to reductions in the number of neurons and to a decrease in concentration, synthesis and turnover of neurotransmitters. Reductions of the levels of transmitter substances and of the activities of enzymes involved in their synthesis have been demonstrated in the ageing brain. These neurotransmitters mediate rapid intracellular communications not only within the central nervous system but also in the peripheral tissues. They exert their function through receptors present in both neuronal and non-neuronal cell surface that trigger second messenger signaling pathways. Neurotransmitters have been reported to show significant alterations during diabetes resulting in altered functions causing neuronal degeneration. Chronic hyperglycaemia during diabetes mellitus is a major initiator of diabetic micro-vascular complications like retinopathy, neuropathy and nephropathy. Age related changes in the capacity of β-cell for proliferation affect the insulin production and contribute to a decrease in glucose tolerance with advance in age.

Acetylcholine is a neurotransmitter that has been implicated in various central neuronal degenerative disorders like Alzheimer's disease, dementia and other age

related memory disorders. An acetylcholine receptor is an integral membrane protein that responds to the binding of the neurotransmitter acetylcholine. There are two main classes of acetylcholine receptor (AChR), nicotinic acetylcholine receptors (nAChR) and muscarinic acetylcholine receptors (mAChR). Muscarinic receptors are metabotropic and affect neurons over a longer period. They are stimulated by muscarine and acetylcholine, and blocked by atropine. Muscarinic receptors are found in both the central nervous system and the peripheral nervous system, in heart, lungs, upper GI tract and sweat glands. Acetylcholine in the central nervous system is involved in the control of motor activity, emotional behaviour, cognition and endocrine regulation. Hyperglycaemia during diabetes is reported to damage cholinergic functions. The progression of diabetes is associated with an impaired ability of the neurons in the CNS to release neurotransmitters resulting in behavioural changes.

Parasympathetic activity plays an important role in insulin secretion from pancreatic β-cells. Cholinergic agonist carbachol increases insulin secretion from isolated rat islets (Zawalich & Zawalich, 2002). The muscarinic receptor stimulation by ACh leads to activation of phospholipase C, which, in turn, hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP2) to produce D-myo-Inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) (Best & Malaisse, 1983; Zawalich *et al.*,1989). 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 on the other hand, activates PKC (Nishizuka, 1995; Renstrom *et al.*, 1996). PKC, like Ca^{2+}/calmodulin, accelerates exocytosis of insulin granules (Nanko *et al.*, 2002).

The mitogenic effect of acetylcholine has been studied in different cell types. Muscarinic acetylcholine receptors activate many downstream signaling pathways,

some of which can lead to mitogen activated protein kinase (MAPK) phosphorylation and activation. Mitogen activated protein kinases play a role in regulating cell growth, differentiation and synaptic plasticity. Both Gi and Gq coupled muscarinic receptors have been shown to activate MAPK in various system. Muscarinic M3 receptors activate MAPK in the oligodendrocyte progenitors (Ragheb *et al.*, 2001). The involvement of M1 receptors has been reported in muscarinic activation of MAPK in PC12 cells (Berkeley *et al.*, 2000). Acetylcholine analogue carbachol stimulated DNA synthesis *via* muscarinic receptors in primary astrocytes derived from perinatal rat brain (Ashkenazi, 1989). Carbachol is also mitogenic in certain brain derived astrocytoma and neuroblastoma, as well as in Chinese hamster ovary (CHO) cells expressing recombinant muscarinic receptors (Ashkenazi, 1989). Proliferation experiments with subtype specific antagonists in astrocytes suggest that cell proliferation is induced by the activation of muscarinic M3 receptors (Guizzetti, 1996, 2002).

Regeneration is a complex interplay of several factors - growth factors, hormones and neurotransmitters. The stimulatory effect of growth hormone on insulin (INS) production and β-cell replication are well documented (Swenne *et al.*, 1987; Nielsen, 1986, Sjoholm *et al.*, 2000). *In vitro* and *in vivo* studies have established the role of insulin in β-cell replication (Chick *et al.*, 1973). Insulin interacts with type1 IGF receptor and stimulates β-cell proliferation. Somatotropin (STH), is an important anabolic hormone which exerts stimulatory effects on protein synthesis and on lipolysis. Pituitary STH release is regulated primarily by the interaction of the hypothalamic peptides, GHRH, which stimulates and somatostatin which inhibits STH production. STH (Growth hormone, GH) regulates body growth and metabolism. STH exerts its biological action by stimulating JAK2, a GH receptor (GHR)-associated tyrosine kinase. Activated JAK2 phosphorylates itself and GHR, thus initiating multiple signaling pathways.

If glucose supply to the brain is not maintained, there may be a decrease in cerebral electrical activity, membrane breakdown with release of free fatty acids, and altered amino acid metabolism. Deterioration in glucose homeostasis that results from hyperglycaemia can trigger neuronal injuries; the molecular basis of this neuronal vulnerability is not yet explored. The reports so far stated did not attempt to emphasis the functional correlation of cholinergic receptors in diabetic brain as a function of age. Studies targeting the exact molecular mechanism of cholinergic receptor subtypes in diabetic rats of different age groups will be useful in improving selective cognitive processes and insulin function with increasing chronological age. In the present study, the alterations of muscarinic M1 and M3 receptor subtypes in the brain regions of streptozotocin induced diabetic rats were carried out in different experimental age groups of rats. Also, the second messengers, IP3 and cGMP, in brain regions of diabetic rats were studied as a function of age. Calcium imaging studies were carried out to observe the role of muscarinic receptor antagonists in intracellular Ca^{2+} release from pancreatic islets of young and old rats *in vitro*. The functional role of long term low dose STH and INS treatment in the regulation of neurotransmitter levels in the ageing brain was studied. Also, the possible linkage between the STH and INS induced changes in second messengers like IP3 and cGMP has been elucidated as a function of age. Electroencepalogram studies were performed to analyse the brain wave signaling pattern in young and old rats. This will have immense therapeutic application in improving cognition, learning and memory and rejuvenating brain functions during ageing.

OBJECTIVES OF THE PRESENT STUDY

1. To study acetylcholine esterase (AChE) activity in the brain regions; cerebral cortex (CC), brainstem (BS) & corpus striatum (CS) of 7 weeks (young) and 90 weeks (old) control, diabetic and insulin treated diabetic rats.

2. To study AChE activity in the pancreas of 7 weeks (young) and 90 weeks (old) control, diabetic and insulin treated diabetic rats.

3. To study acetylcholine receptor changes in the cerebral cortex of 7 weeks and 90 weeks old control, diabetic and insulin treated diabetic rats.

4. To study muscarinic M1 and M3 receptors changes in the brain regions of 7 weeks and 90 weeks old control, diabetic and insulin treated diabetic rats.

5. To study muscarinic M1 and M3 receptor gene expression in the brain regions of 7 weeks and 90 weeks old control, diabetic and insulin treated diabetic rats.

6. To study muscarinic M3 receptors changes in the pancreas of 7 weeks and 90 weeks old control, diabetic and insulin treated diabetic rats.

7. To study muscarinic M3 receptor gene expression in the pancreas of 7 weeks and 90 weeks old control, diabetic and insulin treated diabetic rats.

8. To study glutamate NMDAR1, mGlu-5, α_{2A}, β_2, $GABA_{A\alpha 1}$ and $GABA_B$, DAD2 and 5-HT_{2C} receptors gene expression in the cerebral cortex of 7 weeks and 90 weeks old control, diabetic and insulin treated diabetic rats.

9. To quantify IP3 and cGMP content in the brain regions and pancreas of 7 weeks and 90 weeks old control, diabetic and insulin treated diabetic rats.

10. To study the role of carbachol and muscarinic M1, M3 receptors antagonists on glucose induced insulin secretion by pancreatic islets of young and old rats *in vitro.*

11. To study the role of carbachol and muscarinic M1, M3 receptors antagonists on IP3 and cGMP release by pancreatic islets of young and old rats *in vitro.*

12. To study the role of carbachol and dopamine on IP3 and cGMP release by pancreatic islets of young and old rats *in vitro.*

13. To quantify the triiodothyronine (T3) content in the serum of 7 weeks and 90 weeks old control, diabetic and insulin treated diabetic rats.

14. To quantify epinephrine (EPI), norepinephrine (NE), dopamine (DA) and serotonin (5-HT) content in the brain regions CC, CS, BS & Hypothalamus (Hypo) of long term low dose somatotropin and insulin treated young and old rats using HPLC.

15. To study AChE activity in the cerebral cortex of long term low dose somatotropin and insulin treated young and old rats.

16. To study muscarinic M1, M3, glutamate NMDAR1, mGlu-5, α_{2A}, β_2, $GABA_{A\alpha 1}$ and $GABA_B$, DAD2 and $5\text{-}HT_{2C}$ receptors gene expression in the

cerebral cortex of long term low dose somatotropin and insulin treated young and old rats.

17. To quantify the IP3 and cGMP content in the brain regions of long term low dose somatotropin and insulin treated young and old rats.

18. To quantify the triiodothyronine (T3) content in the serum of long term low dose somatotropin and insulin treated young and old rats.

19. To study the effect of carbachol and muscarinic M1, M3 receptor antagonists on intracellular calcium release in pancreatic islets of young and old rats *in vitro*.

20. To perform neurophysiologic analysis of the electrical activity of the brain using electroencephalogram of experimental rats.

Literature Review

Ageing refers to multidimensional process of physical, psychological, and social change to an individual. Several biochemical pathways appear to be involved in the ageing process, and these include that govern DNA repair, control death and survival of individual cells in a process called apoptosis, control functions of mitochondria in cells, regulate organism's response to oxidative stress and govern metabolic consequences of glucose intake. DNA damage due to oxidation increase as the brain ages is mainly due to impaired mitochondrial function. The human brain shows a decline in function and a change in gene expression as the individual progresses from embryo to old age. This modulation in gene expression is mainly due to oxidative DNA damage at promoter regions in the genome. It has long been known that there is a genetic contribution to long life and to successful ageing. Also, a whole range of genetic alterations have been discovered that promote longer life and avoidance of cognitive decline and neurodegeneration. The up regulated genes in the ageing brain are associated with inflammation and intracellular Ca^{2+} release pathways, whereas genes associated with energy metabolism, biosynthesis and activity-regulated synaptogenesis were down regulated (Sara & Carol, 2006). If specific molecular pathways controlling the rate of ageing can be modulated genetically, then perhaps they can be modulated pharmacologically. These insights have an important impact on the discovery and development of drugs to both treat and prevent a wide range of age related diseases in the future.

Ageing and the brain

Ageing causes changes to the brain size, vasculature, and cognition (Peters, 2006). The brain shrinks with increasing age and there are changes occuring at the molecular level to morphology. The region specific changes in dendritic branching

and spine density are more characteristic of the effects of ageing on neuronal morphology (Sara & Carol, 2006). The brains of individuals, who are cognitively normal, show age-related changes that include an overall reduction in brain volume and weight, which are associated with gyral atrophy and widening of the sulci of the cerebral cortex, and enlargement of the brain ventricles. Microscopically, there are increasing amounts of the age-related pigment, lipofuscin, granulovacuolar degeneration in neurons, hirano bodies, diffused deposits of beta-amyloid in parenchyma, neurofibrillary tangles in hippocampus and amygdala, and sparse numbers of senile plaques in these regions and in other cortical areas of the brain (Anderton, 1997). Of these changes, neurofibrillary tangles and senile plaques are the neuropathological hallmark of Alzheimer's disease (AD) in which they are more abundant and widespread (Hof *et al.*, 1996). AD has therefore been regarded as accelerated brain ageing. Understanding the molecular basis of plaque and tangle formation is advancing greatly and is the main focus of research into the cellular and molecular changes observed in the ageing brain. The nature of the cognitive and neurobiological alterations associated with age-related change is substantially different from that seen in the early stages of a dementing illness, such as Alzheimer's disease (Albert, 1997). The interplay between genetic and environmental factors determines the degree of pathological brain ageing and whether or not individuals develop dementia in later stages.

Neuropathological changes associated with normal brain ageing

The ageing brain shows selective neurochemical changes involving several neuronal cell populations. Ageing and its variants, such as AD, viewed as the result of alterations in the levels of Abeta, metals, cholinesterase enzymes and neuronal gene expression (Lahiri, 2005). Neurofibrillary tangles and senile plaques are common neuropathological features in both normal brain ageing and AD disease. Layer II of

the entorhinal cortex is involved with neurofibrillary tangle formation in all of the cases, while the CA1 field of the hippocampus and the subiculum are less consistently affected. Neocortical area 20 is particularly prone to develop neurofibrillary tangles in intellectually preserved elders, whereas other neocortical areas are relatively spared. Substantial senile plaque formation is seen in the neocortex of non-demented cases. Mild cognitive impairment is correlated with neurofibrillary tangle densities in layer II of the entorhinal cortex, and clinically overt AD with neurofibrillary tangle densities in area 20. In non-demented cases, there is an early development of neurofibrillary tangles in areas usually spared in the course of the degenerative process in younger individuals. These observations demonstrate that mesial and inferior temporal lobe structures are affected more frequently in normal brain ageing. In this respect, neurofibrillary tangle formation in area 20 may represent a crucial step of the degenerative process because it precedes the emergence of the neuropsychological deficits characteristic of age related disorders. In addition, this reveals age-related heterogeneity in the regional vulnerability of the brain region during normal brain ageing (Hof *et al.*, 1996).

Neural plasticity in the ageing brain

Aged animals have alterations in the mechanisms of plasticity that contribute to cognitive functions. One functional alteration that could directly affect plasticity is reduced synapse number, which could make it more difficult to attain the sufficient amount of active synapses that is necessary for the network modification. An early electron microscopic investigation at the perforant path–granule cell synapse showed that aged rats have a 27% decrease in axodendritic synapse number in the middle molecular layer of the dentate gyrus compared with young rats (Bondareff & Geinisman, 1976). Moreover, spatial memory deficits have been shown to correlate with a reduction in perforated synapses at the medial perforant path–granule cell synapse (Geinisman *et al.*, 1986). The total number of synaptic contacts per neuron

was found to be diminished significantly in the middle and inner molecular layer of dentate gyrus of aged rats relative to young adults. Both perforated and non-perforated axospinous synapses showed age-dependent decreases in numbers (Geinisman *et al.*, 1992). Cognitive functions that rely on the medial temporal lobe and prefrontal cortex, such as learning, memory and executive function show considerable age-related decline. Several neural mechanisms in these brain areas also seem to be vulnerable during the ageing process. Age-related changes in the medial temporal lobe and prefrontal cortex results in altered functional plasticity contribute to behavioural impairments in the absence of significant pathology (Burke & Barnes, 2006). The subtle changes in neuronal morphology, cell–cell interactions and gene expression that might contribute to alterations in plasticity in aged animals disrupt the network dynamics of aged neurons that ultimately contribute to selective behavioural impairments (Sara & Carol, 2006).

Memory and ageing

Memory is an organism's ability to store, retain, and subsequently retrieve information. Ageing affect memory by changing the way the brain stores information and recall the stored information. Studies comparing the effects of ageing on episodic memory, semantic memory, short-term memory and priming found that episodic memory is greatly impaired in normal ageing (Nilsson, 2003). These deficits are related to impairments seen in the ability to refresh recently processed information (Johnson *et al.*, 2002). The ability to encode new memories of events or facts and working memory showed decline in both cross-sectional and longitudinal studies (Hedden & Gabrieli, 2004). In addition, older adults tend to be worse at remembering the source of their information for a particular item or fact (Johnson *et al.*, 1993), a deficit that is related to declines in the ability to bind information together in memory (Mitchell *et al.*, 2000). In contrast, implicit or procedural memory typically shows no decline with age (Fleischman *et al.*, 2004), short-term memory shows little decline

(Nilsson, 2003) and semantic knowledge, such as vocabulary improves with age (Verhaeghen, 2003). In addition, the enhancement seen in memory for emotional events is also maintained with age (Mather & Carstensen, 2005). Brain imaging studies have revealed that older adults are more likely to use both hemispheres when completing memory tasks than younger adults (Cabeza, 2002). In addition, older adults show a positive effect when remembering information, which seems to be a result of the increased focus on regulating emotion seen with age (Mather & Carstensen, 2005; Isaacowitz *et al.*, 2006).

In normal ageing, cognitive functions remain unimpaired over the life span whereas sustained decline might represent a pathologic condition (Morris *et al.*, 1991; Linn *et al.*, 1995). AD is the most common cause of dementia demonstrating progressive decline in memory, language and visuospatial abilities. Distinguishing AD from normal ageing has been a recurring nosologic and diagnostic problem (Morris *et al.*, 1991; Berg *et al.*, 1982). However, memory loss is qualitatively different in normal ageing from the kind of memory loss associated with a diagnosis of AD (Budson & Price, 2005). Recent research has identified a transitional state between the cognitive changes of normal ageing and AD; known as mild cognitive impairment (MCI). Many people who experience mild cognitive impairment are at a high risk of developing AD. Several studies have indicated that MCI individuals are at an increased risk for developing AD, ranging from 1% to 25% per year; 24% of MCI patients progressed to AD in 2 years and 20% more over 3 years, whereas a recent study indicated that the progression of MCI subjects was 55% in 4.5 years (Almkvist & Arnáiz, 2003). In neuropathologic studies, Gomez-Isla *et al.*, (1996) reported specific neuronal loss in the entorhinal cortex in persons with very mild AD and no change in the same region in the cognitively intact elderly. These observations imply that AD and normal ageing are dichotomous.

Age related changes in gene expression

Memory loss and similar cognitive associated dysfunctions are commonly recognized as being the most serious symptoms of ageing and other age related memory disorders. The dysregulation in the phosphorylation state of CREB (cAMP-responsive element-binding protein) in the hippocampus of aged rats is considered as a primary biochemical correlate of impaired memory. Other proteins, known to be important for various steps of memory formation and consolidation and linked to CREB, are to some extent altered in their constitutive expression or in the response to learning in the aged hippocampus. In particular, phosphorylated CREB and Arc, a protein functionally related to CREB in memory consolidation, are both present at constitutively higher levels in the hippocampus of aged rats, but they are not susceptible to the learning related up regulation occurring in young adults. Two other CREB-regulated proteins involved in memory consolidation, the neurotrophin BDNF and the transcription factor C/EBPbeta, are expressed at similar levels in the hippocampus of young adult and aged rats but, their response to conditioned fear learning appears dysregulated by ageing. Calcineurin, a protein phosphatase having CREB among its substrates and whose expression negatively correlates with learning, is more expressed in the hippocampus of aged rats. However, while calcineurin expression decreases in the hippocampus of young adults after learning, no changes are observed in the hippocampus of aged, learning-impaired rats (Monti, 2005). Genes that are necessary for learning and memory are only expressed after synaptic activity. Resting levels of expression might not reveal an age difference that occurs in gene expression during behaviour.

Oxidative damage and age-related functional declines

The ageing process is associated with cognitive impairment and dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, as well as with oxidative stress. A

number of studies in a variety of species support a strong link between oxidative damage and life span determination. A significant age-dependent increase in the generation of free radicals was observed in hippocampus, hypothalamus and adrenal glands, as well as on lipid peroxidation in hippocampus and hypothalamus (Rodrigues *et al.*, 2005). The treatments which increase the accumulation of oxidative damage with age frequently exacerbate functional losses in the brain regions. Moreover, treatments that reduce the accumulation of oxidative damage often attenuate or delay the loss of function associated with ageing. These data provide the foundation for a link between oxidative damage and functional senescence, thereby supporting the oxidative damage hypothesis of ageing within the context of age-related functional decline (Martin, 2006). Ageing is associated with high incidence of ischemic diseases, which go along with a drop in tissue oxygenation. At the molecular level the hypoxia inducible factor (HIF) is the master regulator for hypoxia-induced gene expression. Recent studies demonstrated age-related changes in the HIF system, which might explain the reduced ability to cope with hypoxia in elderly. There are also some evidences that HIF is functionally connected to the ageing process itself (Katschinski, 2006). Since this senescence of function is thought to underlie the decrease in quality of life in addition to the increase in susceptibility to disease and death associated with ageing, identifying the mechanisms involved would be highly beneficial in treating oxidative damage in functional senescence.

Age related alterations of neurotransmitters and receptors in the brain

Evidence for age-dependent gradual deterioration of a variety of physiological functions in humans and animals has been indicated in many clinical and experimental reports. Studies on structural, chemical and functional changes of the brain during the life-span shows that the mammalian brain undergoes an early period of growth, remains relatively stable during most of adulthood and then gradually decline in senescence. The qualitative aspect of changes in the nervous system with age have a

greater significance for function and survival. Of all the events that occur in the ageing brain, subtle changes at the synaptic level is of greatest importance. A slight imbalance of putative neurotransmitters or their associated enzymes and receptor sites at the synapse result in a greater impairment of information processing which is evident from the apparent loss of neurons. Thus, age dependent changes in neurotransmitters lead not only to changes in the electrical activity of the brain, but also to changes in behaviour and function. Neurotransmitter functions are altered during ageing in several ways: changes in the amount of precursor substance reaching the neuron, changes in the amount of enzymes present within a neuron or the accumulation of metabolites, decrease in number of synaptic processes or decrease in number or affinity of receptor sites for the neurotransmitter system (Samorajshi, 1981). Previous reports indicate that during normal ageing of mammals prominent alterations occur in the adrenergic, cholinergic, dopaminergic and serotonergic systems related to reductions in the number of neurons and to a decrease in concentration, synthesis and turnover of neurotransmitters in the brain (Finch & Roth, 1999; Fisher *et al.*, 1992; McEntee & Crook, 1991; Morgan & May, 1990; Pradham, 1980). A variety of investigations have examined the effect of ageing on different neurotransmitter receptor sensitivity in the central nervous system.

Neurotransmitters and their receptor subtypes

Enzymes:

Significant decrease with ageing of catecholamine synthesizing enzymes like tyrosine hydroxylase and dopamine decarboxylase has been reported in several brain regions of man (McGeer & McGeer, 1971). However, an increase in tyrosine hydroxylase and dopa-decarboylase activity was reported in the hypothalamus, superior cervical ganglion and in the adrenal glands of rats and mice (Reis *et al.*, 1977). Enzymes for amine disposal, monoamine oxidase and catechol-o-methyl transferase showed age related changes. Monoamine oxidase activity was increased in

brain and heart in man and rats (Robinson, 1975). However, Oreland & Shasken, (1983) have reported a progressive decline in the activity. Catechol-o-methyl transferase activity decreased in brain and liver of rats (Stramentinoli, 1977). Following release of amine transmitters, their substantial portion is taken up into the storage granules in the presynaptic neurons. This uptake process is also affected during ageing. Uptake of norepinephrine and dopamine in the synaptosomes of hypothalamus and striatum of mouse was decreased (Sun, 1976).

Norepinephrine

Norepinephrine content decreased slightly but significantly with age in the hind brain of man (Robinson, 1975) and in rats, NE content in the hypothalamus and brain stem were found to be lower (Sun, 1976). The age-related changes in the noradrenergic system as the reduction in the NE content in old rats was described in the spinal cord, brainstem and limbic areas (Leslie *et al.*, 1985; Miguez *et al.*, 1999; Ponzio *et al.*, 1982; Roubein *et al.*, 1986; Sirvio *et al.* 1994), but opposite results, i.e., an increase in the NE content in aged rats were reported in the hypothalamus, striatum and cerebral cortex (Moretti *et al.*, 1987; Harik & Mc Cracken, 1986; Machado *et al.*, 1986). The levels of β-adrenergic receptors appear to decline during the ageing process. In the cerebellum, striatum, brain stem, pineal gland and cerebral cortex, there is a significant loss of β-adrenergic receptors with age (Paulose & Kanungo., 1982; Misra *et al.*, 1980). Differential alterations in β-adrenergic receptor subtypes during ageing has been reported in the cerebellum with β_1-adrenergic receptors being increased and β_2-adrenergic receptors decreased in older animals (Misra *et al.*, 1980). Greenberg & Weiss, (1979) have studied changes in the adaptability of β-adrenergic receptors with age and they have proposed that the reduced responsiveness observed is related to a lower capability of brain tissue to synthesise β-adrenergic receptors. α-adrenergic receptors in the cortex of Fischer rats were reported to have decreased as a function of age (Misra *et al.*, 1980). cAMP levels in cerebral cortex stimulated by

norepinephrine or electric pulses in anaesthetised rats were also three to four fold lower in older rats compared to the young controls (Berg & Zimerman, 1975).

Dopamine

In man, dopamine content decreased in the caudate nucleus and putamen, hippocampus and mesencephalon (Adolfsson *et al.*, 1979). Homovanillic acid, a dopamine metabolite in cortical areas could also be positively interrelated with age (Adolfsson *et al.*, 1979). Results of age-related alterations in the dopaminergic system are relatively compatible. During normal ageing the following gradual changes have been observed: continuous decline in DA and/or DOPAC levels (Ma *et al.*, 1999; Miguez *et al.*, 1999; Kish *et al.*, 1992; Venero *et al.*, 1991; Santiago *et al.*, 1988; Moretti *et al.*, 1987; Machado *et al.*, 1986; Ponzio *et al.*, 1982; Strong *et al.*, 1982;), impairment of DA synthesis and metabolism (Venero *et al.*, 1991; Moretti *et al.*,. 1987; Carfagna *et al.*, 1985; Ponzio *et al.*, 1978; Reis *et al.*, 1977; Simpkins *et al.*, 1977; Finch, 1976) and a loss of dopaminergic neurons (Gerhardt *et al.*, 2002; Siddigi *et al.* 1999; Brizee *et al.*, 1998). These alterations occur mainly in the striatum, substantia nigra, putamen, nucleus caudatus, amygdala, hippocampus and hypothalamus. In the striatum and substantia nigra, there is a significant decrease in the ability of dopamine to stimulate the formation of cAMP with age. Several brain areas including striatum, nucleus accumbens, tuberculum olfactorium and substantia nigra showed a marked decrease in the activity of adenylate cyclase (both basal and stimulated) to dopamine, apomorphine or LSD during ageing; however, the retina of senescent rats showed an increased activity of dopamine-stimulated adenylate cyclase, which is due to receptor denervation or supersensitivity in light deprivation during ageing (Govoni *et al.*, 1977). A decrease in dopamine receptors in the striatum of rats have been reported (Misra *et al.*, 1980).

Serotonin

Studies indicated age-related decrease in the content, turnover and uptake of 5-HT in the rat limbic areas, striatum, brainstem and frontal cortex (Miguez *et al.*, 1999; Venero *et al.*, 1991; Brunello *et al.*, 1988; Machado *et al.*, 1986; Roubien *et al.*, 1986; Strong *et al.*, 1984). Also evidence for unchanged (Robson *et al.*, 1993) or enhanced (Moretti *et al.*, 1987; Timiras *et al.*, 1982; Simpkins *et al.*, 1977) 5-HT metabolism was observed in the rat hypothalamus, hippocampus and frontal cortex. Moreover, Gozlan *et al.*, (1990) observed age-dependent decreases in 5-HT levels associated with parallel increases in 5-HIAA/5-HT ratio in the hypothalamus, hippocampus, striatum and cerebral cortex suggesting an accelerated 5-HT turnover in aged rats. Serotonin content or turnover has been reported to show usually no change or slight increase (Simpkins *et al.*, 1977), although in occasional experiments a decrease in serotonin content and tryptophan hydroxylase activity in some brain areas (eg. Raphe nucleus, ventral pontine nucleus) has been demonstrated (Meek *et al.*, 1977). Turnover rate of serotonin was found to be increased in the hypothalamus (Simpkins *et al.*, 1977). 5-Hydroxy indole acetic acid (5-HIAA), a metabolite of 5-HT was shown to increase in cerebrospinal fluid (Bowers & Gerbode, 1968), blood and brain (Robinson, 1975). There is consistent preclinical evidence that brain 5-HT and 5-HIAA concentrations are abnormal during natural ageing. 5-HT turnover is positively correlated with natural ageing, suggesting that ageing may alter 5-HT system in humans (Dursun *et al.*, 1977). 5-HT_2 and 5-HT_{1C} receptors have been reported to be markedly increased during ontogeny in mice (Bryan *et al.*, 1991).

Gamma amino butyric acid (GABA)

GABA, an inhibitory neurotransmitter has also been studied in relation to ageing. The GABA synthesising enzyme, glutamic acid decarboxylase (GAD) (McGeer & McGeer, 1976) has been reported to decline in several brain regions of man. Benzodiazepine binding sites and the $GABA_A$ binding sites of $GABA_A$ receptors

complex from rat prefrontal cortical membranes were differentially affected by the ageing process (Ruano *et al.*, 1996). Decreased GABA content with age has been reported in preparations using either synaptosomes (Hare *et al.*, 1982) or microdissected tissues (Banay-Schwartz *et al.*, 1989). GABA transport also decreases significantly with age (Wheeler, 1982). A decrease in $GABA_B$ receptor mediated postsynaptic current has been observed in the aged brain (Krzywkowski *et al.*, 1996). Although significant decreases in the level of mRNA of different $GABA_A$ receptor subunits with age (Gutierrez *et al.*, 1996; Mhatre & Ticku, 1998), an age-related change in protein expression of different $GABA_A$ receptor subunits have not been reported (Gutierrez *et al.*, 1997). Also, no change in the binding of allosteric ligands at $GABA_A$ receptors with age was observed. Studying the binding of benzodiazepine site at the $GABA_A$ receptors also revealed no change with age (Ruano *et al.*, 1993; Tsang *et al.*, 1982). Binding of $GABA_B$ receptors also revealed little modification with age (Turgeon & Albin, 1994). Despite the lack of any modification in the binding of $GABA_A$ agonists and modulators, binding of the $GABA_A$ receptor-coupled ionophore is decreased significantly in the aged brain (Erdö & Wolff, 1989; Mhatre & Ticku, 1992). A decrease in picrotoxin binding, which requires an open receptor/channel, was also observed in the aged cerebral cortex (Mhatre *et al.*, 1991). These findings suggest that the kinetic/structural properties of the $GABA_A$ receptors, instead of its density, are affected in ageing.

Glutamate

Glutamate, an important amino acid mediating excitatory synaptic transmission has also been studied in ageing. There are four major types of glutamate receptors. N-methyl-D-aspartate (NMDA) receptors, amino- 3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, and kainate receptors belong to the family of ligand-gated ion channels. The last group of glutamate receptors is the G protein-coupled metabotropic glutamate (mGluR) receptor. The most consistent age-

related change in the glutamatergic system is the loss of glutamate receptors. Significant decreases in the mRNA level of glutamate receptors were found in the aged cerebral cortex (Carpenter *et al.*, 1992). Among different glutamate receptors, NMDA receptors are preferentially altered in the aged brain. Decrease in NMDA binding was shown in both rodents and mammalian brain (Cohen & Muller, 1992; Wenk *et al.*, 1991). mRNA level of both NR1 and NR2B subunits of the NMDA receptors have been shown to decrease preferentially in the aged cerebral cortex, whereas no age-related change was observed in the NR2A subunit (Magnusson, 2000). The modification of subunit expression alters the receptor composition of NMDA receptor in the aged brain and lead to age-related changes in the binding properties of this receptor (Gallagher *et al.*, 1996; Priestley *et al.*, 1995) and/or physiological properties such as desensitization (Monyer *et al.*, 1992). Binding studies revealed significant decrease in NMDA but not AMPA and kainate receptors (Tamaru *et al.*, 1991). These findings support a significant loss of postsynaptic glutamatergic receptors, especially the NMDA subtype, in the aged brain.

Acetylcholine

Activity of enzymes responsible for biosynthesis, choline acetyl transferase, (CAT) and hydrolysis, acetylcholine esterase (AChE) of acetylcholine were shown to decrease with ageing in various brain areas (Bisso *et al.*, 1991; Michalek *et al.*, 1989). Impairment in the ability to synthesize and release ACh has been consistently reported in aged rodent cortex (Araujo *et al.*, 1990; Vannuchi *et al.*, 1990; Gibson *et al.*, 1981). Age-related memory disorders show a substantial decrease in cholinergic markers, such as ChAT, AChE and the number of muscarinic receptors (mAChR), in the hippocampal formation and other cortical and basal forebrain structures (Whitehouse *et al.*, 1982; Perry *et al.*, 1981; White *et al.*, 1977; Davies & Maloney, 1976;). Also, memory decline during ageing has been extensively documented in rodents (Ingram *et al.*, 1981), nonhuman primates (Presty *et al.*, 1987), and humans (Rowe & Kahn,

1987). Although the ageing process seems to impinge on several neurotransmitter systems, special emphasis has been placed on the role of the cortical cholinergic innervations in geriatric memory dysfunction (Bartus *et al.*, 1982). A decrease in the cholinergic receptors in rat striatum, cortex, hippocampus and cerebellum has been reported (James & Kanungo, 1976). Muscarinic receptor density found to be decreased significantly with ageing in certain brain regions, depending on the ligand used (Norbury *et al.*, 2005; Schwarz *et al.*, 1990; Michalek *et al.*, 1989; Surichamorn *et al.*, 1988). L-QNB showed a decline with age (Briggs *et al.*, 1982) and age-related increase in the affinity of mAChRs was observed in the cortical regions of aged and young rats (Yufu *et al.*, 1994). Also, the selective M1 antagonist, [^{3}H]-pirenzepine ([^{3}H]PZ) reported decreased receptor density in the cortex of aged rats compared to young rats (Schwarz *et al.*, 1990; Ehlert & Tran., 1990). Watson *et al.*, (1988) found an age-related decline in M1-receptor binding in the cortex, striatum, hippocampus and hypothalamus using [^{3}H] pirenzepine. Schwarz *et al.*, (1990) also found an age-related decrease in M1-receptors in the cortex of rats using the same selective M1-receptor antagonist. However, Sirvio *et al.*, (1988) reported that the maximal number of M1 binding sites was unaltered in the cortex and hippocampus as rats aged. Age-related differences in coupling of brain muscarinic receptors to G-proteins and in muscarinic receptor-stimulated phosphoinositide hydrolysis have also been reported.

Ageing and insulin secretion

It is well documented that ageing is associated with a progressive impairment of glucose tolerance (Davidson, 1979). Few laboratories have attributed the alterations in insulin secretion, with age to changes in diet rather than ageing *per se* (Hara *et al.*, 1992). Animals fed with restricted diet showed a prolonged and higher secretory rate during first phase of insulin release when compared to animals fed *ad libitum* (Castro *et al.*, 1997). Total glucose-stimulated insulin secretion has been described as being unchanged (Ruhe *et al.*, 1997) suppressed (Molina *et al.*, 1985) or increased (Curry *et*

al., 1984) as the animal ages. Resting pancreatic insulin stores are not affected by ageing. However in general, when insulin secretion is reported in terms of islet cell mass, ageing appears to result in decreased insulin secretory efficiency (Curry *et al.*, 1984). Glucose induced time dependent potentiation of insulin release is impaired in the islets of mature and old Sprague-Dawley rats, confirming an early loss of sensitivity of β-cells to secretagogues during ageing (Bombara *et al.*, 1995). Intact pancreatic islets from old Wistar rats showed impaired glucose induced insulin release; glucose uptake and oxygen consumption than in the young or adult rats. Moreover, $^{45}Ca^{2+}$ uptake and calmodulin content were decreased in pancreatic islets from older rats, which explained the impairment in glucose induced insulin release in ageing. No age related changes were observed in glucose induced $^{45}Ca^{2+}$ efflux in pancreatic islets (Castro *et al.*, 1993). Alterations in glucose-stimulus/secretion coupling are not associated with changes in K^+/ATP channel mediated responsiveness in aged male Fischer rats (Ruhe *et al.*, 1993). It was suggested that the delay in the first phase of insulin secretion as a function of ageing is due to a defect which lies distal to the elevation of [Ca2$^+$]i (Komatsu *et al.*, 1991).

Despite increases in islet size, β-cell number, the pancreatic insulin content, amount of insulin secreted and insulin mRNA levels decline with age. Glucagon mRNA levels showed a modest decline with age where as somatostatin mRNA levels did not vary significantly (Perfetti *et al.*, 1995). Similar abnormalities were reported in ageing C57 BL/6J mice (Perfetti *et al.*, 1996). This progressive decline represents the biological feature of the age dependent risk for the developmentof diabetes. Studies conducted in ageing human subjects show that alterations of pancreatic β-cell function independent of that seen with NIDDM occurred in relation to ageing. This is a disposing factor to the development of impaired glucose tolerance or NIDDM in elderly subjects (Shimizu *et al.*, 1996). Insulin action was altered in rats between two and twenty months of age. The most pronounced impairment was in insulin binding to

muscle membranes and maximal response of insulin-induced glucose disappearance rate occurs during early life stage (through maturation) and then a coupling defect seems to be superimposed with further ageing (Haruo *et al.*, 1988).

The stimulatory role of ACh in insulin secretion is well established. Pancreatic mAChRs have critical role in stimulating insulin and glucagon secretion from islet cells. Receptor localization studies suggest that multiple muscarinic receptors (M1, M3, M4, and M5) are expressed in pancreatic β-cells or β-cell derived tumor cell lines (Iismaa *et al.*, 2000; Tang *et al.*, 1997; Boschero *et al.*, 1995). The activation of mAChRs located on the pancreatic β-cells mediates the acetylcholine/vagus effects on pancreatic insulin release (Gilon & Henquin, 2001; Ahren, 2000; Satin & Kinard, 1998). Most of the studies with ACh suggest muscarinic M3 receptor as the predominant cholinergic receptor subtype expressed by pancreatic β-cells and in pancreatic insulin and glucagon release (Duttaroy *et al.*, 2004; Gilon & Henquin, 2001;). Muscarinic M1 receptor subtype antagonist, pirenzepine inhibits cholinergic mediated insulin secretion confirming the role of this receptor subtype in insulin synthesis and secretion (Iismaa *et al.*, 2000). Recent studies from our laboratory have reported the regulatory role of mAChRs in glucose induced insulin secretion (Renuka *et al.*, 2006; 2005). The pronounced impairment of insulin binding to muscle membranes and insulin-induced glucose disappearance rate was reported as a function of age (Haruo *et al.*, 1988). McEvoy & Herge, (1978) reported that administration of insulin to diabetic rats implanted with foetal pancreas resulted in a three-fold increase in β-cell mass. Insulin favored regeneration of β-cell by activating the neogenesis of the β-cells from precursor cells (Movassat *et al.*, 1997). Rabinovitch *et al.*, (1982) 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 can directly modulate synaptic plasticity, learning, memory and disturbances in insulin signalling pathways have been implicated in brain ageing.

Insulin signalling controls synaptic plasticity by modulating the activities of AChRs and by triggering signal transduction cascades that is required for memory consolidation (Zhao *et al.*, 2004).

Insulin signalling in brain ageing

Insulin receptor is a heterotetrameric tyrosine kinase receptor which upon binding of insulin, undergoes dimerisation and tyrosine autophosphorylation. Insulin binding activates the A subunit of insulin receptor inducing a conformational change that leads to autophosphorylation of tyrosine residues in the β subunit and tyrosine phosphorylation of phosphotyrosine-binding domains of adaptor proteins. Tyrosine phosphorylated insulin receptor substrates (IRS) adaptor proteins bind their effectors and transduce the insulin signal to multiple insulin response pathways. Insulin/IGF1 receptor signalling has been implicated as an important factor in invertebrate and vertebrate development, nutrient sensing, growth and ageing (Bartke, 2006; Hafen, 2004). In the brain, insulin serves as a neuromodulatory and neuroendocrine molecule in addition to its usual metabolic function, playing a significant role in neuronal growth and survival (Gasparini & Xu, 2003). Insulin is the prototypic trophic factor since trophic factor signalling overlaps the insulin pathways and it is essential not only for neuronal development, but for continued survival *in vitro* and *in vivo*. In neurons, the ERK/MAPK, P13-K, AKT, GSK3β, BAD, FOXO and TOR pathways are for survival signalling, while regulation of glucose uptake in neurons is depends on GLUT3 than GLUT4. IGFs are potent neurotrophic agents (Carson *et al.*, 1993) with increased expression after injury (Guthrie *et al.*, 1995) that protect and rescue hippocampal neurons from amyloid and other toxins (Dore *et al.*, 1997). IGF-1 can cross the blood brain barrier and is neuroprotective *in vivo* (Liu *et al.*, 1995). Further insulin-sensitizing agents such as troglitazones (thiazolidinediones, TZDs) can have potent neurotrophin-like survival activity (Nishijima *et al.*, 2001). Increased insulin-like signalling in the brain would promote neuronal survival and longevity. Insulin

pathway mutations also influence ageing in mice where defects limiting signalling through IGF receptor 1 result in long-lived mice with increase resistance to oxidative stress (Holzenberger *et al.*, 2004).

Ageing and diabetes

Diabetes mellitus is a major global health problem that affects more than 185 million people around the world (Zimmet *et al.*, 2001; Zimmet, 1999; Amos *et al.*, 1997). The disease is an increasingly prevalent metabolic disorder in humans and is characterised by hyperglycaemia (Dunne *et al.*, 2004; Kumar *et al.*, 2002). The number of diabetic patients is expected to reach 300 million by the year 2025. The projected increase in the number of diabetic patients will strain the capabilities of healthcare providers the world over (Adeghate *et al.*, 2006). The pancreatic hormones have an important role in the regulation of glucose metabolism. The secretion of insulin by ß-cells of the endocrine pancreas is regulated by glucose and other circulating nutrients. It is also modulated by several hormones and neurotransmitters, among which acetylcholine plays a prominent role. The progression of diabetes is associated with an impaired ability of the neurons in the CNS to release neurotransmitters (Broderick & Jacoby, 1989). Numerous neurochemical studies using both animals and humans have revealed age-related changes in neurotransmitter enzyme activities and receptor binding (Pradham, 1990; Hepler *et al.*, 1985; McGeer & McGeer, 1982; Smith, 1988). Neurotransmitters show significant alterations during hyperglycaemia and causes degenerative changes in neurons of the central nervous system (Bhardwaj, *et al.*, 1999; Garris, 1990; Lackovic *et al.*, 1990). Studies on STZ-induced diabetic rat models have shown similar results which exhibits morphological, behavioural and electrophysiological alterations on diabetes (Chabot *et al.*, 1997; Biessels *et al.*, 1996; Jakobsen *et al.*, 1987). Learning and memory deficits are associated with Type I and Type II diabetes mellitus (Gispen & Biessels, 2000) and brain morphological abnormalities have been found in diabetic patients, mainly in the

cortical area (Dejgaard *et al.*, 1991). STZ-induced diabetes results in structural alterations of mAChRs in the brain (Latifpour *et al.*, 1991) which in turn alters cholinergic nerve components (Akria *et al.*, 1994) with decrease in the Na^+, K^+-ATPase activity (Gurcharan & Sukwinder, 1994). Studies of Latifpour & McNeill, (1984) on long-term STZ-induced diabetes reported large reduction in muscarinic receptor densities as compared with their age-matched controls. Ageing and diabetes are intimately related at a molecular level and hence diabetes is able to provide the link between disease treatment and the prevention of age-related diseases. If specific molecular pathways controlling the rate of ageing can be modulated genetically, then perhaps they can be modulated pharmacologically (Geesaman, 2006). These insights ultimately have an important impact on the discovery and development of drugs to both treat and prevent a wide range of diseases

β-Cell function: physiology and pathophysiology

Islets of Langerhans are microscopic organelles scattered diffusely throughout the pancreas. Each islet contains approximately 2000 cells, which include four types: α, β, δ and pancreatic polypeptide (PP) cells. The major secretory products of these cells are glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively. The α-cell secretes glucagon primarily in response to hypoglycaemia, but also to amino acids. The β-cell secretes insulin in response to elevated glucose levels and also responds to other substances such as glucagon and acetylcholine. Insulin responses to intravenous glucose are time-dependent and referred to as first- and second-phase responses. The δ-cell releases somatostatin in response to glucose. The PP cell releases pancreatic polypeptide in response to hypoglycaemia and secretin. The functions of these hormones are distinctly different. Glucagon stimulates glycogenolysis in the liver to increase blood glucose levels. Insulin decreases hepatic glucose production and increases glucose entry into muscle and fat cells. Somatostatin inhibits the secretion of many hormones, including insulin and glucagon, and likely is

an intra islet paracrine regulator of α and β cells. The function of pancreatic polypeptide in humans remains unclear (Robertson & Harmon, 2006).

The endocrine pancreas is richly innervated, but the abundance and organization of these innervations are highly variable between species (Kobayashi & Fujita, 1969). Most of the nerve fibers enter the pancreas along the arteries (Miller, 1981; Woods & Porte Jr, 1974). Unmyelinated nerve fibers are found in the neighborhood of all islet cell types at the periphery and within the islet. At some distance from the islets, glial Schwann cells often form a thin sheet around nerve fibers on their travel toward and within the islet. In the vicinity of islet cells, however, it is not rare to see some nerve fibers lacking this glial protection and coming close to or ending blindly 20–30 nm from the endocrine cells (Bock, 1986; Radke & Stach (a), 1986; Radke & Stach (b), 1986; Fujita & Kobayashi, 1979; Shorr & Bloom, 1970; Kobayashi & Fujita, 1969; Watari, 1968; Legg, 1967).

The autonomic innervation of the endocrine pancreas has several origins. Classically, the autonomic nervous system uses two interconnected neurons to control effector functions and is divided into two systems, the sympathetic and the parasympathetic nervous systems, according to the location of the preganglionic cell bodies. However, there are indications suggesting that these two systems are not always independent of each other, but display anatomical interactions (Berthoud & Powley, 1993) or share similar neurotransmitters (Verchere *et al.*, 1996; Liu *et al.*, 1998; Sheikh *et al.*, 1988).

The parasympathetic innervation

The preganglionic fibers of the parasympathetic limb originate from perikarya located in the dorsal motor nucleus of the vagus (Chen *et al.*, 1996; Berthoud & Powley, 1991; Berthoud *et al.*, 1990; Rinaman & Miselis, 1987; Ahrén *et al.*,1986; Louis-Sylvestre, 1987; Luiten *et al.*, 1984; Ionescu *et al.*, 1983) and possibly also in the nucleus ambiguus (Luiten *et al.*, 1986; Luiten *et al.*, 1984; Sharkey *et al.*, 1984;

Sharkey & Williams, 1983; Weaver, 1980) which are both under the control of the hypothalamus. They are organized in well separated branches traveling within the vagus nerves (cranial nerve X), and through the hepatic, gastric (Berthoud & Powley, 1991; Berthoud *et al.*, 1990) and possibly celiac branches of the vagus (Kinami *et al.*, 1997). They reach intrapancreatic ganglia that are dispersed in the exocrine tissue. These ganglia send unmyelinated postganglionic fibers toward the islets (Berthoud & Powley, 1990; Berthoud *et al.*, 1981; Woods & Porte Jr., 1974). Preganglionic vagal fibers release ACh that binds to nicotinic receptors on intraganglionic neurons. Postganglionic vagal fibers release several neurotransmitters: ACh, Vasoactive Intestinal Peptide (VIP), gastrin-releasing peptide (GRP), nitric oxide (NO) and pituitary adenylate cyclase-activating polypeptide (PACAP) (Ahrén, 2000; Myojin *et al.*, 2000; Love & Szebeni, 1999; Wang *et al.*, 1999; Ahrén *et al.*, 1999; Havel *et al.*, 1997; Sha *et al.*, 1995; Ekblad *et al.*, 1994; Knuhtsen *et al.*, 1987; Ahrén *et al.*, 1986; Knuhtsen *et al.*, 1985; Bloom *et al.*,1983; Bloom & Edwards, 1981). Cholinergic terminals are found in the neighborhood of all islet cell types at the periphery and within the islet (Love & Szebeni, 1999; Van der Zee *et al.*, 1992; Radke & Stach, 1986; Stach & Radke, 1982; Esterhuizen *et al.*, 1968; Coupland, 1958). The importance of the cholinergic innervation of the endocrine pancreas is attested by the presence of a 10-fold higher activity of choline acetyl transferase and acetylcholine esterase in the islets than in the surrounding exocrine tissue (Godfrey & Matschinsky, 1975). Cholinergic synapses with endocrine cells have been observed in some species (Golding & Pow, 1990; Voss *et al.*, 1978).

Understanding the organization of the pancreatic innervation permits correct interpretation of some experiments using different cholinergic antagonists. The stimulation of insulin release occurring upon electrical stimulation of vagal nerves in the dog is abolished by both nicotinic and muscarinic antagonists (Ahrén & Taborsky Jr., 1986). In the perfused rat pancreas, nicotine produces an increase of insulin secretion that is blocked by atropine (Miller, 1981). These observations can be

explained by the presence of nicotinic receptors on pancreatic ganglia and nerves (Karlsson & Ahrén, 1998; Kirchgessner & Liu, 1998; Stagner & Samols, 1986) and muscarinic receptors on ß-cells.

Parasympathetic neurotransmission is the sum of various biological effects. The overall effect of a parasympathetic stimulation causes an increase of insulin secretion since postganglionic fibers contain various neurotransmitters in addition to the classic neurotransmitter ACh. VIP and PACAP stimulate insulin secretion by increasing cAMP levels (Ahrén, 2000). They act on the same family of receptors (Jian *et al.*, 1999) and exert their action by two mechanisms, directly by stimulating ß-cells through the PLC-PKC pathway (Ahrén, 2000) and indirectly by activating intrapancreatic postganglionic nerves that stimulate insulin secretion (Karlsson & Ahrén, 1998).

The sympathetic innervation

The sympathetic innervation of the pancreas originates from preganglionic perikarya located in the thoracic and upper lumbar segments of the spinal cord (Furuzawa *et al.*, 1996). The myelinated axons of these cells traverse the ventral roots to form the white communicating rami of the thoracic and lumbar nerves that reach the paravertebral sympathetic chain (Chusid, 1979). Preganglionic fibers communicate with a nest of ganglion cells within the paravertebral sympathetic chain or pass through the sympathetic chain, travel through the splanchnic nerves and reach the celiac (Ahrén, 2000; Furuzawa *et al.*, 1996; Brunicardi *et al.*, 1995; Fox & Powley, 1986; Sharkey & Williams, 1983) and mesenteric ganglia (Furuzawa *et al.*, 1996). Ganglia within the paravertebral sympathetic chain, and the celiac and mesenteric ganglia, give off postganglionic fibers that eventually reach the pancreas. The existence of intrapancreatic sympathetic ganglia has also been reported (Liu *et al.*, 1998; Luiten *et al.*, 1986; Luiten *et al.*, 1984). The preganglionic fibers release

ACh that acts on nicotinic receptors on intraganglionic neurons whereas the postganglionic fibers release several neurotransmitters: norepinephrine, galanin, (Ahrén, 2000; Myojin *et al.*, 2000; Dunning *et al.*, 1988; Ahrén & Taborsky, 1986). A rich supply of adrenergic nerves in close proximity of the islet cells has been observed in several mammalian species (Radke & Stach, 1986; Stach & Radke, 1982; Ahrén *et al.*, 1981; Esterhuizen *et al.*, 1968).

Brain neurotransmitter changes during diabetes

Neurotransmitters have been reported to show significant alterations during hyperglycaemia resulting in altered functions causing neuronal degeneration. A significant increase in the catecholamine contents and activity of metabolising enzymes has been reported in experimental diabetes (Gupta *et al.*, 1992). Norepinephrine has been reported to increase in several brain regions during diabetes (Tassava *et al.*, 1992; Chen & Yang, 1991; Wesselmann *et al.*, 1988; Chu *et al.*, 1986; Fushimi *et al.*, 1984; Oreland & Shasken, 1983) but a significant decrease in NE has been reported in hypothalamus (Ohtani *et al.*, 1997), pons and medulla (Ramakrishna & Namasivayam, 1995). EPI levels were significantly increased in the striatum, hippocampus and hypothalamus of diabetic rats and these changes were reversed to normal by insulin treatment (Ramakrishna & Namasivayam, 1995). Streptozotocin-induced diabetes and acute insulin deficiency were demonstrated to result in increased content of EPI in the supra chiasmatic nucleus. In addition to this, a decreased turnover of dopamine in the ventromedial nucleus in diabetes was found to be reversed by insulin treatment (Oliver *et al.*, 1989). These data indicate that experimental diabetes and acute insulin deficiency result in the rapid onset of detectable alterations in EPI and DA activity in specific hypothalamic nuclei. This can lead to the development of secondary neuroendocrine abnormalities known to occur in the diabetic condition. The DA content was increased in whole brain, (Chen & Yang, 1991; Lackovic *et al.*, 1990) corpus striatum (Chu *et al.*, 1986), cerebral

cortex and hypothalamus of diabetic rats (Ohtani *et al.*, 1997; Tassava *et al.*, 1992). The plasma DA content was decreased in diabetic rats (Eswar *et al.*, 2006). Serotonin (5-HT) content is increased in the brain regions and hypothalamic nuclei (Chen & Yang, 1991; Lackovic *et al.*, 1990) but there are reports suggesting a decrease in brain 5-HT content during diabetes (Jackson & Paulose, 1999; Sandrini *et al.*, 1997; Sumiyoshi *et al.*, 1997). Brain tryptophan was also reduced during diabetes (Jamnicky *et al.*, 1991). Insulin treatment was reported to reverse this reduced tryptophan content to normal (Jamnicky *et al.*, 1993).

Acetylcholine

Cholinergic system plays an important role in physiological and behavioural functions. Acetylcholine acts by binding to specific membrane receptors and can be divided into muscarinic and nicotinic receptors. Cholinergic stimulation of pancreatic β-cells increases insulin secretion (Kaneto *et al.*, 1967). This are mediated by muscarinic cholinergic, rather than nicotinic receptors (Stubbe & Steffens, 1993; Ahren *et al.*, 1990; Zawalich *et al.*, 1989; Henquin & Nenquin, 1988; Morgan *et al.*, 1985; Grill & Ostenson, 1983) and is dependent on extracellular glucose concentration (Henquin *et al.*, 1988). Acetylcholine stimulated insulin secretion coupling is mediated by complex mechanisms of signal transduction. It has been proposed that ACh activates phospholipid turnover and thereby increases the intracellular calcium level. Normal β-cells' voltage-dependent sodium channels are important for membrane depolarisation. ACh increases sodium influx into the cells (Henquin *et al.*, 1988). ACh hyperpolarises the cell by increasing potassium permeability. Quist (1982) reported that carbachol causes Ca^{2+}-dependent stimulation of phosphate incorporation into phosphatidyl inositol phosphates in the canine heart. Cholinergic stimulation of phosphatidyl inositol phosphates synthesis is blocked by muscarinic antagonist atropine (Brown & Brown, 1983).

Muscarinic receptors

Muscarinic receptors are a family of G protein-coupled receptors that have a primary role in central cholinergic neurotransmission. Specific agonists, which activate postsynaptic muscarinic receptors, stimulate cholinergic signalling (Valentin *et al.*, 2006). The muscarinic acetylcholine receptors are widely distributed throughout the body and subserve numerous vital functions in both the brain and autonomic nervous system (Hassal *et al.*, 1993). Activation of muscarinic receptors in the periphery causes decrease in heart rate, relaxation of blood vessels, constriction in the airways of the lung, increase in the secretions and motility of the various organs of the gastrointestinal tract, increase in the secretions of the lacrimal and sweat glands and constriction in the iris sphincter and ciliary muscles of the eye (Wess, 1993). In the brain, muscarinic receptors participate in many important functions such as learning, memory and the control of posture.

Muscarinic receptors are members of a large family of plasma membrane receptors that transduce the intracellular signals *via* coupling to guanine nucleotide binding regulatory proteins (G proteins) (Hulme *et al.*, 1990; Bonner, 1989; Nathanson, 1987). Molecular cloning studies have revealed the existence of five molecularly distinct mammalian muscarinic receptor proteins (Hulme *et al.*, 1990; Bonner, 1989).

All mammalian muscarinic receptor genes share one common feature with several other members of G-protein receptor gene family *i.e.*, their open reading frame contained within a single exon (Bonner *et al.*, 1987). Like all other G protein coupled receptors, the muscarinic receptors are predicted to conform to a generic protein fold consisting of seven hydrophobic transmembrane helices joined by alternating intracellular and extracellular amino-terminal domain and a cytoplasmic carboxy-terminal domain. The five mammalian muscarinic receptors display a high degree of sequence identity sharing about 145 amino acids. Characteristically all muscarinic receptors contain a very large third cytoplasmic loop, except for the proximal portions

which displays virtually no sequence identity among the different subtypes (Bonner, 1989). Agonist binding to muscarinic receptors is thought to trigger conformational changes within the helical bundle, which are then transmitted to the cytoplasmic face where the interaction with specific G proteins is known to occur. Site directed mutagenesis and receptor-modeling studies suggest that a conserved Asp residue present in TM II of almost all G protein coupled receptors plays a pivotal role in mediating the conformational changes associated with receptor activation (Wess, 1993).

The ligand binding to muscarinic receptors is predicted to occur in a pocket formed by the ring like arrangement of the seven transmembrane domains (Wess *et al.*, 1991; Hulme *et al.*, 1990). Ligand binding appears to be initiated by ion-ion interaction between positively charged amino head present in virtually all muscarinic receptor ligands and a conserved Asp residue located in TM III. In addition a previous mutagenesis study has shown that replacement of the conserved TM III Asp residue in the rat muscarinic M1 receptor with Asn results in a receptor unable to bind to [^{3}H]QNB.

Sequence analysis shows that the hydrophobic core of all muscarinic receptors contains a series of conserved Ser, Thr and Tyr residues, most of which do not occur in other G protein coupled receptors. Pharmacological analysis of mutant M3 muscarinic receptors showed that two Thr residues (Thr231 and Thr234) and four Tyr residues (Tyr148, Tyr506, Tyr529 and Tyr533) are important for high affinity acetylcholine binding (Wess *et al.*, 1991). It has been shown that a Pro 201 to Ala mutant M3 muscarinic receptor exhibits affinities for both muscarinic agonists and antagonists 80-450 times less than those of the wild type (Wess *et al.*, 1993).

In the periphery, among other effects, muscarinic receptors mediate smooth muscle contraction, glandular secretion, and modulation of cardiac rate and force. In the central nervous system there is evidence that muscarinic receptors are involved in motor control, temperature regulation, cardiovascular regulation and memory.

Interest in the classification of muscarinic receptors involved in functions at different locations has been heightened by the potential therapeutic application of selective agents in areas such as Alzheimer's disease, Parkinson's disease, asthma, analgesia, and disorders of intestinal motility and cardiac and urinary bladder function (Caulfield & Birdsall, 1998).

Classification

Muscarinic receptors are widely distributed throughout the central and peripheral nervous system. They have critical functions in learning and memory, attention and motor activity (Levey, 1993; Weiner *et al.*, 1990; Bonner, 1989). The five muscarinic receptor subtypes are designated as M1 - M5. The odd-numbered receptors; M1, M3 and M5 couple to Gq/11 and thus activate phospholipase C which initiates the phosphatidyl inositol trisphosphate cascade. This leads to the dissociation of phosphatidyl 4, 5- bisphosphates (PIP2) into two components, i.e., IP3 and DAG. IP3 mediates Ca^{2+} release from the intracellular pool (endoplasmic reticulum), whereas DAG is responsible for activation of protein kinase C. On the other hand, PIP2 is required for the activation of several membrane protein, such as the "M current" channel and Na^{+}/Ca^{2+} exchangers, and muscarinic receptor- dependent depletion of PIP2 inhibits the function of these proteins (Suh & Hille, 2005; Winks *et al.*, 2005; Fuster *et al.*, 2004; Meyer *et al.*, 2001; Caulfield & Birdsall, 1998; Bonner *et al.*, 1988; Bonner *et al.*, 1987;). The M1, M2 and M4 subtypes of mAChRs are the predominant receptors in the CNS. These receptors activate a multitude of signalling pathways important for modulating neuronal excitability, synaptic plasticity and feedback regulation of ACh release (Volpivelli *et al.*, 2004)

Muscarinic M1 receptor

Muscarinic M1 receptors are predominantly expressed in the forebrain, including the cerebral cortex, hippocampus and corpus striatum, where this sub-type

contributes by 50-60% to the total of the muscarinic receptors (Gerber *et al.*, 2001; Miyakawa *et al.*, 2001; Hamilton *et al.*, 1997). The muscarinic M1 receptor subtype, which is also expressed in peripheral tissues, has been implicated in stress adaptive cardiovascular reflexes and central blood pressure control. Studies have shown that central administration of the muscarinic M1 specific antagonist pirenzepine lowered the blood pressure (Buccafusco, 1996; Brezenoff & Xiao, 1986). A putative overexpression of the muscarinic M1 subtype in selected brain areas of spontaneously hypertensive rats has been reported (Scheucher *et al.*, 1991). Muscarinic agonist depolarization of rat isolated superior cervical ganglion is mediated by muscarinic M1 receptors (Brown *et al.*, 1980). Muscarinic M1 is one of the predominant muscarinic receptor subtypes expressed in pancreatic islets (Gilon & Henquin., 2001). Studies in pancreatic islets revealed that activation of muscarinic receptors is pertusis toxin insensitive and Gq mediated. Muscarinic M1 receptor number was shown to be decreased in the brainstem during pancreatic regeneration without any change in the affinity (Renuka *et al.*, 2006).

Muscarinic M2 receptor

Muscarinic receptor activation in guinea pig heart produces a reduction in force of contraction and a decrease in the rate of beating. These effects are probably the consequence of inhibition of voltage-gated Ca^{2+} channels and activation of inwardly rectifying K^{+} channels, respectively. Extensive studies with many antagonists have defined this response as being mediated by the muscarinic M2 receptor (Caulfield, 1993). Muscarinic M2 receptors mediate both negative and positive ionotropic responses in the left atrium of the reserpinized rat, latter effect being insensitive to pertusis toxin (Kenakin & Boselli, 1990). Central cholinergic transmission can also be activated by inhibition of the presynaptic M2 acetylcholine autoreceptor using selective antagonists. The presynaptic M2 auto receptor negatively influenced the release of acetylcholine in several brain regions, including the striatum,

hippocampus, and cerebral cortex (Zhank *et al.*, 2002; Kitaichi *et al.*, 1999; Billard *et al.*, 1995). A direct consequence of brain M2 auto receptor inhibition is an elevation of acetylcholine release in the synaptic cleft. Methoctramine and other M2 receptor antagonists have been shown to enhance the release of acetylcholine in different brain regions (Stillman *et al.*, 1993; 1996).

Muscarinic M3 receptor

M3 muscarinic receptors are broadly expressed in the brain, although the expression level is not high, compared to those of the M1 and M2 receptors (Levey, 1993). Muscarinic M3 receptor is widely distributed in the peripheral autonomic organs with the highest expression found in the exocrine glands (Matsui *et al.*, 2000; Kashihara *et al.*, 1992; Pedder *et al.*, 1991; Candell *et al.*, 1990). Expression of the M3 receptor in the rat pancreatic islets and insulin secreting cell lines has been established (Iismaa, 2000). Muscarinic M3 receptor also triggers direct contractions of smooth muscle; however, it only represents a minor fraction of total muscarinic receptor population in smooth muscle. It is expressed in relatively low density throughout the brain. Studies using knock out mice for muscarinic M3 receptors gave evidences for the primary importance of these receptors in the peripheral cholinergic system. In urinary bladder, pupillary muscles and intestinal smooth muscles the cholinergic contractions are mediated predominately by M3 receptors (Matsui *et al.*, 2000).

Muscarinic M4 receptor

Muscarinic M4 receptor is known to be abundantly expressed in the striatum (Levey, 1993). Muscarinic M4 receptors act as inhibitory muscarinic autoreceptors in the mouse (Zhang *et al.*, 2002). The neuroblastoma-glioma hybrid cell line NG108–15 expresses M4 mRNA and M4 receptors can be detected readily in radioligand binding assays (Lazareno *et al.*, 1990). Inhibition of adenylyl cyclase activity by

muscarinic agonists in rat corpus striatum is mediated by muscarinic M4 receptors (Caulfield, 1993; Olianas *et al.*, 1996).

Muscarinic M5 receptor

The Muscarinic M5 receptor (M5R) was the last muscarinic acetylcholine receptor cloned. Localization studies have revealed that the M5R is abundantly expressed in dopamine-containing neurons of the substantia nigra *pars compacta* (SNc), an area of the midbrain providing dopaminergic innervation to the striatum. Concordantly, oxotremorine-mediated dopamine release in the striatum was markedly decreased in M5R-deficient mice. More intriguingly, in M5R-deficient mice, acetylcholine induced dilation of cerebral arteries and arterioles was greatly attenuated (Yamada *et al.*, 2001), suggesting that the M5 receptor is a suitable target for the treatment of cerebrovascular ischemia. Muscarinic M5 receptor subtype is expressed at low levels in the brain (Hosey, 1992; Hulme *et al.*, 1990).

Studies of the muscarinic M5 receptor have been hampered both by the lack of selective ligands and of tissues or cell lines that endogenously express the native receptor protein. Immunoprecipitation and RT-PCR studies have shown that the muscarinic M5 receptor is expressed at very low densities in the mammalian brain. However, *in situ* hybridization studies have demonstrated that muscarinic M5 transcripts are highly concentrated in the basal ganglia and are the only muscarinic receptor transcripts expressed on dopaminergic neurons in the SNc and ventral tegmental area (VTA) (Reever *et al.*, 1997). Another potentially useful system is the eosinophilic leukemia cell line (EoL-1) where M5 receptors can be induced on differentiation with interferon-γ (Mita *et al.*, 1996).

Signal transduction by muscarinic activation

Gq-protein-coupled receptors (GqPCRs) are widely distributed in the CNS and play fundamental roles in a variety of neuronal processes. Their activation results

in phosphatidyl inositol 4,5-bisphosphate (PIP2) hydrolysis and Ca^{2+} release from intracellular stores *via* the phospholipase C (PLC)-inositol 1,4,5-trisphosphate (IP3) signalling pathway. Because early GqPCR signalling events occur at the plasma membrane of neurons, they are influenced by changes in membrane potential (Billups *et al.*, 2006). Muscarinic receptors, which are G protein coupled, stimulate signalling by first binding to G protein complex ($\alpha\beta\gamma$) which provides specificity for coupling to an appropriate effector. The α subunit interacts with an effector protein or ion channel to stimulate or inhibit release of intracellular second messengers. Mutation analysis showed that the G protein is primarily but not exclusively acts through interaction with the third cytoplasmic loop. It is suggested that the short sequences, N terminal 16-21 amino acids and C terminal 19 amino acids of the loop play a key role in determining the specificity (Wess *et al.*, 1989).

Cyclic adenosine monophosphate

Adenylate cyclase can be either positively or negatively regulated by G protein coupled receptors resulting in an increase or decrease in the generation of the second messenger, Cyclic adenosine monophosphate (cAMP). The stimulation of muscarinic M2 and M4 receptors which are endogenously expressed in cell lines results in the inhibition of adenylate cyclase. G protein reconstitution experiments have shown that M2 receptors inhibit adenylate cyclase through Gi and possibly through the pertusis toxin insensitive Gz. In neuroblastoma SK-N-SH cells which express endogenous muscarinic M3 receptors stimulate adenylate cyclase activity (Baumgold & Fishman, 1988). The muscarinic M1 receptor which ectopically expressed at physiological levels in A9L cells was shown to stimulate adenylate cyclase through an IP3 and Ca^{2+} dependent mechanism (Felder *et al.*, 1989). In contrast, M1 receptors stimulate adenylate cyclase in CHO cells predominantly through an IP3 and Ca^{2+} independent mechanism that also contained a small Ca^{2+} dependent component (Gurwitz *et al.*, 1994).

Phospholipase C

The family of phospholipase C (PLC) enzymes has been grouped into three classes, β, γ and δ (Rhee & Choi, 1992). PLC serves as the primary effector for the muscarinic M1 receptor that is coupled through Gq α subunits (Berstein *et al.*, 1992). Muscarinic M1, M3 and M5 receptors can stimulate the production of IP3, independent of direct PLCβ and G protein interaction (Gusovsky, 1993). This alternate route for the generation of IP3 involves the tyrosine kinase dependent phosphorylation of PLCγ, a mechanism normally stimulated by growth factors and their receptors (Meisenhelder *et al.*, 1989). Expression studies revealed that the cloned muscarinic M2 receptor stimulates PLC through a pertusis toxin-sensitive G protein although with lower efficiency than M1 or M3 receptors (Ashkenazi *et al.*, 1987). Inhibition of PLC by an endogenously expressed M2 receptor has been reported in FRTL5 cells suggesting that negative regulation occur in some cells (Bizzarri *et al.*, 1990).

Phospholipase A2

Phospholipase A2 catalyse the hydrolysis of membrane phospholipids to generate free arachidonic acid and the corresponding lysophospholipid. Muscarinic receptors have been shown to stimulate the release of arachidonic acid and its eicosanoid metabolites in a variety of tissues including heart, brain and muscle (Abdel-Latif, 1986). Ectopic transfection experiments indicate that the muscarinic M1, M3 or M5 receptors, but not M2 or M4 receptors are linked to phospholipase A2 activation (Felder *et al.*, 1990; Liao *et al.*, 1990; Conklin *et al.*, 1988). Muscarinic receptor stimulated release of arachidonic acid occurs predominantly through the activation of phospholipase A2 and phosphatidylcholine serves as the primary substrate. Studies suggested that calcium influx, through voltage independent calcium channel activation, and diacylglycerol, through PLC activation were essential for phospholipase A2 activation (Felder *et al.*, 1990; Brooks *et al.*, 1989). In ileal smooth

muscle cells, carbachol stimulated phospholipase A2 itself caused calcium influx, implicating an amplification mechanism in phospholipase A2 regulation (Wang *et al.*, 1993).

Phospholipase D

Muscarinic receptor stimulated phospholipase D has been reported in a number of cell types including canine synaptosomes (Qian & Drewes, 1989), rat astrocytoma cells (Martinson, 1990), human neuroblastoma cells (Sandmann & Wurtman, 1991) and rat parotid cells (Guillemain & Rossignol, 1992). Association of muscarinic subtypes with phospholipase D has been shown in human embryonic kidney cells transfected with the muscarinic M1-M4 receptors. In most cells studied, phospholipase C and D are usually stimulated simultaneously following receptor activation (Liscovitch, 1991).

Calcium influx and release from intracellular stores

Muscarinic receptors typically stimulate biphasic increases in intracellular Ca^{2+} in most cells. The transient phase represents the release of calcium from IP3 sensitive intracellular calcium stores. Ca^{2+} influx through calcium channels play a central role in the regulation of multiple signalling pathways activated by muscarinic receptors. In excitable cells such as neurons and muscle cells calcium passes predominantly through voltage sensitive Ca^{2+} channels (VOCC). In non-excitable cells, such as fibroblasts and epithelial cells, calcium passes through a family of poorly characterised voltage - insensitive Ca^{2+} channels (VICC) (Fasolato *et al.*, 1994). VICCs open in response to receptor activation and have been classified into (1) receptor operated calcium channels which are second messenger independent, (2) second messenger - operated Ca^{2+} channels (SMOCCs) and (3) depletion operated Ca^{2+} channels which open following IP3 mediated depletion of intracellular stores and provide a source of Ca^{2+} for refilling the stores.

Insulin secretion regulating factors

Glucose

Glucose is an important regulator of various β-cell processes including insulin biosynthesis and release. Glucose, over short intervals stimulates insulin biosynthesis at the level of translation (Permut *et al.*, 1972). Studies have shown that preproinsulin mRNA levels rise 4-10 folds in response to glucose stimulation. Studies of insulin gene expression in primary cultures of rat islets transfected Insulin I gene 5' flanking sequence suggested that metabolic signal from glucose influx is transmitted through the insulin enhancer (German *et al.*, 1990).

Phosphorylation of glucose to glucose-6-phosphate serves as the rate limiting step in glucose oxidation (Schuit, 1996). Glucokinase acts as sensor during this process. The entry of glucose into β-cells is followed by an acceleration of metabolism that generates one or several signals that close ATP-sensitive K^+ channels in the plasma membrane. The resulting decrease in K^+ conductance leads to depolarisation of the membrane with subsequent opening of voltage dependent Ca^{2+} channels. The rise in the cytoplasmic free Ca^{2+} eventually leads to the exocytosis of insulin containing granules (Gembal *et al.*, 1992; Dunne, 1991). Glucose induced insulin secretion is also partly dependent upon the activation of typical isoforms of protein kinase C within the β-cell (Harris, 1996). It is suggested that PKC is tonically active and effective in the maintenance of the phosphorylated state of the voltage-gated L-type Ca^{2+} channel, enabling an appropriate function of this channel in the insulin secretory process (Arkhammar, 1994).

Fatty acids

Short chain fatty acids and their derivatives are highly active stimulators of insulin release in sheep (Horino *et al.*, 1968). Exogenous saturated long chain fatty acids markedly potentiated glucose-induced insulin release and elevated long chain acyl-CoA esters in the clonal β-cell line, HIT (Prentki *et al.*, 1992). A novel ester of

succinic acid 1, 2, 3-tri-(methyl-succinyl) glycerol ester displayed stimulation of insulin release and biosynthetic activity in pancreatic islets of Goto-Kakizaki rats (Laghmich *et al.*, 1997). A monomethyl ester of succinic acid along with D-glucose is required to maintain the β-cell response to D-glucose (Fernandez *et al.*, 1996).

Amino acids

Amino acids act as potent stimulators of insulin release. L-Tryptophan, which is the precursor of 5-HT can act as a stimulator of insulin release (Bird *et al.*, 1980). L-Arginine also stimulates insulin release from pancreatic β-cells. Several *in vitro* studies have suggested the production of nitric oxides from islet nitric oxide system may have a negative regulation of the L-arginine induced secretion of insulin in mice.

Substrates derived from nutrients

Substrates like pyruvate (Lisa, 1994), citrate, ATP (Tahani *et al.*, 1979), NADH and NADPH involve in the indirect reflux stimulation triggered by food intake or local islet stimulation through the production of metabolites. The NADH acts as an intracellular regulator of insulin secretion. Heterotrimeric GTP-binding protein Gαi is involved in regulating glucose induced insulin release (Konrad *et al.*, 1995). GTP analogues are also important regulators of insulin secretion (Lucia *et al.*, 1987). Glucose induced insulin secretion is accompanied by an increase in the islet content of cAMP (Rabinovitch *et al.*, 1976).

Glucagon

Glucagon is the hormone secreted by pancreatic α-cells. It has been shown that glucagon has a striking stimulatory effect on insulin release in the absence of glucose (Sevi, 1966). The presence of specific glucagon receptors on isolated rat pancreatic β-cells as well as a subpopulation of α- and δ-cells shows the relevance of glucagon on regulation of insulin secretion. Intra-islet glucagon appears to be a

paracrine regulator of cAMP *in vitro* (Schuit, 1996). Glucagon stimulates insulin release by elevating cAMP. cAMP through activation of protein kinase A, increases Ca^{2+} influx through voltage dependent L-type Ca^{2+} channels, thereby elevating Ca^{2+} and accelerating exocytosis (Carina, 1993). Protein phosphorylation by Ca^{2+}/Calmodulin and cAMP dependent protein kinase play a positive role in insulin granule movement which results in potentiation of insulin release from the pancreatic β-cell (Hisatomi, 1996).

Somatostatin

This hormone is secreted by the pancreatic δ-cells of the islets of Langerhans. Somatostatin inhibits insulin release. Its action is dependent on the activation of G-proteins but not associated with the inhibition of the voltage dependent Ca^{2+} currents or adenylate cyclase activity (Renstrom *et al.*, 1996).

Pancreastatin

Pancreastatin is known to be produced in islet β-cells and to inhibit insulin secretion. Pancreastatin is a modulator of the early changes in insulin secretion after increase of glucose concentration within the physiological range (Ahren *et al.*, 1996). It is reported to increase Ca^{2+} in insulin secreting RINm5F cells independent of extracellular calcium (Sanchez *et al.*, 1992).

Amylin

Amylin is a 37-amino acid peptide hormone co-secreted with insulin from pancreatic β-cells. Amylin appears to control plasma glucose *via* several mechanisms that reduce the rate of glucose appearance in the plasma. Amylin limits nutrient inflow into the gut and nutrient flux from the gut to blood. It is predicted to modulate the flux of glucose from liver to blood by its ability to suppress glucagon secretion. Amylin is absolutely or relatively deficient in type I - diabetes and in insulin requiring

type II - diabetes (Young, 1997). It inhibits insulin secretion *via* an autocrine effect within pancreatic islets. Amylin fibril formation in the pancreas cause islet cell dysfunction and cell death in type II - diabetes mellitus (Alfredo *et al.*, 1994).

Adrenomedullin

Adrenomedullin is a novel hypotensive adrenal polypeptide isolated from a human pheochromocytoma and is structurally related to calcitonin gene related peptide and amylin. It has been suggested that besides being an adrenal hypotensive peptide, adrenomedullin, a gut hormone has potential insulinotropic function (Mulder *et al.*, 1996).

Galanin

Galanin is a 29 amino acid neuropeptide localised in the intrinsic nervous system of the entire gastrointestinal tract and the pancreas of man and several animal species (Scheurink *et al.*, 1992). It inhibits insulin secretion in rat, mouse, and also in isolated human islets pig. In isolated rat and mouse islets galanin inhibits insulin secretion by increasing the K^+ permeability and interfering with activation of adenylate cyclase and the activity of protein kinase C and cAMP. Among other functions, galanin inhibits insulin release (Ahren *et al.*, 1991), *via* activation of G proteins (Renstrom, 1996) by the mediation of activated galanin receptors.

Macrophage migration inhibitory factor

Macrophage migration inhibitory factor (MIF), originally identified as cytokines, secreted by T lymphocytes. It was found recently to be both a pituitary hormone and a mediator released by immune cells in response to glucocorticoid stimulation. Recently it has been demonstrated that insulin secreting β-cells of the islets of Langerhans express MIF and its production is regulated by glucose in a time and concentration dependent manner. MIF and insulin were both present within the

secretory granules of the pancreatic β-cells and once released, MIF appears to regulate insulin release in an autocrine fashion. MIF is therefore a glucose dependent islet cell product that regulates insulin secretion in a positive manner and play an important role in carbohydrate metabolism (Waeber *et al.*, 1997).

Nerve growth factor

Nerve growth factor (NGF) is a neurotropic growth factor that promotes neurite outgrowth during development. This growth factor is capable of modulating β-cell plasticity because it promotes neurite-like outgrowth in fetal and adult pancreatic β-cells from primary cultures (Vidaltamayo *et al.*, 1996) and in RINm5F and insulinoma cells (Polak *et al.*, 1993). In adult rat β-cells, *in vitro* NGF stimulates glucose induced insulin secretion. The presence of the high affinity receptor for NGF has been described in insulinoma cell lines as well as in foetal and adult β-cells. The adult β-cells synthesise and secrete NGF in response to increasing extra cellular glucose concentration (Vidaltamayo *et al.*, 1996). The effect of NGF on insulin secretion is partly mediated by an increase in Ca^{2+} current through Ca^{2+} channels (Rosenbaum *et al.*, 2001).

Neuropeptides

Immunocytochemistry has revealed the presence of three neuropeptides in the nerve terminals of pancreatic ganglia and islets of different species: Vasoactive intestinal peptide (VIP), gastrin releasing peptide (GRP) and pituitary adenylate cyclase activating polypeptide (PACAP).

Gastrin releasing peptide

Gastrin releasing peptide (GRP) consists of a 27 amino acid residue. It is localised to pancreatic nerves, including islet nerve terminals of several species. GRP released from the pancreas after vagal nerve activation and stimulates insulin secretion

(Sundler & Bottcher, 1991; Knuhtsen *et al.*, 1987). In islets, activation by GRP receptors is coupled to PLC and phospholipase D (Gregersen & Ahren, 1996, Wahl *et al.*, 1992).

Vasoactive intestinal peptide

Vasoactive intestinal peptide (VIP) stimulates insulin secretion in a glucose dependent manner accompanied by increased action of adenylate cyclase with increased formation of cAMP (Klinteberg *et al.*, 1996). VIP increases activity of sympathetic system, including release of catecholamines from the adrenal medulla and lead to the release of the pancreatic glucagon and inhibition of insulin release, by the activation of adrenergic receptors (Jarrhult & Holst, 1978).

Pituitary adenylate cyclase activating polypeptide

Pituitary adenylate cyclase activating polypeptide (PACAP) is localised to the parasympathetic nerves and released by the activation of the vagus nerve (Ahren, 2000). It exists in two forms consisting of 27 and 38 amino acids and show 68% homology (Arimura & Shioda, 1995). PACAP stimulates insulin secretion in a glucose dependent manner accompanied by increased action of adenylate cyclase with increased formation of cAMP (Klinteberg *et al.*, 1996).

Role of neurotransmitters in insulin regulation & secretion

Acetylcholine

Acetylcholine is one of the principal neurotransmitters of the parasympathetic system. Acetylcholine, through vagal muscarinic and non-vagal muscarinic pathways (Greenberg & Pokol, 1994) increases insulin secretion (Tassava *et al.*, 1992). They function through muscarinic receptors present on pancreatic islet cells (Ostenson *et al.*, 1993). Acetylcholine agonist, carbachol, at low concentration (10^{-7} M) stimulated insulin secretion at 4 mM and 20 mM concentrations of glucose (Renuka *et al.*, 2006).

Dopamine

Dopamine is reported to inhibit glucose stimulated insulin secretion from pancreatic islets (Tabeuchi *et al.*, 1990). Eswar *et al.*, (2006) reported that dopamine significantly stimulated insulin secretion at a concentration of 10^{-8} M in the presence of high glucose (20mM). Reports show that experimental diabetes and insulin deficiency result in the rapid onset of detectable alterations in dopaminergic activity in specific hypothalamic nuclei. The uptake affinity and velocity of dopamine in synaptosomes decreased significantly during diabetes. The dopamine content was increased in the cerebral cortex and hypothalamus of diabetic rats (Ohtani *et al.*, 1997; Tassava *et al.*, 1992; Shiimzu, 1991). The altered turnover ratio in the limbic forebrain is reported to cause enhanced spontaneous locomotor activity in diabetic rats (Kamei *et al.*, 1994).

High concentrations of dopamine in pancreatic islets can decrease glucose stimulated insulin secretion (Tabeuchi *et al.*, 1990). L-DOPA, the precursor of dopamine had similar effect to that of dopamine (Lindstrom & Sehlin, 1983). Dopamine D3 receptors are implicated in the control of blood glucose levels (Alster & Hillegaart, 1996). Dopamine D1 receptors have also been reported to be present on pancreatic β-cells (Tabeuchi *et al.*, 1990). These clearly indicate the role of dopamine in the regulation of pancreatic function.

Gamma-Aminobutyric acid

Gamma aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system. GABA is reported to present in the endocrine pancreas at concentrations comparable with those found in central nervous system. The highest concentration of GABA within the pancreatic islet is confined to β-cells (Sorenson *et al.*, 1991). Glutamate decarboxylase, the primary enzyme that is involved in the synthesis of GABA, has been identified as an early target antigen of the T-lymphocyte

mediated destruction of pancreatic β-cells causing insulin-dependent diabetes mellitus (Baekkeskov *et al.*, 1990). GABA, through its receptors has been demonstrated to attenuate the glucagon and somatostatin secretion from pancreatic α--cells and δ-cells respectively (Gaskins, 1995). It is present in the cytoplasm and in synaptic-like microvesicles (Reetz, 1991) and is co-released with insulin from β-cells in response to glucose. The released GABA inhibits islet α-and δ-cell hormonal secretion in a paracrine manner. During diabetes the destruction of β-cells will lead to decrease in GABA release resulting in the enhancement of glucagon secretion from α-cells leading to hyperglycaemia. The brain GABAergic mechanisms also play an important role in glucose homeostasis. Inhibition of central $GABA_A$ receptors increases plasma glucose concentration (Lang, 1995). $GABA_A$ receptors in brainstem have a regulatory role in pancreatic regeneration (Kaimal *et al.*, 2007). Thus, any impairment in the GABAergic mechanism in the central nervous system and/or in the pancreatic islets is important in the pathogenesis of diabetes.

Serotonin

Serotonin content is increased in the brain regions and hypothalamic nuclei (Chen & Yang, 1991; Lackovic *et al.*, 1990), but there are reports suggesting a decrease in brain 5-HT content during diabetes (Jackson & Paulose, 1999; Sumiyoshi *et al.*, 1997; Sandrini *et al.*, 1997). Ohtani *et al.*, (1997) have reported a significant decrease in extracellular concentrations of NE, 5-HT and their metabolites in the ventro medial hypothalamus (VHM). The ratio of 5-HIAA/5-HT was increased. A similar observation was reported by (Ding *et al.*, 1992) with a decrease in 5-HT in cortex (19%) and 5-HT turnover (5-HIAA/5-HT) that increased by 48%. Chu *et al.*, (1986) has reported lower 5-HT levels in both hypothalamus and brainstem but not in corpus striatum. Insulin treatment brought about an increase in the cerebral concentration of 5-HIAA and accelerated the cerebral 5-HT turnover (Juszkiewicz, 1985). The 5-HIAA concentration was reported to be approximately twice as high as

the controls regardless of duration of treatment. Brain tryptophan, the precursor of 5-HT, was also reduced in brain regions during diabetes (Jamnicky *et al.*, 1991). Insulin treatment was reported to reverse this reduced tryptophan content to normal (Jamnicky *et al.*, 1993). There was a significant increase in 5-HIAA observed at 2-6 hours after insulin administration (Kwok & Juorio, 1987).

Epinephrine and Norepinephrine

These are secreted by the adrenal medulla. Norepinephrine (NE) is a principal neurotransmitter of sympathetic nervous system. These hormones inhibit insulin secretion, both *in vivo* and *in vitro* (Renstrom *et al.*, 1996; Porte, 1967). Epinephrine exerts opposite effects on peripheral glucose disposal and glucose stimulated insulin secretion (Avogaro *et al.*, 1996). NE and EPI - the flight and fright hormones - are released in all stress conditions and are the main regulators of glucose turnover in strenuous exercise (Simartirkis *et al.*, 1990). In severe insulin-induced hypoglycaemia, a 15 to 40 -fold increase of epinephrine plays a pivotal role in increasing glucose production independently of glucagon (Gauthier *et al.*, 1980). It is already known that, when used in high doses *in vivo* or *in vitro*, epinephrine reduces the insulin response to stimulators (Malaisse, 1972). *In vitro* studies with yohimbine showed that the insulin secretion from the pancreatic islets increased significantly suggesting that when the alpha 2-adrenergic receptors are blocked, it enhances islet cell proliferation and insulin secretion (Ani *et al.*, 2006). EPI and NE have an antagonistic effect on insulin secretion and glucose uptake (Porte *et al.*, 1966). They also inhibit insulin -stimulated glycogenesis through inactivation of glycogen synthase and activation of phosphorylase with consequent accumulation of glucose-6-phosphate. In addition, it has been reported that epinephrine enhances glycolysis through an increased activation of phospho-fructokinase. In humans, adrenaline stimulates lipolysis, ketogenesis, thermogenesis and glycolysis and raises plasma glucose concentrations by stimulating both glycogenolysis and gluconeogenesis.

Adrenaline is, however, known to play a secondary role in the physiology of glucose counter-regulation. Indeed, it has been shown to play a critical role in one pathophysiological state, the altered glucose counter-regulation in patients with established insulin-dependent diabetes mellitus (Cryer, 1993). The inhibitory effect of EPI upon insulin secretion induced by glucose was reported by Coore & Randle, (1964), who incubated pancreatic tissue from the rabbit. As judged by Malaisse *et al.*, (1967) the inhibitory effect of EPI on glucose-induced insulin secretion is mediated through the activation of α-adrenoreceptors.

Central muscarinic regulation of glucose homeostasis

The acetylcholine esterase inhibitor soman induced marked and sustained hypertension in rats (Letienne *et al*, 1999). Stimulation of muscarinic receptors in the nucleus tractus solitarius (NTS) of the rat decreases arterial blood pressure and heart rate. Atropine injected into the NTS of rats produced a dose-dependent inhibition of cardiovascular response elicited by injection of acetylcholine into the same site. It is suggested that cholinergic mechanisms in the NTS are not involved in the tonic regulation of cardiovascular function or the baroreceptor reflex (Tsukamoto *et al.*, 1994).

When carbachol, muscarine, bethanechol, methacholine, or neostigmine were injected into the third cerebral ventricle, it caused a dose-dependent increase in the hepatic venous plasma glucose concentration. However, in the case of 1, 1-dimethylphenyl-4-piperazinium iodide (DMPP) or nicotine, the level of hepatic venous glucose did not differ from that of the saline-treated control rats. The increase in glucose level caused by neostigmine was dose-dependently suppressed by co-administration of atropine. These facts suggest that cholinergic activation of muscarinic receptors in the central nervous system plays a role in increasing hepatic glucose output. Injection of neostigmine, an inhibitor of cholinesterase, into the ventricle resulted in the increase of not only glucose, but also glucagon, epinephrine,

and norepinephrine in the hepatic venous plasma. Neostigmine-induced increments in glucose did not occur in adrenalectomized rats. This suggests that the secreted epinephrine acts directly on the liver to increase hepatic glucose output (Iguchi *et al.*, 1986).

The injection of adrenaline and carbachol into the third cerebral ventricle resulted in a marked hyperglycaemia associated with increased immunoreactive glucagon. Adrenaline-induced hyperglycaemia was not affected by bilateral adrenalectomy, while carbachol-induced hyperglycaemia was completely inhibited by adrenalectomy. The injection of somatostatin with adrenaline into the third cerebral ventricle did not influence adrenaline-induced hyperglycaemia, while carbachol-induced hyperglycaemia was inhibited by co-administration with somatostatin (Iguchi *et al.*, 1985).

Atropine injected into the third cerebral ventricle suppressed epinephrine secretion and dose-dependently inhibited hepatic venous hyperglycaemia induced by neostigmine in intact rats. The neostigmine-induced glucagon secretion which occurs in adrenalectomised rats was suppressed by atropine. Atropine also prevented the neostigmine-induced hyperglycaemia in adrenalectomised rats receiving constant somatostatin infusion through femoral vein. Phentolamine, propranolol and hexamethonium showed no significant inhibitory effect on neostigmine-induced hyperglycaemia, epinephrine and glucagon secretion in intact rats, glucagon secretion in adrenalectomised rats or hyperglycaemia in adrenalectomised-Somato rats. These results suggest that neostigmine-induced epinephrine and glucagon secretion and increased hepatic glucose output stimulated by direct neural innervation to liver is mediated by central muscarinic receptor in fed rats (Iguchi *et al.*, 1990)

Studies by Iguchi *et al.*, (1992) suggest that the glucoregulatory hippocampal activity evoked by the acetylcholine esterase inhibitor, neostigmine transmitted to peripheral organs *via* the ventromedial hypothalamus. The ventromedial hypothalamus, lateral hypothalamus, paraventricular hypothalamus and median site of

the lateral-preoptic area were involved in increasing the plasma levels of glucose and epinephrine by cholinergic stimulation (Honmura *et al.*, 1992).

Atropine in a dose-dependent manner suppressed the hyperglycaemia induced by hippocampal administration of neostigmine whereas hexamethonium had no significant effect. These observations suggest that the pathway for this experimental hyperglycaemia involves, at least in part, the muscarinic cholinergic neurons in the ventromedial hypothalamus (Iguchi *et al.*, 1991). Takahashi *et al.*, (1993) reported that neostigmine induced hyperglycaemia affects not only the cholinergic system but also the noradrenergic and dopaminergic systems in the hypothalamus (Takahashi *et al.*, 1993). Muscarinic cholinergic system is reported to participate in the $HgCl_2$-induced central hyperglycemic effect through the function of the adrenal medulla. Norepinephrine and dopamine content were found to be decreased suggesting that their neurons have hypothalamic glycoregulation (Takahashi *et al.*, 1994).

Microinjections of carbachol or neostigmine into the ventromedial nucleus of the hypothalamus of fed, conscious rats produced marked increases in plasma glucose and lactate, which were suppressed or markedly reduced by previous adrenodemedullation. The reports suggest that cholinergic synapses in the ventromedial hypoythalamus participate in a central glucoregulatory system that increases hepatic glucose production mainly through a stimulation of adrenal medulla epinephrine secretion (Brito *et al.*, 1993).

Neostigmine caused significant increases in serum glucose concentrations, hypothalamic noradrenergic and dopaminergic neuronal activities and significantly suppressed hypothalamic serotonergic neuronal activity. All these responses to neostigmine were completely inhibited by the co-administration of atropine. These observations emphasize the important role of the interactions between cholinergic (muscarinic) and monoaminergic neurons in the brain (Gotoh & Smythe, 1992). In the ventromedial hypothalamic nucleus, lateral hypothalamus and paraventricular nucleus

the cholinergic activity is increased after 2-D glucose administration (Takahashi *et al.*, 1994; 1996).

Central cholinergic-muscarinic activation with neostigmine stimulates sympathetic nervous activity in the liver, heart, pancreas and interscapular brown adipose tissue (Gotoh & Smythe, 1992). Histamine induction of central nervous system-mediated hyperglycaemia involves neuronal transmission not only *via* H1 receptors but also, at least in part, by muscarinic cholinergic neurons (Nonogaki *et al.*, 1993). The action of acetylcholine within the hypothalamus on the pancreatic hormone secretions is mediated to a large part through sympatho-adrenomedullary activity. However, a part of the decreased insulin response to glucose mediated by direct innervation of the pancreas (Ishikawa *et al.*, 1982).

Intravenous 2-D glucose induced a marked increase in plasma glucose that was not affected by intracerebroventricular administration. However, the hyperglycaemia induced by intracerebroventricular 2-D glucose was significantly reduced by previous intracerebroventricular injection of atropine. Central cholinergic neurons participate in the complex neural events responsible for the hyperglycemic response to neurocytoglucopenia and to stressful situations (Brito *et al.*, 2001). Intravenous administration of 2-D glucose caused neuroglycopenia and marked hyperglycaemia. The cholinergic activity was increased after 2-D glucose administration (Takahashi *et al.*, 1996).

Peripheral muscarinic receptor alterations in diabetes

Autonomic neuropathy is a major complication of chronic diabetes and is responsible for disturbances in the cardiovascular system and other organs. Early cardiac disturbances have been attributed to defective vagal control of the heart (Carrier *et al.*, 1984). Streptozotocin (STZ) induced diabetes caused a variety of abnormalities including alterations in the muscarinic receptors (Latifpour *et al.*, 1991).

Muscarinic acetylcholine receptors are reported to be decreased in the atrium of STZ induced diabetic rats (Mardon *et al.*, 1999).

Tonq *et al.*, (2006) reported that STZ-induced diabetes increases mRNA and protein expression of the M2-mAChR in the urothelium as well as the muscle layer. The myocardium of STZ induced diabetic rats exhibited an increase in Gi function by the increased inhibition of guanyliminodiphosphate-mediated adenylyl cyclase and the superhigh affinity for carbachol of the muscarinic receptors. This functional alteration of G_i is suggested to be related to the cardiac dysfunction that is associated with diabetes. The cerebral blood flow response to muscarinic receptor agonist decreased in the brain regions of diabetic rats (Pelligrino *et al.*, 1992).

Bladder dysfunction is a common complication of diabetes mellitus and is attributed in part to peripheral neuropathy. [^{3}H]quinuclidinyl benzylate (QNB) binding studies revealed that the receptor number is higher in the diabetic animals showing a direct correlation between the diabetes-induced biochemical and functional alterations in muscarinic receptor properties of rat bladder (Latifpour *et al.*, 1989). In STZ induced diabetes, inositol phosphate production in the bladder is found to be enhanced after muscarinic agonist stimulation (Mimata *et al.*, 1995). The bladder contractile response to muscarinic agonist, arecaidine propargyl ester (APE), was significantly increased in the diabetic rats. The M2 receptor is the dominant muscarinic subtype in animal bladders. There was an over-expression of M2 receptor resulting in hyper-contractility in the bladder of diabetic rats (Tong *et al.*, 1999; 2002). The M3 and M2-recptor protein and mRNA in the bladder tissue were significantly increased in diabetic rats (Tong & Cheng, 2002; Tong *et al.*, 2002). STZ-induced diabetes caused a variety of abnormalities including a down regulation in the density of M3 muscarinic receptors in the rat prostrate and insulin, but myo-inositol could not prevent the development of these abnormalities (Latifpour *et al.*, 1991; Fukumoto *et al.*, 1993).

The inhibitory M2 receptors on parasympathetic nerves in the trachea and ileum are hyperfunctional in diabetic rats. In the trachea the function of post-junctional M3 muscarinic receptors is also increased in diabetes (Coulson *et al.*, 2002). In [^{3}H]QNB binding studies for muscarinic receptor of the STZ rats, in the parotid gland the receptor number was decreased and the affinity of receptors decreased in the submandibular gland. The decrease in salivary secretion of STZ rats is not only induced by a water loss, but also closely associated with the lowered susceptibility of the muscarinic receptors (Watanabe *et al.*, 2001). Studies of Latifpour & McNeill, (1984) on long-term STZ-induced diabetes revealed that ventricular β adrenergic and muscarinic receptors demonstrated a large reduction in their densities as compared with their age-matched controls.

Insulin-induced net hepatic glucose uptake depends on the sensing by muscarinic, intrahepatic nerves of a glucose concentration gradient between portal vein and hepatic artery. The function of these intrahepatic nerves is impaired in diabetic animals (Stumpel *et al.*, 1998). Muscarinic receptor number increased in the pancreatic islets of diabetic rats. Cholinergic-induced insulin release was also higher in STZ induced diabetes than in normal islets (Ostenson & Grill, 1987).

Insulin partly reversed the changes observed in the STZ-treated rats. There was a decrease in the muscarinic receptor number and axonal transport of receptor-bound opiate in STZ induced hyperglycaemia suggesting that impaired axonal transport of receptors partly involved in the neurological disturbance which is seen in diabetic patients (Laduron & Janssen, 1986).

Alterations of glucose transport during diabetes

In diabetes mellitus, apart from raised blood glucose levels, disturbances in the metabolism of a number of other biomolecules such as glycogen, lipids, proteins and glycoproteins have also been reported (Randle *et al.*, 1963; Williamson *et al.*, 1968). Treatment with insulin generally rectifies these disturbances in diabetic state as

it increases the peripheral utilisation of glucose by influencing key enzymes of glucose metabolic pathways (Exton *et al.*, 1966; Jafferson *et al.*, 1968; Lenzen *et al.*, 1990). The liver plays a major role in insulin-regulated glucose homoeostasis through the balance between glucose utilization and glucose production, both processes being tightly coordinated (Nevado *et al.*, 2006). More recently, it has been shown that glucose uptake and release required a family of membrane facilitated-diffusion glucose transporters which are expressed in a tissue-specific manner. In muscle and fat, GLUT-4 is the main isoform of glucose transporters (Burant *et al.*, 1991). In adipose tissue the concentrations of GLUT-4 protein and mRNA are markedly decreased after 2-3 weeks of diabetes and they are restored by insulin therapy (Berger *et al.*, 1989; Garvey *et al.*, 1989), whereas in skeletal muscle the concentrations of GLUT-4 protein and mRNA are marginally altered (Bourey *et al.*, 1990; Garvey *et al.*, 1989; Kahn *et al.*, 1989). In liver, GLUT-2 is the main isoform of glucose transporters (Thorens *et al.*, 1988). Much less information is available concerning the expression of GLUT-2 in liver of diabetic rats, and the results are somewhat contradictory.

Effect of age on calcium dynamics

Ca^{2+} is widely recognized as a universal second messenger within neuronal cells and integrates multiple cellular functions. These include release of neurotransmitters, gene expression, proliferation, excitability and regulation of cell death or apoptotic pathways (Wuytack *et al.*, 2002; Ginty, 1997; Clapham, 1995; Berridge, 1995, 1998; Choi, 1992; Malenka *et al.*, 1989). Ca^{2+} signalling is initiated by calcium influx by the rapid release of calcium from smooth endoplasmic reticulum (SER) and is mediated by ryanodine receptor (RyR) channels (Akita & Kuba, 2000; Usachev & Thayer, 1999a, b; 1997; Verkhratsky & Petersen, 1998; Verkhratsky & Shmigol, 1996; Belan *et al.*, 1993). The complex buffering systems include multiple Ca^{2+}-buffering proteins, smooth endoplasmic reticulum Ca^{2+} ATPases (SERCA), mitochondrial calcium uptake, plasmalemma calcium ATPases (PMCA) and the

sodium– Ca^{2+} exchanger (Na^{+}/Ca^{2+}) reduces rapid rise in [Ca^{2+}] to resting levels (Wuytack *et al.*, 2002; Pottorf *et al.*, 2002, 2000a,c; Usachev & Thayer, 1999a; Buchholz *et al.*, 1996; Werth *et al.*, 1996; Werth & Thayer, 1994).

Subtle age-related declines in mechanisms that modulate stimulation-evoked increases in [Ca^{2+}] have been hypothesized to contribute to age-related neuronal dysfunction and degeneration (Verkhratsky & Toescu, 1998; Kirischuk & Verkhratsky, 1996). The mechanism of Ca^{2+}-mediated cell death suggests that calcium overload results in mitochondrial dysfunction leading to mitochondrial calcium overload and activation of caspases that mediate cell apoptosis (Begley *et al.*, 1999; Ichas & Mazat, 1998; Thibault *et al.*, 1998). An important issue in ageing studies is a tendency to assume a general age-related deterioration of Ca^{2+} regulatory processes leading to increased susceptibility to pathology and cell death (Porter *et al.*, 1997). This assumption does not take into account compensatory mechanisms that serve to regulate [Ca^{2+}] homeostasis, thus maintaining some degree of neuronal function in senescent neurones or during acute insults such as stroke (Pottorf *et al.*, 2002, 2000a; Griffith *et al.*, 2000; Lee *et al.*, 1999; Verkhratsky & Toescu, 1998; Murchinson & Griffith, 1998). Ca^{2+} dysregulation and changes in synaptic connectivity might affect plasticity and gene expression, resulting in altered dynamics of neuronal circuits in ageing brain (Geinisman *et al.*, 1992; Nicholson *et al.*, 2004).

Inositol 1, 4, 5-trisphosphate (IP3) and activation of calcium release

Cytosolic Ca^{2+} is a focal point of many signal transduction pathways and modulates a diverse array of cellular activities ranging from fertilization to cell death (Berridge *et al.*, 2000). In most cell types, the major internal [Ca^{2+}] stores are the endoplasmic reticulum/sarcoplasmic reticulum (ER/SR). One mechanism for mobilizing such stores involves the phosphoinositide pathway. The binding of many hormones to specific receptors on the plasma membrane leads to the activation of an enzyme (phosphoinositidase C) that catalyses the hydrolysis of phospholipids to

produce the intracellular messenger inositol 1,4,5-trisphosphate (IP3). Although derived from a lipid, IP3 is water soluble and diffuses into the cell interior where it encounters IP3 receptors (IP3Rs) on the ER/SR. The binding of IP3 changes the conformation of IP3Rs such that an integral channel is opened, thus allowing the $[Ca^{2+}]$ stored at high concentrations in the ER/SR to enter the cytoplasm. A critical feature of IP3Rs is that their opening is regulated by the cytosolic Ca^{2+}concentration. This sensitivity to cytosolic $[Ca^{2+}]$ allows them to act as Ca^{2+}-induced calcium release (CICR) channels that promote the rapid amplification of smaller trigger events.

G-protein signalling and regulation of cell growth

Heterotrimeric guanine nucleotide binding proteins, commonly known as G proteins, form the super-family of signal transduction proteins that provide signal coupling mechanisms to seven transmembrane receptors. The requirement of GTP for the signal coupling of glucagon receptor to adenylyl cyclase (Rodbell *et al.*, 1971) and subsequent characterization of a GTP- binding protein identified the role of G proteins in diverse signal transduction processes (Hepler & Gilman, 1992; Gilman, 1987; Ross & Gilman, 1977). G proteins are peripherally associated with the cytoplasmic phase of the plasma membrane and are composed of monomers of α, β and γ subunits. Upon activation by an appropriate signal, the receptor interacts with the G protein and catalyzes the exchange of `bound GDP' for GTP in the α-subunit (Gα). Subsequently, the GTP-bound α-subunit and the βγ-subunits (Gβγ) dissociate from the receptor as well as from each other. The `active' α-subunit and the `free' βγ-subunits initiate cellular responses by altering the activity of intracellular effector molecules. Meanwhile, the intrinsic GTPase activity of the Gα hydrolyzes the bound-GTP to GDP thus deactivating itself. The Gα - GDP re-associates with Gβγ, possibly attenuating the βγ-effector interaction as well. The re-associated Gαβγ-GDP heterotrimer resets itself to interact with another molecule of receptor (Hepler & Gilman, 1992). It has been reported that in the ageing process several components of

the signal transduction pathways, including phosphoinositide, protein kinase C, protein kinase A and reactive oxygen intermediate (ROI) generation, are altered. a differential functional metabolic balance of cAMP and cGMP in relation to the lack of modulation of the endogenous or exogenous contents of cAMP or cGMP on ROI generation altered the age-related functional metabolic balance (Bernardo *et al.*, 2005). The N-methyl-D-aspartate (NMDA) receptor/nitric oxide synthase/guanylate cyclase pathway was studied during ageing by monitoring extracellular cGMP in the rat hippocampus and cerebellum during *in vivo* microdialysis. In the hippocampus the basal cGMP efflux decreased by 50% from 3 to 12 months of age, whereas it remained constant with age in the cerebellum. The nitric oxide donor S-nitroso-N-penicillamine (1 mM) elicited cGMP responses which slightly decreased from 3 to 12-24 months in the hippocampus, while no significant decrement with age could be seen in the cerebellum (Vallebuona & Raiteri, 1995).

Triiodothyronine (T3) regulation on diabetes and ageing

Diabetes mellitus and thyroid diseases are the two common endocriniopathies seen in the adult population. Insulin and thyroid hormones being intimately involved in cellular metabolism and excess/ deficit of either of these hormones could result in the functional derangement of the other. In euthyroid individuals with diabetes mellitus, the serum T3 levels, basal TSH levels and TSH response to thyrotropin releasing hormone (TRH) is influenced by the glycemic status (Schlienger *et al.*, 1982). Poorly controlled diabetes, both Type 1 and Type 2, induce a "Low T3 state" characterized by low serum total and free T3 levels, increase in reverse T3 (rT3) but near normal serum T4 and TSH concentrations (Donckier, 1993). Low serum T3 is due to reduced peripheral conversion of thyroxine (T4) to tri-iodothyronine (T3) *via* 5' monodeiodination reaction. Studies indicate that long term diabetic control determines the plasma T3 levels. TSH responses and low T3 state normalized with

improvement in glycaemic status but even with good diabetes control, the normal nocturnal TSH peak may not be restored in C-peptide negative patients i.e. those with totally absent pancreatic β cell function (Coiro *et al.*, 1997). Studies show decreased insulin secretion (Taylor *et al.*, 1985; Ahren *et al.*, 1985) as well as normal or increased levels of insulin is reported in the peripheral and portal circulation in hyperthyroidism (Dimitriadis *et al.*, 1985). Long term thyrotoxicosis has been shown to cause beta cell dysfunction resulting in reduced pancreatic insulin content, poor insulin response to glucose and decreased rate of insulin secretion (Bech *et al.*, 1996).

In hyperthyroidism, the endogenous glucose production is greatly increased by a variety of mechanisms: (a) an increase in the availability of gluconeogenic precursors in the form of lactate, glutamine and alanine from skeletal muscles and glycerol from adipose tissue, (b) an increase in the concentration of plasma FFA stimulating hepatic gluconeogenesis (Dimitriadis & Raptis, 2001); (c) an increase in glycogenolysis due to inhibition of glycogen synthesis resulting in hepatic glucose output even in fed state (Holness & Sugden, 1987); (d) an upregulation of GLUT-2 glucose transporters protein expression in the hepatocyte plasma membrane. This permits increased glucose efflux to occur without intracellular glucose accumulation which would limit hepatic glucose production (Mokuno *et al.*, 1990); and (e) an increased secretion and exaggerated effects of glucagon and adrenaline on liver cells (Dimitriadis & Raptis, 2001). In skeletal muscle, there is a preferential increase in glucose uptake and lactate formation relative to glucose oxidation and storage in hyperthyroid state. This is due to increase in both basal and insulin stimulated GLUT1 and GLUT-4 transporters (Haber *et al.*, 1995), increased responsiveness of glycogenolysis to beta adrenergic stimulation (Dimitriadis & Raptis, 2001), increased activity of hexokinase and 5- phosphofructokinase and decreased sensitivity of glycogen synthesis to insulin (Dimitriadis *et al.*, 1997). In hypothyroidism, the synthesis and release of insulin is decreased (Ahren *et al.*, 1985). The rate of hepatic glucose output is decreased probably due to reduced gluconeogenesis. A post receptor

defect has been proposed to explain the decrease in insulin stimulated glucose utilization in peripheral tissues (Dimitriadis & Raptis, 2001), results in recurrent hypoglycaemia in diabetic individual (Mohanan *et al.*, 2002). A reduced secretion of thyroid hormones with age has been documented in humans and animals with no substantial increase in TSH secretion, which is indicative of an age-related impairment of the pituitary sensitivity to the negative control exerted by thyroid hormones. Studies in young animals of both sexes showed an inverse correlation between the density of pituitary T3 receptors and plasma TSH whereas in old animals an age-related impairment of T3 action was reported on the thyrotrophs or changes pertaining to others factors modulating TSH secretion (Donda *et al.*, 1987).

Growth hormone (GH) and ageing

Ageing in humans is associated with a progressive loss of function which leads to a decreased capacity to maintain homeostasis. Certain aspects of the ageing process are similar to the states of hormone deficiency/excess. GH has gained much attention because of its potential clinical applications in reversing metabolic correlates of ageing (Rudman *et al.*, 1990) and disease (Salomon *et al.*, 1989; Herndon *et al.*, 1990; Perrieio *et al.*, 1990). The decreased muscle and bone mass, increased body fat, reduced rate of protein synthesis and issue healing occur in normal ageing as well as in pathological growth hormone deficiency. Previous studies suggest that administration of recombinant GH to non-elderly GH deficient adult leads to improvements in body composition, metabolic variables, muscle strength, cardiac endurance and psycho social outcomes. As normal ageing is associated with a decline in GH secretion and serum level of insulin like growth factor-1 (IGF-1) (Kelijman, 1991; Corpas *et al.*, 1993c), it has been suggested that alterations in the body composition and function in older persons is due to the decrements in GH-IGF-1 axis.

Growth hormone signalling in brain ageing

Growth hormone (GH) is a major growth-promoting and metabolic regulatory hormone. The release of GH is pulsatile and mainly controlled by two hypothalamic factors: somatostatin, which acts as an inhibitor, and GHRH, which stimulates secretion (Muller *et al.*, 1999). Hypersecretion of GH results in gigantism or acromegaly and hyposecretion causes dwarfism (Cheek & Hill, 1974). The effects of GH are mediated *via* the GH receptor (GHR), a 620-amino acid type I transmembrane glycoprotein (Mr 130,000) that is ubiquitously expressed with high levels in liver and adipose tissue (Leung *et al.*, 1987; Kelly *et al.*, 1994). GHR has been identified as a member of the cytokine hematopoietin receptor superfamily (Bazan, 1990; Argetsinger & Carter-Su, 1996). The GHR extracellular domain contains seven cysteine residues, of which six are paired. The unpaired cysteine at position 241 is intermediary in GH-dependent disulfide linkage of two adjacent GHRs but not essential for GHR internalization and signalling (Zhang *et al.*, 1999). Two forms exist for the GHR: the full-length membrane-bound human receptor is a protein of 620 amino acids with a single transmembrane region; and the GH binding protein (GHBP) is a short soluble from corresponding to the extracellular domain of the full-length receptor. In rodents, GHBP is encoded by a specific mRNA of 1.2-1.5 kb, whereas in man and other species GHBP is believed to result from proteolytic cleavage of the membrane receptor. Growth hormone binding protein prolongs the half-life of GH but other functions for GHBP remain to be demonstrated (Postel-Vinay & Finidori, 1995). Some mechanisms regulate receptor abundance and/or availability while others alter the responsiveness of downstream signalling molecules to receptor engagement (Frank, 2001).

GH signalling initiates with the binding of a single GH molecule by a pair of GH receptors (GHRs). The dimerization of GHRs leads to the activation of Janus kinase 2 (JAK2), a nonreceptor tyrosine kinase that associates with the cytoplasmic

domain of GHR. Once activated, JAK2 tyrosyl-phosphorylates both itself and the cytoplasmic domain of GHR. The signalling molecules that are recruited and activated by the GHR-JAK2 complex include signal transducers and activators of transcription (Stat) factors, the adapter protein Shc, and the insulin receptor substrates (IRSs) 1 and 2. The recruitment and activation of these signalling intermediates leads to the activation of enzymes such as MAP kinase, phosphatidylinositol-3'-kinase, protein kinase C, phospholipase A2 and to the release of various second messengers such as diacylglycerol, calcium, and nitric oxide. Ultimately, these pathways modulate cellular functions such as gene transcription, metabolite transport and enzymatic activities that affect the GH-dependent control of growth and metabolism (Campbell, 1997; Liang *et al.*, 1999; Zych *et al.*, 2006).

Growth hormone in the nervous system

The presence of GH-like proteins in the central nervous system is well established (Harvey *et al.*, 1993; Harvey & Hull, 2003). GH immunoreactivity has been detected in in the midbrain, cortex, hippocampus, striatum, olfactory bulb and cerebellum of the rat brain (Mustafa *et al.*, 1995) and in chickens, turkeys, doves (Ramesh *et al.*, 2000). In addition to the brain, GH immunoreactivity is present in the neural tube particularly in marginal layers of the mantle and in the dorsal and ventral horns, as well as the dorsal- and ventral-root ganglia (Harvey *et al.*, 1998, 2000*a*, 2001; Murphy & Harvey, 2001). The presence of GH in the nervous system suggests an extrapituitary site of GH production. This possibility is supported by the presence of GH immunoreactivity in the brain prior to the ontogeny of the pituitary gland and somatotroph differentiation, as demonstrated in the rat (Hojvat *et al.*, 1982), human (Costa *et al.*, 1993), and chicken (Harvey *et al.*, 2001) brain. The presence of GH in the brain also occurs before the appearance of GH in systemic circulation (Harvey *et al.*,1998) and persists after the maturation of the blood–brain barrier (BBB) (Harvey & Hull, 2003). The abundance of GH and GH-binding proteins in the choroid plexus

(Harvey *et al.*, 2001; Harvey & Hull, 2003; Ramesh *et al.*, 2000) suggest some of the GH in the brain might enter through a receptor-mediated transport system (Harvey *et al.* 2002). This possibility is supported by correlations between GH concentrations in peripheral plasma and cerebrospinal fluid (CSF) (Johansson *et al.*, 1995; Schaub *et al.*, 1997; Prahalada *et al.*, 1999), and by the actions of systemically administered GH on brain function (Harvey *et al.* 1998; Harvey & Hull, 2003).

Modulation of growth hormone secretion by neurotransmitters

The central neurotransmitters play a key role in modulating GH secretion. Pharmacological studies in humans reveal that activation of α-2 adrenergic receptors and muscarinic cholinergic receptors in GH secretion; antagonists of these receptors suppress GH release (Muller, 1987; Guistina & Veldhuis, 1998). The influence of α_2 adrenergic neurons appears to be dominant since co-administration of clonidine (α_2 adrenergic antagonist) and atropine (muscarinic cholinergic antagonist) stimulate GH release. Furthermore, treatment with yohimbine (α_2 adrenergic antagonist) can completely block the stimulatory effects on GH secretion of enhancing cholinergic tone with pyridostigmine, a cholinesterase inhibitor (Devassa *et al.*, 1991). In contrast, β- adrenergic receptors appear to mediate significant inhibitory effects on GH release. Blockade of β-adrenergic receptors enhances the GH response to GHRH and other provocative stimulus appears to have no effect on spontaneous GH secretion in boys with constitutional delay of growth (Muller, 1987; Guistina & Veldhuis, 1998; Martha *et al.*, 1988). Administration of salbutamol, a β_2 adrenergic agonist, inhibits GH secretion and is able to block the stimulation of GH release by L-arginine or pyridostigmine (Ghigo *et al.*, 1994). Nicotinic cholinergic and α_2 adrenergic receptors appear to have lesser effects on GH secretion (Muller, 1987; Guistina & Veldhuis, 1998).

Although α-adrenergic and cholinergic neurotransmission have important roles in regulating GH secretion in humans, it is still unknown whether the stimulatory effect on GH secretion on these pathways is mediated by suppression of somatostatin release or stimulation of GHRH secretion or both. In rats, passive immunisation with antiserum to GHRH but not to somatostatin suppresses the stimulatory effects of clonidine, suggesting that clonidine stimulates GHRH release (Miki *et al.*, 1984). In sheep, clonidine increases the hypophyseal-portal blood concentrations of GHRH (Magnan *et al.*, 1994). In humans, administration of a GHRH antagonist significantly suppresses the stimulatory effect of clonidine on GH release (Jaffe *et al.*, 1996). However, the fact that clonidine potentiates the GH response to GHRH in both rats and humans suggest that clonidine decreases somatostatin secretion (Devasa *et al.*, 1991; Lima *et al.*, 1993). In rabbits, yohimbine suppresses spontaneous and GHRH-stimulated GH secretion in anti-somatostatin immunised animals suggesting that α_2 adrenergic receptors may affect both GHRH and somatostatin secretion (Minamitani *et al.*, 1989). Most experimental evidence supports the hypothesis that activation of β-adrenergic receptors increases hypothalamic somatostatin secretion (Guistina & Veldhuis, 1998).

Cholinergic pathways suppress the hypothalamic somatostatin release. GHRH-stimulated GH release is potentiated by cholinergic agonist and blocked by cholinergic antagonists in rats and humans (Locatelli *et al.*, 1986; Massara *et al.*, 1986; Kelijman & Frohman, 1991). Pyridostigmine significantly reverses the inhibitory effect of intravenous GH infusions on the GH responses to GHRH or insulin-induced hypoglycaemia (Kelijman & Frohman, 1991). The depletion of hypothalamic somatostatin content by antero lateral deafferentiation of the medio basal hypothalamus or treatment with cystamine eliminates the effect of cholinergic agonist and antagonist on GH secretion in rats (Locatelli *et al.*, 1986). In contrast, administration of neostigmine to sheep increases hypophyseal- portal blood concentrations of GHRH (Magnan *et al.*, 1993). In humans, administration of a

GHRH antagonist significantly suppresses the stimulatory effect of pyridostigmine on GH release (Jaffe *et al.*, 1996). These results suggest that pyridostigmine stimulates GH release by suppressing somatostatin release, which triggers an increase in GHRH release *via* hypothalamic neuronal interactions (Guistina & Veldhuis, 1998).

Dopaminergic agonist stimulates spontaneous GH release and enhances the GH response to GHRH in normal subjects (Muller 1987; Vans *et al.*, 1987). Although some studies suggest that dopaminergic agonist stimulates the GH release *via* suppression of somatostatin (Guistina & Veldhuis, 1998), administration of GHRH antagonist significantly suppresses the stimulatory effect of L-dopa on GH release (Jaffe *et al.*, 1996). In contrast, bromocriptine and other dopaminergic agonists inhibit GH release in patients with GH-secreting pituitary tumours (Jaffe & Barkan, 1992). In normal subjects, prior infusion of dopamine inhibits the GH response to L-dopa, L-arginine and insulin induced hypoglycaemia (Woolf *et al.*, 1979; Bansal *et al.*, 1981a). Administration of bromocriptine to normal subjects also inhibits the GH response to insulin induced hypoglycaemia (Bansal *et al.*, 1981b). These observations suggest that GH stimulation tests being performed for establishing the diagnosis of GH deficiency affected by concomitant therapy with dopaminergic agonists for pituitary tumours.

Other neurotransmitters which stimulate GH secretion include serotonin, gamma-hydroxy butyrate (GHB), and excitatory aminoacids, such as N-methyl-D, L-aspartate (NMDA). The effects of histamine on GH secretion appear to be inhibitory in rats (Muller, 1987; Guistina & Veldhuis, 1998). However, in humans blockade of histamine type I receptors reduces the GH response to other pharmacological stimuli (Guistina & Veldhuis, 1998). Adrenergic, cholinergic and serotonergic pathways mediate the effects of physiological factors that regulate GH secretion. Alpha adrenergic pathways mediate the GH response to insulin-induced hypoglycaemia, exercise and certain stresses since these responses can be blocked by the administration of phentolamine (Martin, 1973). The GH response to stress involve α-adrenergic pathways in the limbic system because blockade of catecholamine

synthesis in the rat inhibits GH release induced by electrical stimulation of the hippocampus and the baso-lateral amygdala (Martin, 1973). Also, serotonergic and cholinergic pathways have been implicated in the increase in GH secretion associated with sleep (Martin, 1984; Muller, 1987).

Growth hormone in the brain: role in neurogenesis

Neural growth and development is a complex process involving both cellular proliferation and differentiation and can be induced by GH action. GH stimulates neuronal and glial proliferation and increases brain size in young animals (Harvey *et al.*, 1993; Harvey & Hull, 2003). Roles for GH in neural development, independent of insulin-like growth factor-1 (IGF-1), are indicated by the transgenic over expression of the GH gene, which increases brain and spinal weights and increases the size (nuclear and cell body) of lumbar spinal neurons (Chen *et al.*, 1997). GH-deficient (*dw/dw*) mice, have a microcephalic cerebrum that is hypomyelinated with retarded neuronal growth and poor synaptogenesis (Noguchi, 1996). The hypomyelination is due to arrested glial proliferation, suggesting a role for GH in the proliferation and maturation of both glial and neuronal cells. This possibility is supported by the ability of exogenous GH to accelerate glial cell division and myelinogenesis in *dw/dw* mice and to increase the content of gangliosides, a marker of neuronal maturation in neurons (Noguchi, 1996). Exogenous GH has been shown to induce preoligodendrocyte proliferation and oligodendrocyte myelination in cerebrocortical cells of embryonic rats, *via* an activation of the mitogenactivated protein (MAP) - kinase signal-transduction pathway (Ajo *et al.* 2001; Palacios *et al.* 2001). GH is also thought to have neuroprotective roles in neurogenesis. Exogenous GH has been shown to reduce neuronal necrosis induced by local hypoxic-ischemic injury (Scheepens *et al.* 1999, 2000, 2001) or by pilocarpine induced epilepsy (Yu *et al.* 2001). This neuroprotective role may reflect an autocrine or paracrine action of GH, since GH immunoactivity is increased in cortical pyramidal neurons in hemispheres injured by

focal hypoxic–ischemic injury and is more marked in regions that constitutively express GH receptors (e.g., in thalamic, hippocampal and cortical pyramidal neurons, but not in striatal neurons; Scheepens *et al.* 1999, 2001). It also appears to occur by an IGF-1 independent mechanism, since it occurs before the induction of IGF-1 expression and in brain regions with low IGF-1 tissue levels. In the brain, neurogenesis involves increased communication between its component cells (astrocytes, endothelial cells, leptomeningeal cells, ependymocytes and neurons), through the formation of gap junctions. These structures are induced by the expression of connexin genes and the formation of gap junctions in the brain is thought to be induced by GH, since the peripheral administration of GH increases connexin-43 mRNA and protein in the rat cerebral cortex and hypothalamus (Åberg *et al.* 2000). Brain growth and development are dependent upon adequate vascularization. GH has angiogenic activity (Struman *et al.* 1999) and increases vascular density in the cortex of ageing rats (Sonntag *et al.* 1989) in which the decreased cerebral microvasculature has been correlated with an age-related decline in pituitary GH secretion (Sonntag *et al.* 1989).

Calcium imaging

Intracellular free Ca^{2+} concentration plays a pivotal role in the regulation of various cellular functions as an intracellular messenger system. Since the development of digital video imaging of Ca^{2+} novel findings including Ca^{2+} oscillations (Berridge & Galione, 1988; Berridge, 1990) and Ca^{2+} waves (Berridge, 1993) have been described in many different cultured cell types. Ca^{2+} spots were reported as an elementary Ca^{2+} influx event through mechanosensitive channels directly coupled with the initial step in mechanotransduction in cultured endothelial (Ohata et al., 2001a,b; Tanaka & Takamatsu, 2001) and cultured lens epithelial cells (Ohata *et al.*, 2001b,c). The Ca^{2+} spots, which develop sporadically, exhibit a spatiotemporal pattern distinct

from Ca^{2+} sparks, the elementary Ca^{2+} release events from intracellular stores (Cheng *et al.*, 1993; Nelson *et al.*, 1995).

Electrophysiological changes during diabetes and ageing

Neuroelectrophysiological recordings represent a non-invasive and reproducible method of detecting central and peripheral nervous system alterations in diabetes mellitus (Morano *et al.*, 1996). Diabetes mellitus is associated with chronic complications such as nephropathy, angiopathy, retinopathy and peripheral neuropathy. In diabetic patients, hyperglycaemia precipitate seizures and in experimental diabetes, indications for an increased neuronal excitability have been found (Anderson *et al.*, 2006). Neurophysiological alterations have also been described in animal models of diabetes, in particular in rats. The morphological alterations of the autonomic nervous system in diabetes, either in rats or humans, are reported to be related to degenerative processes observed in the nervous fibers and their myelin sheaths (Monckton & Pehowich, 1980; Powell *et al.*, 1976; Yasaki & Dyck, 1990). Electrophysiological and morphological studies suggest that the axon is the initial site of damage in the peripheral nerve (Clements & Junior, 1979). In the peripheral nervous system (PNS) of diabetic rats the time course of neurophysiological changes is well established.

Deficits in both motor and sensory nerve conduction velocity (MNCV and SNCV, respectively) can be detected within weeks after the onset of diabetes and increase up to 2–3 months after diabetes onset, remaining relatively stable thereafter (Moore *et al.*, 1980; Cameron *et al.*, 1986; Brismar *et al.*, 1987; Kappelle *et al.*, 1993). Studies of MNCV and SNCV in diabetic rats have made important contributions to the elucidation of the pathogenesis of the effects of diabetes on the PNS, as well as in the development of putative pharmacotherapy. Neurophysiological alterations have also been reported in the CNS of diabetic rats. Less is known about the underlying mechanisms of alterations in the CNS in diabetic rats. Cerebral

metabolic (Knudsen *et al.*, 1989; Kumar & Menon, 1993) and vascular (Duckrow *et al.*, 1987; Jakobsen *et al.*, 1990) disturbances have been demonstrated within weeks after diabetes induction. However, the severity of these disturbances appears to be limited compared with the PNS (Biessels *et al.*, 1994), possibly leading to a less hostile neuronal microenvironment. EEG recordings showed changes in the brain activity of 14 day diabetic rats compared to control rats (Gireesh, 2007). It is reported that metabolic control influences the EEG and improvement of glucose metabolism is an important factor in avoiding EEG abnormalities in young diabetic patients (Hauser *et al.*, 1995).

The latencies of auditory and visual potentials were found to be prolonged in STZ-diabetic rats (Biessels *et al.*, 1999; Rubini *et al.*, 1992) and BB/W diabetic rats (Chakrabarti *et al.*, 1991; Sima *et al.*, 1992), indicating impaired CNS conduction velocities in diabetes. In hippocampal slices from STZ-diabetic rats, long-term potentiation (LTP) induced by 100-Hz stimulation was impaired, whereas long-term depression (LTD) was enhanced compared with control rats (Biessels *et al.*, 1998; Gispen & Biessels, 2000; Kamal *et al.*, 2000), indicating that altered hippocampal synaptic plasticity occurs in type 1 STZ-diabetic rats. Because changes in hippocampal synaptic plasticity in STZ-diabetic rats occur in association with impairments of spatial learning function (Gispen & Biessels, 2000; Kamal *et al.*, 2000), these studies provide a mechanism for cognitive dysfunction in diabetic animals.

Materials and Methods

CHEMICALS USED AND THEIR SOURCES

Biochemicals

Acetylthiocholine iodide, (±)norepinephrine, (±)epinephrine, dopamine, 5-hydroxytryptamine, sodium octyl sulfonic acid, ethylene diamine tetra acetic acid-EDTA, HEPES-[n′ (2-hydroxy ethyl)] piperazine-n′-[2-ethanesulfonic acid], streptozotocin, foetal calf serum (heat inactivated), citric acid, Tris HCl, D-glucose, calcium chloride, collagenase type XI, bovine serum albumin fraction V, Triton X 100, acetylcholine iodide, pirenzepine, 4-DAMP mustard (4- deoxy acetyl methyl piperidine mustard), and RPMI-1640 medium (Sigma Chemical Co., St. Louis, MI, USA). All other reagents were of analytical grade purchased locally. HPLC solvents were of HPLC grade obtained from SRL and Merck, India.

Radiochemicals

[^{3}H]Acetylcholine iodide (Sp. Activity 82 mCi/mmol), 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, U.S.A. [^{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, UK. Radioimmunoassay kits for Insulin and Triiodothyronine was purchased from Bhabha Atomic Research Centre (BARC), Mumbai, India.

Molecular Biology Chemicals

Tri-reagent kit was purchased from Sigma Chemical Co., St. Louis, MI, USA. ABI PRISM High Capacity cDNA Archive kit, Primers and Taqman probes for Real-Time PCR were purchased from Applied Biosystems, FosterCity, CA, USA. Taqman probes used were muscarinic M1 (Rn_00589936), M3 (Rn_00560986), NMDAR1 (Rn_00433800), mGlu-R5 (Rn_00566628), dopamine D_2 (Rn_00561126), adrenergic α_{2A} (Rn_00562488), adrenergic β_2 (Rn_0050650), $GABA_{A\alpha1}$ (Rn_00788315), $GABA_B$ (Rn_00578911), 5-HT_{2C} (Rn_00562748). Ca^{2+} fluorescent dye, fluo 4-AM was purchased from Molecular Probes, Eugene, Oregon, USA.

ANIMALS

Adult male Wistar rats of different age groups were purchased from Kerala Agriculture University, Mannuthy and Amrita Institute of Medical Sciences, Kochi and used for all experiments. They were housed in separate cages under 12 hours light and 12 hours dark periods and were maintained on standard food pellets and water *ad libitum*.

INDUCTION OF DIABETES

Animals were divided into the following groups as i) Control ii) Diabetic iii) Insulin treated diabetics of 7 weeks old and 90 weeks old rats. Each group consisted of 4-6 animals. Diabetes was induced by a single intrafemoral dose (50-60 mg/kg body weight) of streptozotocin freshly dissolved in citrate buffer (pH 4.5) under anaesthesia (Junod *et al.*, 1969; Hohenegger & Rudas, 1971; Arison *et al.*, 1967). The insulin treated diabetic group (Group iii) received subcutaneous injections (1Unit/kg body weight) of insulin daily during the entire period of the experiment. A mixture of both Lente and Plain insulin (Abbott India) were given for the better control (Sasaki & Bunag, 1983). The last injection was given 24 hr before sacrificing the diabetic rats. Control rats were injected with citrate buffer. Rats were sacrificed by decapitation on

the 15th day of the experiment. The brain regions; cerebral cortex, brain stem and corpus striatum were dissected out quickly over ice according to the procedure of Glowinski and Iversen, (1966). The tissues were stored at -70^0 C until assay.

ESTIMATION OF BLOOD GLUCOSE

The diabetic state of animals was assessed by measuring blood glucose concentrations at 72 hours after streptozotocin treatment. The rats with a blood sugar level above 250 mg/dl were selected as diabetic rats. Blood glucose was estimated using Glucose estimation kit (Merck). The spectrophotometric method using glucose oxidase-peroxidase reactions is as follows:

Principle: Glucose oxidase (GOD) catalyses the oxidation of glucose in accordance with the following equation:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{(GOD)} \text{Gluconic acid} + H_2O_2.$$

The hydrogen peroxide formed in this reaction reacts with 4-aminoantipyrine and 4-hydroxybenzoic acid in the presence of peroxidase (POD) to form N-(-4-antipyryl)-p-benzo quinoneimine. The addition of mutarotase accelerates the reactions. The amount of dye formed is proportional to the glucose concentration. The absorbance was read at 500nm in (Milton Roy Genesys 5 Spectronic) spectrophotometer.

LONG TERM LOW DOSE INSULIN AND SOMATOTROPIN TREATMENT

Animals were divided into following groups as (group I- aged 4 weeks continued to 16 weeks old and group II- aged 60 weeks continued to 90 weeks old). Each group consisted of eight male Wistar rats. The animals were injected twice in a week with (1) subcutaneous saline injections (2) subcutaneous GH injections (0.05 IU/kg) and (3) subcutaneous INS injections (0.05 IU/kg). A mixture of both Lente and

Plain insulin (Abbott India) were given for the insulin treated animals. Rats were sacrificed by decapitation and cerebral cortex was dissected out quickly over ice according to the procedure of Glowinski and Iversen, (1966) and stored at -70^0 C until assay.

QUANTIFICATION OF BRAIN MONOAMINES AND THEIR METABOLITES

The monoamines were assayed according to Paulose *et al.*, (1988). The cerebral cortex (CC), brain stem (BS), corpus striatum (CS) and Hypothalamus (Hypo) of saline, insulin and somatotropin treated young and old rats was homogenised in 0.4N perchloric acid. The homogenate was then centrifuged at 5000xg for 10 minutes at 4°C (Sigma 3K30 refrigerated centrifuge) and the clear supernatant was filtered through 0.45 μm HPLC grade filters and used for HPLC analysis.

Epinephrine (EPI), norepinephrine (NE), dopamine (DA) and serotonin (5-HT) contents were determined in high performance liquid chromatography (HPLC) with electrochemical detector (ECD) (Waters, USA) fitted with CLC-ODS reverse phase column of 5 μm particle size. The mobile phase consisted of 0.05M sodium phosphate dibasic, 0.03M citric acid, 0.1mM EDTA, 0.6mM sodium octyl sulfonate, 15% methanol. The pH was adjusted to 3.25 with orthophosphoric acid, filtered through 0.22 μm filter (Millipore) and degassed. A Waters (model 515, Milford, USA) pump was used to deliver the solvent at a rate of 1 ml/minute. The neurotransmitters and their metabolites were identified by amperometric detection using an electrochemical detector (Waters, model 2465) with a reduction potential of +0.80 V. Twenty microlitre aliquots of the acidified supernatant were injected into the system for detection. The peaks were identified by relative retention times compared with external standards and quantitatively estimated using an integrator (Empower

software) interfaced with the detector. Data from different brain regions of the experimental and control rats were statistically analysed and tabulated.

ACETYLCHOLINE ESTERASE ASSAY

Acetylcholine esterase assay was done in the brain regions; CC, BS, CS and pancreas of both control and experimental rats using spectrophotometric method of Ellman *et al.*, (1961). The homogenate (10%) was prepared in 30mm sodium phosphate buffer, pH 7.0. One ml of 1% Triton X 100 was added to the homogenate to release the membrane bound enzyme and centrifuged at 10,000 rpm for 30 minutes at 4^0C. Different concentrations of acetylthiocholine iodide was used as substrate. The mercaptan formed as a result of the hydrolysis of the ester reacts with an oxidising agent 5, 5' -dithiobis (2-nitrobenzoate) which absorbs at 412 nm.

ACETYLCHOLINE RECEPTOR BINDING STUDIES USING [^{3}H] RADIOLIGANDS IN THE BRAIN REGION OF CONTROL AND EXPERIMENTAL RATS

Acetylcholine receptor binding studies using [^{3}H]Acetylcholine iodide and unlabelled acetylcholine iodide

[^{3}H]Acetylcholine iodide binding assay in the CC, BS and CS was done according to the modified procedure of Yamamura and Snyder (1981). The brain regions were homogenised in a polytron homogeniser with 50 volumes of cold 50mM Tris-HCl buffer, containing 1mM EDTA, pH 7.4. One ml of 1% Triton X 100 was added to the homogenate to release the membrane bound enzyme. The homogenate was then centrifuged at 30,000xg for 30 minutes. The pellets were again resuspended in Tris-HCl-EDTA buffer and centrifuged at 30,000xg for 30 minutes. The final pellet was resuspended in appropriate volume of Tris-HCl-EDTA buffer and used for binding studies.

Binding assays were done using different concentrations i.e., 1.0 nM to 10.0 nM of [^{3}H]Acetylcholine iodide in the incubation buffer, pH 7.4 in a total incubation volume of 250µl containing appropriate protein concentrations (200-250µg). Non specific binding was determined using 100µM acetylcholine iodide. Tubes were incubated at 22^{0}C for 60 minutes and filtered rapidly through GF/C filters (Whatman). Competition studies were carried out with 2.0nM [^{3}H]Acetylcholine iodide in each tube with unlabelled ligand concentrations varying from 10^{-9}M to 10^{-4}M of acetylcholine iodide. The filters were washed quickly by three successive washing with 5.0 ml of ice cold 50mM Tris-HCl buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non specific binding determined showed 10% in all our experiments.

MUSCARINIC RECEPTOR BINDING STUDIES USING [^{3}H] RADIOLIGANDS IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

Muscarinic M1 receptor binding studies using [^{3}H]QNB and unlabelled pirenzepine

Muscarinic M1 receptor binding assays in CC, were done using different concentrations i.e., 0.5nM to 2.5nM of [^{3}H]QNB in the incubation buffer, pH 7.4 in a total incubation volume of 250µl containing appropriate protein concentrations (200-250µg). Non specific binding was determined using 100µM pirenzepine. Competition studies were carried out with 1.0nM [^{3}H]QNB in each tube with unlabelled ligand concentrations varying from 10^{-9}M to 10^{-4}M of pirenzepine. Tubes were incubated at 22^{0}C for 60 minutes and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 5.0 ml of ice cold 50mM Tris-HCl buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non specific binding determined showed 10% in all our experiments.

Muscarinic M3 receptor binding studies using [^{3}H]DAMP and unlabelled 4-DAMP mustard

[^{3}H]DAMP binding assay in CC, BS and CS was done according to the modified procedure of Yamamura and Snyder (1981). Brain tissues were homogenised in a polytron homogeniser with 20 volumes of cold 50mM Tris-HCl buffer, pH 7.4, containing 1mM EDTA. The supernatant was then centrifuged at 30,000xg for 30 minutes and the pellets were resuspended in appropriate volume of the buffer.

. Binding assays were done using different concentrations i.e., 0.1nM to 1.0nM of [^{3}H]DAMP in the incubation buffer, pH 7.4 in a total incubation volume of 250µl containing appropriate protein concentrations (200-250µg). Non specific binding was determined using 100µM 4-DAMP mustard. Competition studies were carried out with 0.5nM [^{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^{0}C for 60 minutes and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 5.0 ml of ice cold 50mM Tris-HCl buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non specific binding determined showed 10% in all our experiments.

BINDING STUDIES IN THE PANCREATIC ISLETS

[^{3}H]DAMP binding

Pancreatic islets were isolated as described in insulin secretion experiments *in vitro* by collagenase digestion method. Islets were then homogenised for 30 seconds in a polytron homogeniser with 10ml medium consisting of 50mM Na_2HPO_4/NaH_2PO_4 , pH 7.4, and 2mM $MgCl_2$ with the addition of BSA (1mg/ml), bacitracin (0.2mg/ml), aprotinin (500 kallikrein inhibitor units/ml). The homogenate was then centrifuged at 30,000xg for 30 minutes, the pellets were resuspended in

appropriate volume of the same buffer. Binding assays were done using different concentrations i.e., 0.1nM to1.0nM of [^{3}H]DAMP in the incubation buffer, pH 7.4 in a total incubation volume of 250µl containing 250-300µg protein. Non specific binding was determined using 100µM unlabelled 4-DAMP mustard. Competition studies were carried out with 0.5nM [^{3}H]DAMP in each tube with unlabelled ligand concentrations varying from 10^{-9}M to 10^{-4}M of 4-DAMP mustard. The tubes were incubated at 22^0C for 2 hours and filtered rapidly through GF/C filters (Whatman). The filters were washed with ice cold phosphate assay buffer. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non specific binding determined showed 30-40% in all our experiments.

Protein determination

Protein was measured by the method of Lowry *et al.*, (1951) using bovine serum albumin as standard. The intensity of the purple blue colour formed was proportional to the amount of protein, which was read in Spectrophotometer (Milton Roy Genesys 5 Spectronic) at 660nm.

ANALYSIS OF THE RECEPTOR BINDING DATA

Linear regression analysis for Scatchard plots

The data was analysed according to Scatchard, (1949). 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. 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.

Nonlinear regression analysis for displacement curve

The displacement data were analysed by nonlinear regression using Graphpad Prism software, GraphPad, Inc., USA. The concentration of the competing drug that competes for half the specific binding was defined as EC_{50}, which is same as IC_{50} (Unnerstall, 1990). The affinity of the receptor for the competing drug is designated as K_i and is defined as the concentration of the competing ligand that will bind to half the binding sites at equilibrium in the absence of radioligand or other competitors (Cheng & Prusoff, 1973).

GENE EXPRESSION STUDIES OF NEUROTRANSMITTER RECEPTORS IN DIFFERENT BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS.

Isolation of RNA

RNA was isolated from the different brain regions of control and experimental rats using the Tri reagent from Sigma Aldrich. Tissue (25-50 mg) homogenates were made in 0.5ml Tri Reagent and was centrifuged at 12,000x*g* for 10 minutes at 4^0C. The clear supernatant was transferred to a fresh tube and it was allowed to stand at room temperature for 5 minutes. 100µl of chloroform was added to it, mixed vigorously for 15 seconds and allowed to stand at room temperature for 15 minutes. The tubes were then centrifuged at 12,000x*g* for 15 minutes at 4^0C. Three distinct phases appear after centrifugation. The bottom red organic phase contained protein, interphase contained DNA and a colourless upper aqueous phase contained RNA. The upper aqueous phase was transferred to a fresh tube and 250µl of isopropanol was added and the tubes were allowed to stand at room temperature for 10 minutes. The tubes were centrifuged at 12,000x*g* for 10 min at 4^0C. The supernatants were removed and the RNA pellet was washed with 500µl of 75% ethanol, vortexed and centrifuged at 12,000x*g* for 5 min at 4^0C. The pellets were semi

dried and dissolved in minimum volume of DEPC-treated water. 2µl of RNA was made up to 1ml and absorbance was measured at 260nm and 280nm. For pure RNA preparation the ratio of absorbance at 260/280 was ≥ 1.7. The concentration of RNA was calculated as 1 OD at 260 = 42µg.

cDNA Synthesis

Total cDNA synthesis was performed using ABI PRISM cDNA Archive kit in 0.2ml microfuge tubes. The reaction mixture of 20µl contained 0.2µg total RNA, 10X RT buffer, 25X dNTP mixture, 10X Random primers, MultiScribe RT (50U/µl) and RNase free water. The cDNA synthesis reactions were carried out at 25^0C for 10 minutes and 37^0C for 2 hours using an Eppendorf Personal Cycler. The primers and probes were purchased from Applied Biosystems, FosterCity, CA, USA designed using Primer Express Software Version (3.0).

REAL-TIME PCR ASSAY

Real–Time PCR assays were performed in 96-well plates in ABI 7300 Real–Time PCR instrument (Applied Biosystems). To detect gene expression, a TaqMan quantitative 2-step reverse transcriptase polymerase chain reaction (RT-PCR) was used to quantify mRNA levels. First-strand cDNA was synthesized with use of a TaqMan RT reagent kit, as recommended by the manufacturer. PCR analyses were conducted with gene-specific primers and fluorescently labeled Taq probe (designed by Applied Biosystems). Endogenous control, β-actin, was labeled with a reporter dye (VIC). All reagents were purchased from Applied Biosystems. The probes for specific gene of interest were labeled with FAM at the 5' end and a quencher dye at the 3' end. The real-time data were analyzed with Sequence Detection Systems software version 1.7. All reactions were performed in duplicate.

The TaqMan reaction mixture of 20µl contained 25ng of total RNA-derived cDNAs, 200nM each of the forward primer, reverse primer and TaqMan probe for

specific neurotransmitter receptor gene, endogenous control, β-actin and 12.5μl of TaqMan 2X Universal PCR MasterMIX (Applied Biosystems). The volume was made up with RNAse free water.

The thermocycling profile conditions were as follows:

50°C -- 2 minutes ---- Activation

95°C -- 10 minutes ---- Initial Denaturation

95°C -- 15 seconds ---- Denaturation 40 cycles

50°C -- 30 seconds --- Annealing

60°C -- 1 minutes --- Final Extension

Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence. The ΔΔCT method of relative quantification was used to determine the fold change in expression. This was done by first normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control β-actin in the same samples ($\Delta CT = CT_{Target} - CT_{\beta\text{-actin}}$). It was further normalized with the control ($\Delta\Delta CT = \Delta CT - CT_{Control}$). The fold change in expression was then obtained ($2^{-\Delta\Delta CT}$).

ISOLATION OF PANCREATIC ISLETS

Pancreatic islets were isolated from male weanling Wistar rats by standard collagenase digestion procedures using aseptic techniques (Howell, 1968). The islets were isolated in HEPES-buffered sodium free Hanks Balanced Salt Solution (HBSS) (Pipeleers, 1985) with the following composition: 137mM Choline chloride, 5.4mM KCl, 1.8mM $CaCl_2$, 0.8mM $MgSO_4$, 1mM KH_2PO_4, 14.3mM $KHCO_3$ and 10mM HEPES. Autoclaved triple distilled water was used in the preparation of the buffer.

The pancreas from both young and old rats was aseptically dissected out into a sterile petridish containing ice cold HBSS and excess fat and blood vessels were removed. The pancreas was cut into small pieces and transferred to a sterile glass vial containing 2ml collagenase type XI solution (1.5 mg/ml in HBSS, pH 7.4). The collagenase digestion was carried out for 15 minutes at 37°C in an environmental shaker with vigorous shaking (300rpm/minute). The tissue digest was filtered through 500μm nylon screen and the filtrate was washed with three successive centrifugations and resuspensions in cold HBSS. The pancreatic islet preparation having a viability of >90% as assessed by Trypan Blue exclusion which was chosen for cell culture and other experiments.

In vitro insulin secretion study in the pancreatic islets

Pancreatic islets were isolated from the pancreas of both young and old rats by collagenase digestion method and islets were suspended in Krebs Ringer Bicarbonate buffer (KRB), pH 7.3, of following composition: 115mM NaCl, 4mM KCl, 2.56mM $CaCl_2$, 1.2mM KH_2PO_4, 1.2mM $MgSO_4$ and 20mM $NaHCO_3$. The islets were pre-incubated for 1 hr in KRB at 37^0C. The cells were then harvested and resuspended in fresh KRB (100islets/ml medium) with glucose - 4mM and 20mM and carbachol - 10^{-8} to 10^{-4}M. After incubation, the supernatant was transferred to fresh tubes for insulin assay. Insulin assay was done according to the procedure of BARC radioimmunoassay kit. Insulin concentration in the samples was determined from the standard curve plotted using MultiCalc™ software (Wallac, Finland).

In vitro insulin secretion in the presence of carbachol, muscarinic M1 and M3 receptor antagonists

The isolated islets from both young and old rats were incubated for 1hour at 37^0C with 10^{-7}M carbachol, 10^{-6}M pirenzepine - muscarinic M1 receptor antagonist

and 10^{-6}M 4-DAMP mustard - M3 receptor antagonist in two different concentrations of glucose i.e., (i) 4mM glucose and (ii) 20mM glucose to study the effect of carbachol and muscarinic receptor subtypes on glucose induced insulin secretion. Islets were centrifuged after incubation at 1,500xg for 10 min at 4^0C and the supernatant was transferred to fresh tubes for insulin assay by radioimmunoassay.

ESTIMATION OF CIRCULATING INSULIN BY RADIOIMMUNOASSAY

Principle of the assay

The assay was done according to the procedure of BARC radioimmunoassay kit. The radioimmunoassay method is based on the competition of unlabelled insulin in the standard or samples and [^{125}I] insulin for the limited binding sites on a specific antibody. At the end of incubation, the antibody bound and free insulin were separated by the second antibody-polyethylene glycol (PEG) aided separation method. Measuring the radioactivity associated with bound fraction of sample and standards quantitates insulin concentration of samples.

Assay Protocol

Standards, ranging from 0 to 200μU/ml, insulin free serum and insulin antiserum (50μl each) were added together and the volume was made up to 250μl with assay buffer. Samples of appropriate concentration from experiments were used for the assay. They were incubated overnight at 2°C. Then [^{125}I]insulin (50μl) was added and incubated at room temperature for 3 hrs. The second antibody was added (50μl) along with 500μl of PEG. The tubes were then vortexed and incubated for 20 minutes and they were centrifuged at 1500 x g for 20 minutes. The supernatant was aspirated out and the radioactivity in the pellet was determined in a gamma counter.

A standard curve was plotted with $\%B/B_0$ on the Y-axis and insulin concentration/ml on the X-axis of a log-logit graph. $\%B/B_0$ was calculated as:

$$\frac{\text{Corrected average count of standard or sample}}{\text{Corrected average count of zero standard}} \times 100$$

Insulin concentration in the samples was determined from the standard curve plotted using MultiCalc™ software (Wallac, Finland).

IP3 CONTENT IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS *IN VIVO*

Brain tissues- CC, BS and CS were homogenised in a polytron homogeniser with 20 volumes of cold 50mM Tris-HCl buffer, pH.7.4, containing 1mM EDTA. The homogenate was then centrifuged at 30,000xg for 30 minutes and the supernatant was transferred to fresh tubes for IP3 assay using [^{3}H]IP3 Biotrak assay system kit.

Principle of the assay

The assay was based on competition between [^{3}H]IP3 and unlabelled IP3 in the standard or samples for binding to a binding protein prepared from bovine adrenal cortex. The bound IP3 was then separated from the free IP3 by centrifugation. The free IP3 in the supernatant was then discarded by simple decantation, leaving the bound fraction adhering to the tube. Measurement of the radioactivity in the tube enables the amount of unlabelled IP3 in the sample to be determined.

Assay Protocol

Standards, ranging from 0.19 to 25pmol/tube, [^{3}H]IP3 and binding protein were added together and the volume was made up to 100µl with assay buffer. Samples of appropriate concentration from experiments were used for the assay. The tubes were then vortexed and incubated on ice for 15 minutes and they were centrifuged at 2000 x g for 10 minutes at 4^{0}C. The supernatant was aspirated out and the pellet was resuspended in water and incubated at room temperature for 10

minutes. The tubes were then vortexed and decanted immediately into scintillation vials. The radioactivity in the suspension was determined using liquid scintillation counter.

A standard curve was plotted with $\%B/B_o$ on the Y-axis and IP3 concentration (pmol/tube) on the X-axis of a semi-log graph paper. $\%B/B_o$ was calculated as:

$$\frac{\text{(Standard or sample cpm} - \text{NSB cpm)}}{(B_0 \text{ cpm} - \text{NSB cpm})} \times 100$$

NSB- non specific binding and B_0 - zero binding. IP3 concentration in the samples was determined by interpolation from the plotted standard curve.

cGMP CONTENT IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS *IN VIVO*

Brain tissues- CC, BS and CS were homogenised in a polytron homogeniser with 20 volumes of cold 50mM Tris-HCl buffer, pH 7.4, containing 1mM EDTA. The homogenate was then centrifuged at 30,000xg for 30 minutes and the supernatant was transferred to fresh tubes for cGMP assay using [^{3}H]cGMP Biotrak assay system kit.

Principle of the assay

The assay is based on the competition between unlabelled cGMP and a fixed quantity of the tritium labelled compound for binding to an antiserum, which has a high specificity and affinity for cGMP. The amount of [^{3}H]cGMP bound to the antiserum is inversely related to the amount of cGMP present in the assay sample. Measurement of the antibody bound radioactivity enables the amount of unlabelled cGMP in the sample to be calculated. Separation of the antibody bound cGMP from the unbound nucleotide was done by ammonium sulphate precipitation, followed by centrifugation. The precipitate which contains the antibody bound complex was dissolved in water and its activity was determined by liquid scintillation counting. The

concentration of unlabelled cGMP in the sample was determined from a linear standard curve.

Assay Protocol

Standards, ranging from 0.5 to 4.0pmol/tube, and [^{3}H]cGMP were added together and the volume was made up to 100µl with assay buffer. Samples of appropriate concentration from experiments were used for the assay. The antiserum was added to all the assay tubes and then vortexed. The tubes were incubated for 90 minutes at 2-8^{0}C. Ammonium sulphate was added to all tubes, mixed and allowed to stand for 5 minutes in ice bath. The tubes were centrifuged at 12000xg for 2 minutes at room temperature. The supernatant was aspirated out and the pellet was dissolved in water and decanted immediately into scintillation vials. The radioactivity in the suspension was determined using liquid scintillation counter.

A standard curve was plotted with Co/Cx on the Y-axis and cGMP concentration (pmol/tube) on the X-axis of a linear graph paper. Co- the cpm bound in the absence of unlabelled cGMP; Cx- the cpm bound in the presence of standard/unknown cGMP. cGMP concentration in the samples was determined by interpolation from the plotted standard curve.

IP3 AND cGMP CONTENT IN THE PANCREAS OF CONTROL AND EXPERIMENTAL RATS *IN VIVO*

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

EFFECT OF CARBACHOL AND MUSCARINIC ANTAGONISTS ON IP3 LEVELS IN ONE HOUR PANCREATIC ISLETS *IN VITRO*

Pancreatic islets were prepared from both young and old rats by collagenase digestion method as mentioned earlier. The isolated islets were incubated for one hour at 37^0C with 10^{-7}M carbachol, 10^{-6}M pirenzepine - muscarinic M1 receptor antagonist and, 10^{-6}M 4-DAMP mustard - M3 receptor antagonist at two different concentrations of glucose i.e., (i) 4mM glucose and (ii) 20mM glucose. Islets were centrifuged after incubation at 1,500 x g for 10 min at 4^0C and the supernatant was transferred to fresh tubes for IP3 assay using [^{3}H]IP3 Biotrak assay system kit.

Similarly the islets were incubated with combinations of carbachol and dopamine at different concentrations to study their effect on IP3 release using [^{3}H]IP3 Biotrak assay system kit.

EFFECT OF CARBACHOL AND MUSCARINIC ANTAGONISTS ON cGMP LEVELS IN ONE HOUR PANCREATIC ISLETS *IN VITRO*

Pancreatic islets were prepared from both young and old rats by collagenase digestion method as mentioned earlier. The isolated islets were incubated for 1hour at 37^0C with 10^{-7}M carbachol, , 10^{-6}M pirenzepine - muscarinic M1 receptor antagonist and, 10^{-6}M 4-DAMP mustard - M3 receptor antagonist at two different concentrations of glucose i.e., (i) 4mM glucose and (ii) 20mM glucose. Islets were centrifuged after incubation at 1,500 x g for 10 min at 4^0C and the supernatant was transferred to fresh tubes for cGMP assay using [^{3}H]cGMP Biotrak assay system kit.

Similarly the islets were incubated with combinations of carbachol and dopamine at different concentrations to study their effect on cGMP release using [^{3}H]cGMP Biotrak assay system kit.

ESTIMATION OF CIRCULATING TRIIODOTHYRONINE (T3) BY RADIOIMMUNOASSAY

Principle of the assay

The assay was done according to the procedure of BARC radioimmunoassay kit. The radioimmunoassay method is based on the competition of unlabelled endogenous T3 with [^{125}I]T3 for the limited binding sites on the antibody (Ab1) made specially for T3. The antibody was in the form of a complex with second antibody (Ab2). At the end of incubation, the T3 (Ag) bound to the antibody- second antibody complex (Ag-Ab1-Ab2) and free T3 was separated by the addition of PEG. The amount bound to the antibody complex in the assay tubes were compared with values of known T3 standards and the T3 concentration in the sample was calculated.

Assay Protocol

Standards, ranging from 0.15 to 2.5ng, T3 free serum, [^{125}I]T3 and antiserum complex were added together and the volume was made up to 275µl with assay buffer. Samples of appropriate concentration from experiments were used for the assay. They were incubated at 37°C for 45 minutes. The PEG was added to all tubes and they were centrifuged at 1500 x g for 20 minutes. The supernatant was aspirated out and the radioactivity in the pellet was determined in a gamma counter.

A standard curve was plotted with $\%B/B_0$ on the Y-axis and T3 concentration (ng /ml) on the X-axis of a log-logit graph. $\%B/B_0$ was calculated as:

$$\frac{\text{Corrected average count of standard or sample}}{\text{Corrected average count of zero standard}} \times 100$$

T3 concentrations in the samples were determined from the standard curve plotted using MultiCalc™ software (Wallac, Finland).

CALCIUM IMAGING

Pancreatic islets were prepared from both young and old rats by collagenase digestion method as mentioned earlier. The isolated islets were incubated for 4 hours at room temperature in 1ml of RPMI medium containing 5μM of Ca^{2+} fluorescent dye, fluo 4-AM (Molecular Probes, Eugene, OR), to monitor the changes in the intracellular Ca^{2+}. After incubation cells were washed twice in indicator free RPMI medium to remove excess dye that was non specifically associated with the cell surface, and then incubated for further 30 minutes to allow complete de esterification of intra cellular AM esters. The 35mm plates, containing pancreatic islet cells were placed on the stage of a Leica TCS SP5 laser scanning confocal microscope equipped with a HC PL FLUOTAR 20.0x 0.50 dry objective (NA 0.5). Fluo 4-AM was excited with 514nm laser lines from an argon laser, with laser intensity set at 38% of available power. For visualization of Fluo 4-AM, the emission window was set at 508.4 nm – 571.5nm. The images were continuously acquired before and after addition of 10^{-7}M carbachol, 10^{-6}M pirenzepine (muscarinic M1 receptor antagonist) and 10^{-6}M 4-DAMP mustard (M3 receptor antagonist), at time intervals of 1.318 seconds, for a total of 600 seconds. Time series experiments were performed collecting 512x512 pixel images at 400Hz. Fluorescence intensity was analysed using the quantitation mode in LAS-AF software from Leica Microsystems, Germany. A region of interest (ROI) was drawn within a field of view. For each ROI, the pixel intensity was calculated for each image in the 600 seconds sequence to analyse the intracellular Ca^{2+} release from the pancreatic islet cells in experimental conditions.

EEG ANALYSIS

Brain activity changes of the control and experimental rats were studied using EEG recorder. The electroencephalograph was analysed according to the procedure of Hughes *et al.*, (1983) and recorded using Neurocare™ Wingraph Digital EEG system.

The brain waves recorded on the EEG were used to understand the slow waves and neurophysiological mechanisms in the experimental groups of rats. The frequency of the brain waves reflects the responsiveness of the neurons to the stimulus. Spontaneous electrical activities was measured by placing electrodes in the left and right lobes of different brain regions of the scalp of experimental rats, reference electrodes were placed on the ear and ground reference on the trunk. Each electrode was placed 10-20 percent away from the neighbouring electrodes. Brain wave activity was analysed from the EEG recorded data of control and experimental rats.

STATISTICS

Statistical evaluations were done by ANOVA using InStat (Ver.2.04a) computer programme. Linear regression Scatchard plots were made using SIGMA PLOT (Ver 2.03). Sigma Plot software (version 2.0, Jandel GmbH, Erkrath, Germany). Competitive binding data were analysed using non-linear regression curve-fitting procedure (GraphPad PRISM™, San Diego, USA). Empower software was used for HPLC analysis. Relative Quantification Software was used for analyzing Real-Time PCR results.

Results

BODY WEIGHT AND BLOOD GLUCOSE LEVEL OF EXPERIMENTAL RATS

The body weight was significantly decreased ($p<0.001$) in 7 weeks and 90 weeks old diabetic rat groups when compared to their respective controls. With insulin treatment from 3rd day continued till sacrifice, the body weight was reversed to near the initial level in both groups (Table-1, 2; Fig-1,2). Blood glucose level of all rats before streptozotocin administration was within the normal range. Streptozotocin administration led to a significant increase ($p<0.001$) in blood glucose level of 7 weeks and 90 weeks old diabetic rat groups when compared to their respective controls. In both groups, insulin treatment significantly reduce ($p<0.001$) the increased blood glucose level to near the control value when compared to diabetic group (Table-3, 4; Fig-3, 4).

ACETYLCHOLINE ESTERASE ACTIVITY IN BRAIN REGIONS AND PANCREAS OF CONTROL AND EXPERIMENTAL RATS

Cerebral cortex

Acetylcholine esterase activity in the cerebral cortex showed a significant increase in both 7 weeks ($p<0.001$) and 90 weeks old diabetic rats ($p<0.05$) with no significant change in K_m when compared to control. Insulin treatment significantly decreased the enzyme activity in 7 weeks ($p<0.001$) and 90 weeks old groups ($p<0.05$) compared to the diabetics. In control old rats, V_{max} significantly decreased ($p<0.001$) compared to the young rats with no significant change in the affinity. V_{max} of 90 weeks old diabetic and D+I groups decreased significantly ($p<0.001$) when compared to 7 weeks diabetic and D+I groups respectively (Table-5; Fig-5).

Brainstem

Acetylcholine esterase activity in the brainstem showed a significant decrease ($p<0.001$) in V_{max} of 7 weeks diabetic with a significant decrease in K_m ($p<0.05$) whereas the enzyme activity increased significantly ($p<0.001$) in 90 weeks diabetic with no change in K_m when compared to respective control. Insulin treated diabetics showed a significant increase ($p<0.001$) in the V_{max} in 7 weeks group whereas in 90 weeks group, V_{max} decreased significantly ($p<0.05$) compared to the diabetics. In control old rats, V_{max} significantly decreased ($p<0.001$) compared to the young rats with no significant change in the K_m. V_{max} of 90 weeks diabetic, increased significantly ($p<0.001$) with a significant increase in the K_m ($p<0.05$) whereas D+I group showed significant decrease ($p<0.001$) in the activity with no change in K_m when compared to 7 weeks diabetic and D+I groups (Table-6; Fig-6).

Corpus striatum

Acetylcholine esterase activity in the corpus striatum showed a significant increase ($p<0.001$) in both 7 weeks and 90 weeks old diabetic groups ($p<0.01$) with a significant change in K_m ($p<0.05$) when compared to control. In insulin treated diabetics, enzyme activity decreased significantly ($p<0.001$) in 7 weeks and 90 weeks old groups compared to the diabetic groups. In control old rats, V_{max} significantly decreased ($p<0.001$) compared to the young rats with a significant decrease in the K_m ($p<0.05$). V_{max} of 90 weeks old diabetic and D+I decreased significantly ($p<0.001$) with a significant decrease in K_m ($p<0.01$, $p<0.05$) when compared to 7 weeks diabetic and D+I groups respectively (Table-7; Fig-7).

Pancreas

Acetylcholine esterase activity in the pancreas showed a significant decrease in both 7 weeks ($p<0.01$) and 90 weeks ($p<0.05$) old diabetic groups when compared to control. In 7 weeks old diabetic group, K_m was increased ($p<0.001$) significantly

whereas it was decreased significantly in ($p<0.001$) 90 weeks old diabetic groups compared to respective control. In insulin treated diabetics, enzyme activity decreased significantly ($p<0.05$) in 7 weeks and 90 weeks old groups compared to the diabetic groups with a significant decrease in K_m in 7 weeks group ($p<0.001$) whereas the K_m increased in 90 weeks ($p<0.05$) old groups. In control old rats, V_{max} decreased significantly ($p<0.01$) compared to the young rats with no significant change in affinity. V_{max} of 90 weeks old diabetic ($p<0.001$) and D+I group ($p<0.01$) decreased significantly with a significant decrease in K_m in 7 weeks ($p<0.001$) and 90 weeks ($p<0.05$) old groups when compared to 7 weeks diabetic and D+I groups respectively (Table-8; Fig-8).

BRAIN ACETYLCHOLINE RECEPTOR CHANGES IN CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

Cerebral cortex

Acetylcholine receptor analysis

i) Scatchard analysis of [^{3}H]Acetylcholine iodide binding against acetylcholine iodide in the cerebral cortex of 7 weeks and 90 weeks old Control, Diabetic and Insulin treated diabetic rats

Scatchard analysis of [^{3}H]Acetylcholine iodide against acetylcholine iodide in the cerebral cortex showed a significant increase ($p<0.001$) in the B_{max} of 7 weeks and 90 weeks old diabetic rats compared to their respective controls. Insulin treatment significantly reversed ($p<0.001$) the B_{max} to the control with a significant decrease ($p<0.05$) in the affinity in 7 weeks ($p<0.05$) and 90 weeks ($p<0.01$) old rats when compared to diabetic group. In control old rats, B_{max} significantly decreased ($p<0.001$) compared to the young rats with a significant decrease in the K_d ($p<0.05$). B_{max} decreased significantly ($p<0.001$) in 90 weeks old diabetic rats with a significant decrease in K_d ($p<0.01$). Also, in D+I group, B_{max} decreased significantly ($p<0.001$)

with a significant decrease in K_d ($p<0.001$) when compared to 7 weeks old diabetic and D+I groups (Table-9 & Fig-9-11).

ii) Displacement analysis of [^{3}H]Acetylcholine iodide against acetylcholine iodide

The competition curve for [^{3}H]Acetylcholine iodide fitted for one sited model in 7 weeks old control, diabetic and D+I groups with unity as Hill slope value. There was no shift in affinity of the receptor as indicated by the unchanged K_i and log (EC_{50}) in all these groups. The competition curve fitted for one sited model in 90 weeks old rat groups with unity as Hill slope value. Both diabetic and D+I groups show an increased log (EC_{50}) and K_i values compared to that of control showing a shift in affinity from higher to lower state (Table-10, 11 & Fig-12,13).

BRAIN MUSCARINIC RECEPTOR CHANGES IN CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

Cerebral cortex

Muscarinic M1 receptor analysis

i) Scatchard analysis of [^{3}H]QNB binding against pirenzepine in the cerebral cortex of 7 weeks and 90 weeks old Control, Diabetic and Insulin treated diabetic rats

Scatchard analysis of [^{3}H]QNB against pirenzepine in the cerebral cortex showed a significant decrease ($p<0.001$) in the B_{max} of 7 weeks diabetic rats whereas 90 weeks old diabetic rats showed a significant increase in the B_{max} ($p<0.001$) compared to their respective controls. Insulin treatment significantly reversed ($p<0.001$) the B_{max} to the control with a significant increase ($p<0.05$) in the K_d in 7 weeks old rats whereas 90 weeks old rats showed a significant decrease ($p<0.05$) in the K_d when compared to diabetic group. In control old rats, B_{max} significantly decreased ($p<0.001$) compared to the young rats with a significant decrease in the K_d

($p<0.05$). B_{max} increased significantly ($p<0.001$) in 90 weeks old diabetic rats with a significant decrease in K_d ($p<0.01$) whereas in 90 weeks D+I group, B_{max} decreased significantly ($p<0.001$) with a significant decrease in K_d ($p<0.05$) when compared to 7 weeks old diabetic and D+I groups respectively (Table-12& Fig-14-16).

ii) Displacement analysis of [^{3}H]QNB using pirenzepine

The competition curve for [^{3}H]QNB against pirenzepine fitted for one sited model in 7 weeks control group with unity as Hill slope value. The diabetic and D+I groups showed two sited model with Hill slope value away from unity. The log (EC_{50}) and K_i value decreased and an additional low affinity site appeared in 7 weeks diabetic and D+I groups when compared to control. However, an increased log (EC_{50})-1 and $K_{i(H)}$ was observed in 7 weeks D+I group compared to diabetic indicating a shift in high affinity towards lower affinity. The competition curve fitted for one sited model in 90 weeks old control, diabetic and D+I groups with unity as Hill slope value. The K_i and (EC_{50}) value showed no change in diabetic and D+I groups compared to control indicating no shift in affinity (Table-13, 14 & Fig-17, 18).

iii) Real Time-PCR analysis

The Real-Time PCR analysis in the cerebral cortex showed significant decrease ($p<0.001$) in the expression of muscarinic M_1 receptor mRNA in 7 weeks diabetic groups whereas 90 weeks old diabetic groups showed an increased expression ($p<0.001$) when compared to control. Insulin treated diabetics showed decreased expression ($p<0.001$) in 7 weeks group whereas 90 weeks old group showed an increased expression ($p<0.001$) compared to the diabetic. Also, an increased expression ($p<0.001$) of muscarinic M_1 receptor mRNA was observed in 90 weeks old diabetic and D+I groups compared to 7 weeks diabetic and D+I groups respectively (Table-15 & Fig-19a,b).

Muscarinic M3 receptor analysis

i) Scatchard analysis of [^{3}H]DAMP binding against 4-DAMP mustard in the cerebral cortex of 7 weeks and 90 weeks old Control, Diabetic and Insulin treated diabetic rats

Scatchard analysis of [^{3}H]DAMP against 4-DAMP mustard in the cerebral cortex showed a significant increase ($p<0.001$) in the B_{max} of 7 weeks and 90 weeks old diabetic groups with no change in the affinity compared to their respective controls. Insulin treatment significantly reversed ($p<0.001$) the B_{max} to the control in both groups when compared to the diabetic. The control old rats showed no significant change in the B_{max} and K_d compared to the young rats. B_{max} decreased significantly ($p<0.01$, $p<0.05$) in 90 weeks old diabetic and D+I group with no significant change in K_d when compared to 7 weeks diabetic and D+I groups respectively (Table-16 & Fig-20-22).

ii) Displacement analysis of [^{3}H]DAMP using 4-DAMP mustard

The competition curve for [^{3}H]DAMP fitted for one sited model in 7 weeks control, diabetic and D+I groups with unity as Hill slope value. There was no shift in affinity of the receptor as indicated by no change in K_i and log (EC_{50}) in all these groups. The competition curve fitted for one sited model in 90 weeks old rat groups with unity as Hill slope value and no shift in affinity of the receptor was observed as indicated by no change in K_i and log (EC_{50}) values (Table-17,18 & Fig-23,24).

iii) Real Time-PCR analysis

The Real-Time PCR analysis in the cerebral cortex showed a significant increase ($p<0.001$) in the expression of muscarinic M3 receptor mRNA in 7 weeks diabetic groups when compared to control whereas 90 weeks old diabetic groups showed decreased ($p<0.001$) expression compared to control. Insulin treated diabetics showed decreased expression ($p<0.001$) in both 7 weeks and 90 weeks old rat groups

compared to their diabetic groups respectively. Also, decreased expression ($p<0.001$) of muscarinic M3 receptor mRNA was observed in 90 weeks old diabetic and D+I groups compared to 7 weeks old diabetic and D+I groups respectively (Table-19 & Fig- 25a,b).

Brainstem

Muscarinic M1 receptor analysis

i) Scatchard analysis of [^{3}H]QNB binding against pirenzepine in the brainstem of 7 weeks and 90 weeks old Control, Diabetic and Insulin treated diabetic rats

Scatchard analysis of [^{3}H]QNB against pirenzepine in the brainstem showed a significant decrease ($p<0.001$) in the B_{max} of 7 weeks and 90 weeks old diabetic rats compared to their respective controls. K_d showed no significant change in 7 weeks old diabetic rats whereas 90 weeks diabetic rats showed a significant decrease ($p<0.05$) in the K_d compared to their respective controls. Insulin treatment significantly reversed ($p<0.001$) the B_{max} to the control with no significant change in the affinity in 7 weeks whereas 90 weeks old rats showed a significant decrease ($p<0.01$) in the affinity when compared to diabetic group. In control old rats, B_{max} significantly increased ($p<0.001$) compared to the young rats with a significant decrease in the affinity ($p<0.01$). B_{max} and K_d increased significantly ($p<0.001$, $p<0.01$)) in 90 weeks old diabetic and D+I group when compared to 7 weeks diabetic and D+I groups respectively (Table-20 & Fig-26-28).

ii) Displacement analysis of [^{3}H]QNB using pirenzepine

The competition curve for [^{3}H]QNB fitted for two sited model in 7 weeks control, diabetic and D+I groups with Hill slope value away from unity. The competition curve fitted for one sited model in 90 weeks old rat groups with unity as Hill slope value. D+I group show decreased log (EC_{50}) and K_i values compared to

control and diabetic groups showing a shift from lower affinity to further lower affinity state (Table-21,22 & Fig-29,30).

iii) Real Time-PCR analysis

The Real-Time PCR analysis in the brainstem showed a significant decrease in the expression of muscarinic M1 receptor mRNA in 7 weeks diabetic groups ($p<0.001$) when compared to control. An increased M1 receptor expression was observed in 90 weeks old diabetic ($p<0.001$) rats compared to control. Insulin treated diabetics showed an increased expression ($p<0.001$) in both 7 weeks and 90 weeks old groups compared to the diabetic. 90 weeks old diabetic groups showed an increased expression ($p<0.001$) whereas insulin treated diabetic rats showed a decreased expression ($p<0.001$) of muscarinic M1 receptor mRNA compared to 7 weeks diabetic and insulin treated diabetic rats respectively (Table-23 & Fig- 31a,b).

Muscarinic M3 receptor analysis

i) Scatchard analysis of [^{3}H] DAMP binding against 4-DAMP mustard in the brain stem of 7 weeks and 90 weeks old Control, Diabetic and Insulin treated diabetic rats

Scatchard analysis of [^{3}H]DAMP against 4-DAMP mustard in the brainstem showed a significant increase in the B_{max} ($p<0.001$) of 7 weeks and 90 weeks old diabetic groups with a significant decrease ($p<0.05$) in the affinity compared to their respective controls. Insulin treatment significantly reversed ($p<0.001$) the B_{max} to the control with a significant increase ($p<0.05$) in the affinity in both groups when compared to the diabetic. B_{max} showed a significant decrease ($p<0.001$) in control old rats compared to the young rats with no significant change in the affinity. B_{max} and K_d decreased significantly ($p<0.001$, $p<0.05$) in 90 weeks old diabetic and D+I group when compared to 7 weeks diabetic and D+I groups respectively (Table-24 & Fig-32-34).

ii) Displacement analysis of [^{3}H]DAMP using 4-DAMP mustard

The competition curve for [^{3}H]DAMP fitted for two sited model in 7 weeks control, diabetic and D+I groups with Hill slope value away from unity. 7 weeks diabetics showed increased log (EC_{50}) and K_i value indicating shift in affinity from higher affinity state to lower affinity state. The competition curve fitted for two sited model in 90 weeks old rat groups with Hill slope value away from unity. Diabetic groups showed an increased log (EC_{50}) and K_i values compared to control showing a shift in affinity from higher to lower state (Table-25,26 & Fig-35,36).

iii) Real Time-PCR analysis

The Real-Time PCR analysis in the brainstem showed a significant decrease in the expression of muscarinic M3 receptor mRNA in 7 weeks diabetic group ($p<0.001$) when compared to control. An increased expression was observed in 90 weeks old diabetic ($p<0.001$) rats compared to control. 7 weeks insulin treated diabetics rats showed decreased expression ($p<0.001$) whereas 90 weeks old group showed increased expression ($p<0.001$) compared to the diabetic. 90 weeks old diabetic and D+I groups showed increased ($p<0.001$) muscarinic M3 receptor mRNA expression compared to 7 weeks diabetic and D+I groups respectively (Table-27 & Fig-37a,b).

Corpus striatum

Muscarinic M1 receptor analysis

i) Scatchard analysis of [^{3}H] QNB binding against pirenzepine in the corpus striatum of 7 weeks and 90 weeks old Control, Diabetic and Insulin treated diabetic rats

Scatchard analysis of [^{3}H]QNB against pirenzepine in the corpus striatum showed a significant increase ($p<0.001$) in the B_{max} of 7 weeks diabetic group with a significant increase ($p<0.05$) in the K_d compared to control. B_{max} decreased

significantly ($p<0.001$) in 90 weeks old diabetic rats with a significant decrease ($p<0.01$) in the K_d compared to control. Insulin treatment significantly reversed ($p<0.001$) the B_{max} to the control with a significant increase ($p<0.05$) in the affinity in 7 weeks rats whereas 90 weeks old rats showed a significant decrease ($p<0.01$) in the affinity when compared to diabetic group. An increased B_{max} ($p<0.001$) was observed in control old rats compared to the young rats with a significant decrease ($p<0.01$) in the affinity. B_{max} decreased significantly ($p<0.001$) in 90 weeks old diabetic rats with a significant decrease in K_d ($p<0.01$) when compared to 7 weeks diabetic group. 90 weeks old D+I group showed a significant increase in the B_{max}($p<0.01$) and K_d ($p<0.01$) compared to 7 weeks D+I group (Table-28 & Fig-38-40).

ii) Displacement analysis of [^{3}H]QNB using pirenzepine

The competition curve for [^{3}H]QNB fitted for two sited model in 7 weeks control, diabetic and D+I groups with Hill slope value away from unity. 7 weeks diabetics showed increased log (EC_{50}) and K_i value indicating shift in affinity from higher affinity state to lower affinity state. The competition curve fitted for one sited model in 90 weeks old control, diabetic and insulin treated diabetic groups with unity as Hill slope value and no shift in affinity of the receptor was observed as indicated by unchanged K_i and log (EC_{50}) values (Table-29,30 & Fig-41,42).

iii) Real Time-PCR analysis

The Real-Time PCR analysis in the corpus striatum showed that muscarinic M1 receptor mRNA decreased significantly ($p<0.001$) in 7 weeks and 90 weeks old diabetic groups when compared to respective control. Insulin treated diabetics showed a decreased expression ($p<0.001$) in 7 weeks group whereas an increased expression ($p<0.001$) was observed in 90 weeks old group compared to the diabetic. Also, an increased expression of muscarinic M1 receptor mRNA was observed in 90 weeks old

diabetic ($p<0.001$) and D+I ($p<0.001$) groups compared to 7 weeks diabetic and D+I groups respectively (Table-31 & Fig-43a,b).

Muscarinic M3 receptor analysis

i) Scatchard analysis of [^{3}H] DAMP binding against 4-DAMP mustard in the corpus striatum of 7 weeks and 90 weeks old Control, Diabetic and Insulin treated diabetic rats

Scatchard analysis of [^{3}H]DAMP against 4-DAMP mustard in the corpus striatum showed a significant decrease ($p<0.001$) in the B_{max} of 7 weeks diabetic groups whereas B_{max} increased significantly ($p<0.001$) in 90 weeks old diabetic groups with a significant decrease in the affinity ($p<0.01$) compared to control. Insulin treatment significantly reversed ($p<0.001$) the B_{max} to the control when compared to the diabetic. B_{max} showed a significant decrease ($p<0.001$) in control old rats compared to the young rats with a significant increase ($p<0.001$) in the affinity. B_{max} and K_d decreased significantly ($p<0.001$, $p<0.01$) in 90 weeks old diabetic and D+I group when compared to 7 weeks diabetic and D+I groups respectively (Table-32 & Fig-44-46).

ii) Displacement analysis of [^{3}H]DAMP using 4-DAMP mustard

The competition curve for [^{3}H]DAMP fitted for one sited model in 7 weeks control, diabetic and D+I groups with unity as Hill slope value. There was no shift in affinity of the receptor as indicated by no change in K_i and log (EC_{50}) in all these groups. The competition curve fitted for one sited model in 90 weeks old rat groups with unity as Hill slope value. Also, there was no change in log(EC_{50}) and K_i values compared to that of control showing no shift in affinity of the receptor (Table-33,34 & Fig-47,48).

iii) Real Time-PCR analysis

The Real-Time PCR analysis in the corpus striatum showed that muscarinic M3 receptor mRNA decreased significantly ($p<0.001$) in both 7 weeks and 90 weeks old diabetic groups when compared to control. Insulin treated diabetics showed a decreased expression ($p<0.001$) in 7 weeks group whereas an increased expression ($p<0.001$) was observed in 90 weeks old group compared to the diabetic. Also, decreased expression of muscarinic M3 receptor mRNA was observed in 90 weeks old diabetic ($p<0.001$) whereas D+I group showed increased expression ($p<0.001$) compared to 7 weeks diabetic and D+I groups respectively (Table-35 & Fig-49a,b).

PANCREATIC MUSCARINIC RECEPTOR CHANGES IN CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

Muscarinic M3 receptor analysis

i) Scatchard analysis of [^{3}H]DAMP binding against 4-DAMP mustard in the pancreas of 7 weeks and 90 weeks old control, Diabetic and Insulin treated diabetic rats

Scatchard analysis of [^{3}H]DAMP against 4-DAMP mustard in the pancreas showed a significant increase ($p<0.001$) in the B_{max} of both 7 weeks and 90 weeks old diabetic groups with a significant increase in the affinity ($p<0.05$) compared to control. Insulin treatment significantly increase ($p<0.001$) the B_{max} in both 7 weeks and 90 weeks old groups when compared to the diabetic. B_{max} showed a significant decrease ($p<0.001$) in control old rats compared to the young rats with no significant change in the affinity. B_{max} decreased significantly ($p<0.001$) in 90 weeks old diabetic and D+I group with a significant increase in affinity ($p<0.01$) when compared to 7 weeks diabetic and D+I groups respectively (Table-36 & Fig-50-52).

ii) Real Time-PCR analysis

The Real-Time PCR analysis in the pancreas showed that muscarinic M3 receptor mRNA decreased significantly ($p<0.001$) in both 7 weeks and 90 weeks old diabetic groups when compared to control. Insulin treated diabetics showed a decreased expression ($p<0.001$) in 7 weeks group whereas an increased expression ($p<0.001$) was observed in 90 weeks old group compared to the diabetic. Also, decreased expression of muscarinic M3 receptor mRNA was observed in 90 weeks old diabetic ($p<0.001$) whereas D+I group showed increased expression ($p<0.001$) compared to 7 weeks diabetic and D+I groups respectively (Table-37 & Fig-53a,b).

GENE EXPRESSION STUDIES OF GLUTAMATE RECEPTOR, NMDAR1 IN DIFFERENT BRAIN REGIONS OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

Cerebral cortex

Real-Time PCR analysis in the cerebral cortex showed that NMDAR1 receptor mRNA decreased significantly ($p<0.001$) in 7 weeks diabetic group when compared to control. There was an increased expression ($p<0.001$) in 90 weeks old diabetic group compared to control. Insulin treated diabetics showed a decreased expression ($p<0.001$) in 7 weeks group whereas an increased expression ($p<0.001$) was observed in 90 weeks old group compared to the diabetic. An increased NMDAR1 receptor mRNA expression was observed in 90 weeks old diabetic ($p<0.001$) and D+I ($p<0.001$) groups compared to 7 weeks diabetic and D+I groups respectively (Table-38 & Fig-54a,b).

Brainstem

Real-Time PCR analysis in the brainstem showed that NMDAR1 receptor mRNA increased significantly ($p<0.001$) in 7 weeks diabetic group when compared to

control. There was an increased expression ($p<0.001$) in 90 weeks old diabetic group compared to control. Insulin treated diabetics showed increased expression ($p<0.001$) in 7 weeks group whereas decreased expression ($p<0.001$) was observed in 90 weeks old group compared to the diabetic. The NMDAR1 receptor mRNA expression was increased in 90 weeks old diabetic ($p<0.001$) and decreased in D+I ($p<0.001$) groups compared to 7 weeks diabetic and D+I groups respectively (Table-39 & Fig-55a,b).

Corpus striatum

Real-Time PCR analysis in the corpus striatum showed that NMDAR1 receptor mRNA decreased significantly ($p<0.001$) in 7 weeks and 90 weeks old diabetic group when compared to control. Decreased expression ($p<0.001$) was observed in 7 weeks insulin treated diabetic group whereas it was increased ($p<0.001$) in 90 weeks old group compared to diabetic. In 90 weeks old diabetic ($p<0.001$) and D+I ($p<0.001$) groups, NMDAR1 receptor mRNA expression was increased compared to 7 weeks diabetic and D+I groups respectively (Table-40 & Fig-56a,b).

Hippocampus

Real-Time PCR analysis in the hippocampus showed that NMDAR1 receptor mRNA decreased significantly ($p<0.001$) in 7 weeks and 90 weeks old diabetic groups when compared to control groups. Insulin treated diabetics showed increased expression ($p<0.001$) in both 7 weeks and 90 weeks old group compared to the diabetic. NMDAR1 receptor mRNA expression was decreased in 90 weeks old diabetic group ($p<0.001$) and decreased in D+I ($p<0.001$) group compared to 7 weeks diabetic and D+I groups respectively (Table-41& Fig-57a,b).

GENE EXPRESSION STUDIES OF GLUTAMATE RECEPTOR, mGlu-5 IN DIFFERENT BRAIN REGIONS OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

Cerebral cortex

Real-Time PCR analysis in the cerebral cortex showed that glutamate receptor (mGlu-5) mRNA in the cerebral cortex was increased significantly ($p<0.001$) in 7 weeks diabetic rats compared to control. 90 weeks old diabetic rats showed decreased expression ($p<0.001$) compared to control. Also, the expression was decreased significantly in 90 weeks old diabetic rats ($p<0.001$) whereas an increased mGlu-5 receptor mRNA expression ($p<0.001$) was observed in D+I groups when compared to 7 weeks diabetic and D+I groups respectively (Table-42 & Fig-58a,b).

Brainstem

Real-Time PCR analysis in the brainstem showed that mGlu-5 mRNA expression was significantly decreased ($p<0.001$) in 7 weeks diabetic group whereas 90 weeks old diabetic rats showed an increased expression ($p<0.001$) compared to respective controls. Also, an increased expression of mGlu-5 receptor mRNA was observed in 90 weeks old diabetic ($p<0.001$) whereas D+I groups showed decreased expression ($p<0.001$) compared to 7 weeks diabetic and D+I groups respectively (Table-43 & Fig-59a,b).

Corpus striatum

Real-Time PCR analysis in the corpus striatum showed that mGlu-5 mRNA expression decreased significantly ($p<0.001$) in 7 weeks and 90 weeks old diabetic groups when compared to control. In 90 weeks old diabetic group, a significant decrease ($p<0.001$) in the expression was observed whereas D+I group showed an

increased mGlu-5 receptor mRNA expression ($p<0.001$) compared to 7 weeks diabetic and D+I groups respectively (Table-44 & Fig-60a,b).

Hippocampus

Real-Time PCR analysis in the hippocampus showed that mGlu-5 receptor mRNA expression significantly decreased ($p<0.001$) in 7 weeks and 90 weeks old diabetic groups compared to control. 7 weeks D+I groups showed significant increase in the expression ($p<0.001$) whereas it was decreased in 90 weeks D+I groups ($p<0.001$) when compared to diabetic. Also, a significant decrease in mGlu-5 receptor mRNA expression was observed in 90 weeks old diabetic ($p<0.001$) and D+I ($p<0.001$) groups compared to 7 weeks diabetic and D+I groups respectively (Table-45 & Fig-61a,b).

DA D2 RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

DA D2 receptor gene expression in the cerebral cortex was increased significantly ($p<0.001$) in both 7 weeks and 90 weeks old diabetic rat groups compared to control. Insulin treated diabetics showed decreased expression ($p<0.001$) in 7 weeks group whereas it was increased ($p<0.001$) in 90 weeks old rat group compared to diabetic groups. Also, an increased DAD2 receptor mRNA expression ($p<0.001$) was observed in both 90 weeks old diabetic and D+I groups compared to 7 weeks diabetic and D+I groups respectively (Table-46 & Fig-62a,b).

α_{2A}-ADRENERGIC RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

α_{2A}-adrenergic receptor gene expression in the cerebral cortex was decreased significantly ($p<0.001$) in 7 weeks rat groups whereas the expression increased significantly ($p<0.001$) in 90 weeks old rat groups compared to control. Insulin treated

diabetics showed increased expression ($p<0.001$) in both 7 weeks and 90 weeks old group compared to diabetic groups. Also, an increased α_{2A}.adrenergic receptor mRNA expression ($p<0.001$) was observed in both 90 weeks old diabetic and D+I groups compared to 7 weeks diabetic and D+I groups respectively (Table-47 & Fig-63a,b).

β_2-ADRENERGIC RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

β_2-adrenergic receptor gene expression in the cerebral cortex was increased significantly ($p<0.001$) in 7 weeks diabetic groups whereas it was decreased in 90 weeks old diabetic groups compared to control. Insulin treated diabetics showed decreased expression ($p<0.001$) in both 7 weeks and 90 weeks old rat groups compared to diabetic groups. Also, decreased β_2-adrenergic receptor mRNA expression ($p<0.001$) was observed in 90 weeks old diabetic and D+I groups compared to 7 weeks diabetic and D+I groups respectively (Table-48 & Fig-64a,b).

$GABA_{A\alpha1}$ RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

$GABA_{A\alpha1}$ receptor gene expression in the cerebral cortex was decreased significantly ($p<0.001$) in both 7 weeks and 90 weeks old diabetic groups compared to control. Insulin treated diabetics showed decreased expression ($p<0.001$) in 7 weeks old rats whereas it increased significantly ($p<0.001$) in 90 weeks old rat groups compared to diabetic groups respectively. In 90 weeks old diabetic group, a significant decrease ($p<0.001$) in the expression was observed whereas D+I group showed an increased $GABA_{A\alpha1}$ receptor mRNA expression ($p<0.001$) compared to 7 weeks diabetic and D+I groups respectively (Table-49 & Fig-65a,b).

$GABA_B$ RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

$GABA_B$ receptor gene expression in the cerebral cortex was decreased significantly ($p<0.001$) in both 7 weeks and 90 weeks old diabetic groups compared to control. Insulin treated diabetics showed decreased expression ($p<0.001$) in both 7 weeks and 90 weeks old rat groups compared to diabetic groups. In 90 weeks old diabetic group, a significant decrease ($p<0.001$) in the expression was observed whereas D+I group showed an increased $GABA_B$ receptor mRNA expression ($p<0.01$) compared to 7 weeks diabetic and D+I groups respectively (Table-50 & Fig-66a,b).

5-HT_{2C} RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

5-HT_{2C} receptor gene expression in the cerebral cortex was decreased significantly ($p<0.05$) in 7 weeks rat groups whereas the expression increased significantly ($p<0.001$) in 90 weeks old rat groups compared to control. Insulin treated diabetics showed decreased expression ($p<0.001$) in 7 weeks group whereas it increased significantly ($p<0.001$) in 90 weeks old group compared to diabetic groups. Also, an increased 5-HT_{2C} receptor mRNA expression ($p<0.001$) was observed in both 90 weeks old diabetic and D+I groups compared to 7 weeks diabetic and D+I groups respectively (Table-51& Fig-67a,b).

IP3 CONTENT IN DIFFERENT BRAIN REGIONS OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

Cerebral cortex

IP3 content in the cerebral cortex was increased significantly ($p<0.001$) in 7 weeks diabetic groups whereas it decreased significantly ($p<0.001$) in 90 weeks old diabetic groups compared to respective controls. Insulin treatment significantly

reversed ($p<0.001$) the IP3 content near to control. In control old rats, IP3 content increased significantly ($p<0.001$) compared to the young rats. In 90 weeks old diabetic group, we observed decreased IP3 content ($p<0.001$) whereas D+I group showed an increased IP3 content ($p<0.001$) compared to 7 weeks diabetic and D+I groups respectively (Table-52 & Fig-68).

Brainstem

IP3 content in the brainstem was increased significantly ($p<0.001$) in 7 weeks diabetic groups whereas it decreased significantly ($p<0.001$) in 90 weeks old diabetic groups compared to control. Insulin treatment significantly reversed ($p<0.001$) the IP3 content near to control. In control old rats, IP3 content increased significantly ($p<0.001$) compared to the young rats. In 90 weeks old diabetic group, we observed decreased IP3 content ($p<0.001$) whereas D+I group showed an increased IP3 content ($p<0.001$) compared to 7 weeks diabetic and D+I groups respectively (Table-53 & Fig-69).

Corpus striatum

IP3 content in the corpus striatum was increased significantly ($p<0.001$) in 7 weeks diabetic groups whereas it decreased significantly ($p<0.001$) in 90 weeks old diabetic groups compared to respective control. Insulin treatment significantly reversed ($p<0.001$) the IP3 content near to control. In control old rats, IP3 content increased significantly ($p<0.001$) compared to the young rats. In 90 weeks old diabetic group, we observed decreased IP3 content ($p<0.001$) whereas D+I group showed an increased IP3 content ($p<0.001$) compared to 7 weeks diabetic and D+I groups respectively (Table-54 & Fig-70).

IP3 content in the pancreas was increased significantly in both 7 weeks ($p<0.001$) and 90 weeks ($p<0.0$ 1) old diabetic groups compared to control. Insulin treatment significantly reversed the increased IP3 content in 7 weeks ($p<0.001$) and 90 weeks ($p<0.01$) old diabetic groups near to control. In control old rats, IP3 content decreased significantly ($p<0.001$) compared to the young rats. Also, 90 weeks old diabetic and D+I groups showed decreased IP3 content ($p<0.001$) compared to 7 weeks diabetic and D+I groups respectively (Table-55 & Fig-71).

cGMP CONTENT IN DIFFERENT BRAIN REGIONS OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

Cerebral cortex

cGMP content in the cerebral cortex was increased significantly ($p<0.001$) in 7 weeks diabetic groups whereas it decreased significantly ($p<0.001$) in 90 weeks old diabetic groups compared to control. Insulin treatment significantly reversed ($p<0.001$) the cGMP content near to control. In control old rats, cGMP content increased significantly ($p<0.001$) compared to the young rats. Also, 90 weeks old diabetic and D+I groups showed increased cGMP content ($p<0.001$) compared to 7 weeks diabetic and D+I groups respectively (Table-56 & Fig-72).

Brainstem

cGMP content in the brainstem was increased significantly ($p<0.001$) in 7 weeks diabetic groups whereas it decreased significantly ($p<0.001$) in 90 weeks old diabetic groups compared to control. Insulin treatment significantly reversed ($p<0.001$) the cGMP content near to control. In control old rats, cGMP content increased significantly ($p<0.01$) compared to the young rats. In 90 weeks old diabetic

and D+I group, we observed decreased cGMP content ($p<0.001$), ($p<0.05$) compared to 7 weeks diabetic and D+I groups respectively (Table-57 & Fig-73).

Corpus striatum

cGMP content in the corpus striatum was increased significantly ($p<0.001$) in both 7 weeks and 90 weeks old diabetic groups compared to control. Insulin treatment significantly reversed ($p<0.001$) the increased cGMP content near to control. In control old rats, cGMP content increased significantly ($p<0.001$) compared to the young rats. Also, 90 weeks old diabetic and D+I groups showed increased cGMP content ($p<0.001$) compared to 7 weeks diabetic and D+I groups respectively (Table-58 & Fig-74).

Pancreas

cGMP content in the pancreas was increased significantly ($p<0.001$) in both 7 weeks and 90 weeks old diabetic groups compared to control. Insulin treatment significantly reversed ($p<0.001$) the increased cGMP content near to control. In control old rats, cGMP content showed no significant change compared to the young rats. 90 weeks old diabetic and D+I groups showed decreased cGMP content ($p<0.001$), ($p<0.05$) compared to 7 weeks diabetic and D+I groups respectively (Table-59 & Fig-75).

INSULIN SECRETION STUDIES IN PANCREATIC ISLETS

EFFECT OF CARBACHOL AND MUSCARINIC ANTAGONISTS ON INSULIN SECRETION IN ONE HOUR PANCREATIC ISLETS *IN VITRO*

The isolated islets from 7 weeks and 90 weeks old rats were incubated for one hour with carbachol and two different concentrations of glucose, 4mM and 20mM. carbachol at 10^{-7} M significantly increased glucose induced insulin secretion

in both 7 weeks ($p<0.001$) and 90 weeks ($p<0.001$) old rats at 4mM and 20mM glucose concentrations. Stimulatory effect of carbachol on glucose-induced insulin secretion was reversed by the addition of muscarinic M1 and M3 receptor antagonists such as pirenzepine and 4-DAMP mustard respectively. Pirenzepine at 10^{-6}M, inhibited carbachol induced insulin secretion in 90 weeks rats ($p<0.001$) whereas 4-DAMP mustard at 10^{-6}M inhibited the insulin secretion in 7 weeks old rats ($p<0.001$) at 4mM glucose concentration. In the presence of high glucose concentration (20mM), carbachol induced insulin secretion was inhibited by pirenzepine in 7 weeks ($p<0.001$) and 4-DAMP mustard in 90 weeks ($p<0.001$) old rats respectively (Fig-76,77).

EFFECT OF CARBACHOL AND MUSCARINIC ANTAGONISTS ON IP3 LEVELS IN ONE HOUR PANCREATIC ISLETS *IN VITRO*

Carbachol, the cholinergic agonist, at 10^{-7}M significantly increased glucose induced IP3 release in both 7 weeks ($p<0.001$) and 90 weeks ($p<0.001$) old rats at 4mM and 20mM glucose concentrations. Pirenzepine, the M1 receptor antagonist at 10^{-6}M concentration inhibited carbachol induced IP3 release in both 7 weeks and 90 weeks ($p<0.001$) old rats at 4mM glucose concentration. In the presence of high glucose concentration (20mM), carbachol induced IP3 release was inhibited by pirenzepine in 7 weeks rats whereas 4-DAMP mustard at 10^{-6}M inhibited the IP3 release in 90 weeks old rats (Fig-78, 79).

Dopamine at 10^{-4}M significantly increased glucose induced IP3 release in both 7 weeks ($p<0.001$) and 90 weeks ($p<0.001$) old rats at 4mM and 20mM glucose concentrations. Carbachol (10^{-7}M) in combination with dopamine at 10^{-4}M significantly increased glucose induced IP3 release in 7 weeks at 20mM glucose concentrations ($p<0.001$) and 90 weeks ($p<0.001$) old rats at 4mM glucose concentrations (Fig-80,81).

EFFECT OF CARBACHOL AND MUSCARINIC ANTAGONISTS ON cGMP LEVELS IN ONE HOUR PANCREATIC ISLETS *IN VITRO*

Carbachol, at 10^{-7}M concentration significantly ($p<0.001$) stimulated cGMP release in the pancreatic islets of young rats. Stimulatory effect of carbachol in cGMP release was reversed by pirenzepine at 10^{-6}M concentration in the presence of both 4 mM and 20 mM glucose in young rats. In old rats, carbachol induced cGMP release was inhibited ($p<0.001$) by pirenzepine in 4mM glucose whereas it was inhibited ($p<0.001$) by 4-DAMP mustard in 20mM glucose concentration (Fig-82, 83).

Dopamine at 10^{-8}M significantly increased glucose induced cGMP release in both 7 weeks and 90 weeks ($p<0.001$) old rats at 4mM ($p<0.001$) and 20mM ($p<0.01$) glucose concentrations. Carbachol (10^{-7}M) in combination with dopamine at 10^{-4}M significantly increased glucose induced cGMP release in 7 weeks at 20mM glucose concentrations ($p<0.001$).In 90 weeks old rats, carbachol (10^{-7}M) in combination with dopamine at 10^{-8}M significantly increased ($p<0.001$) glucose induced cGMP release at 4mM and 20mM glucose concentrations (Fig-84,85).

TRIIODOTHYRONINE (T3) CONTENT IN SERUM OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

T3 content in the serum was increased significantly ($p<0.001$) in 7 weeks and 90 weeks old diabetic groups compared to control. Insulin treatment significantly reversed ($p<0.001$) the increased T3 content near to control. In control old rats, T3 content decreased significantly ($p<0.001$) compared to the young rats. Also, 90 weeks old diabetic and D+I groups showed decreased T3 content ($p<0.001$) compared to 7 weeks diabetic and D+I groups respectively (Table-60 & Fig-86).

NE, EPI, DA AND 5-HT CONTENT IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

Cerebral cortex

NE content decreased significantly in the cerebral cortex of STH treated ($p<0.001$) and INS treated ($p<0.001$) young rats when compared to saline treated rats. In old rats, NE content showed no change in STH treated rats whereas INS treated rats showed significant decrease ($p<0.001$) when compared to saline treated rats. NE content decreased significantly ($p<0.001$) in saline treated old rats compared to young rats. STH treated old rats showed no significant change in the NE content compared to young rats. However, insulin treated old rats showed significant increase ($p<0.001$) in NE content compared to young rats.

EPI content showed significant decrease in both STH treated rats ($p<0.05$) and INS treated ($p<0.001$) young rats when compared to saline treated young rats. In old rats, EPI content decreased significantly in both STH treated ($p<0.001$) and INS treated rats ($p<0.001$) when compared to saline treated rats. EPI content decreased significantly ($p<0.05$) in saline treated old rats compared to young rats. STH treated groups showed significant decrease ($p<0.001$) whereas INS treated groups showed significant increase ($p<0.001$) in the EPI content in old rats compared to young rats.

DA content in the cerebral cortex of young rats decreased significantly in both STH treated ($p<0.001$) and INS treated rats ($p<0.001$) compared to saline treated rats. STH treated and INS treated rats old rats showed significant decrease in DA content ($p<0.05$), ($p<0.001$) when compared to saline treated rats. DA content decreased significantly ($p<0.001$) in saline treated old rats compared to young rats. STH treated old rats showed significant decrease ($p<0.001$) in DA content compared to young rats. However, insulin treated old rats showed no significant change in the DA content compared to young rats.

5-HT content decreased significantly in both STH treated ($p<0.001$) and INS treated young rats ($p<0.001$) compared to saline treated rats. In old rats, 5-HT content increased in STH treated rats ($p<0.05$) whereas INS treated rats showed significant decrease ($p<0.001$) in the 5-HT content when compared to saline treated rats. 5-HT content decreased significantly ($p<0.001$) in saline treated old rats compared to young rats. STH treated old rats showed no significant change in 5-HT content compared to young rats. Insulin treated old rats showed significant increase ($p<0.01$) in 5-HT content compared to young rats (Table-61).

Corpus striatum

NE content showed no significant change in the corpus striatum of both STH treated and INS treated young rats when compared to saline treated rats. In old rats, NE content increased significantly in both STH treated ($p<0.001$) and INS treated rats ($p<0.001$) when compared to saline treated rats. NE content decreased significantly ($p<0.001$) in saline treated old rats compared to young rats. STH treated and insulin treated old rats showed significant decrease in the NE content ($p<0.05$) compared to young rats.

EPI content increased significantly in both STH treated ($p<0.001$) and in INS treated young rats ($p<0.001$) compared to saline treated rats. STH treated old rats showed significant decrease in the EPI content ($p<0.05$) whereas INS treated rats showed significant increase ($p<0.001$) when compared to saline treated rats. EPI content decreased significantly in saline treated ($p<0.001$), STH treated ($p<0.001$) and INS treated old rats ($p<0.001$) compared to young rats.

DA content in the corpus striatum of young rats decreased significantly in both STH treated ($p<0.001$) and INS treated rats ($p<0.001$) compared to saline treated rats. STH treated old rats showed no significant change in DA content whereas INS treated rats showed significant increase ($p<0.001$) when compared to saline treated rats. DA content decreased significantly ($p<0.001$) in saline treated old rats compared

to young rats. Also, STH treated and INS treated old rats showed significant decrease ($p<0.001$) in DA content compared to young rats respectively.

5-HT content increased significantly in STH treated ($p<0.01$) whereas INS treated young rats showed no significant change compared to saline treated rats. STH treated old rats showed no significant change in 5-HT content whereas INS treated rats showed significant increase ($p<0.01$) when compared to saline treated rats. 5-HT content decreased significantly ($p<0.001$) in saline treated old rats compared to young rats. Also, STH treated and Insulin treated old rats showed significant decrease ($p<0.001$) in 5-HT content compared to young rats (Table-62).

Brainstem

NE content decreased significantly in the brainstem of STH treated young rats ($p<0.001$) whereas INS treated young rats showed no significant change compared to saline treated rats. In old rats, NE content decreased significantly in both STH treated ($p<0.001$) and INS treated rats ($p<0.01$) compared to saline treated rats. NE content decreased significantly ($p<0.01$) in saline treated old rats compared to young rats. NE content decreased significantly in both STH treated ($p<0.001$) and INS treated old rats ($p<0.001$) compared to young rats.

EPI content decreased significantly in both STH treated ($p<0.001$) and INS treated young rats ($p<0.001$) compared to saline treated young rats respectively. EPI content decreased significantly in STH treated old rats ($p<0.001$) whereas INS treated old rats showed no significant change when compared to saline treated old rats. EPI content decreased significantly ($p<0.001$) in saline treated old rats compared to young rats. STH treated old rats showed significant decrease ($p<0.001$) in the EPI content whereas INS treated rats showed no significant change compared to young rats.

DA content in the brainstem decreased significantly in both STH treated ($p<0.001$) and INS treated young rats ($p<0.001$) compared to saline treated young rats. STH treated old rats showed significant decrease ($p<0.001$) in DA content whereas

INS treated rats showed significant increase ($p<0.001$) when compared to saline treated rats. DA content decreased significantly ($p<0.001$) in saline treated old rats compared to young rats. STH treated old rats showed significant decrease ($p<0.001$) in DA content compared to young rats. However, insulin treated old rats showed no significant change in the DA content compared to young rats.

5-HT content decreased significantly in both STH treated ($p<0.05$) and INS treated young rats ($p<0.05$) compared to saline treated rats. STH treated old rats showed significant decrease ($p<0.001$) in 5-HT content whereas INS treated rats showed no significant change when compared to saline treated old rats. Saline treated old rats showed no significant change in 5-HT content compared to young rats. 5-HT content decreased significantly ($p<0.001$) in STH treated old rats compared to young rats. Insulin treated old rats showed significant increase ($p<0.01$) in 5-HT content compared to young rats (Table-63).

Hypothalamus

NE content in the hypothalamus of both STH treated and INS treated young rats decreased significantly ($p<0.001$) when compared to saline treated young rats. However, in both STH treated and INS treated old rats, NE content decreased significantly ($p<0.001$) when compared to saline treated old rats. NE content decreased significantly ($p<0.001$) in saline treated old rats compared to young rats. STH treated old rats showed significant decrease ($p<0.001$) in NE content compared to young rats. However, NE content increased significantly ($p<0.05$) in insulin treated old rats compared to young rats.

EPI content decreased significantly in both STH treated ($p<0.001$) and in INS treated young rats ($p<0.001$) compared to saline treated rats. Both STH treated and INS treated old rats showed significant increase in the EPI content ($p<0.001$) when compared to saline treated rats. EPI content decreased significantly in saline treated

($p<0.001$) and STH treated ($p<0.001$) old rats whereas in INS treated old rats EPI content increased significantly ($p<0.001$) compared to young rats.

DA content in the hypothalamus of young rats decreased significantly in both STH treated ($p<0.001$) and INS treated rats ($p<0.001$) compared to saline treated rats. However, in both STH treated and INS treated old rats, DA content increased significantly ($p<0.001$) when compared to saline treated old rats. DA content decreased significantly ($p<0.001$) in saline treated old rats compared to young rats. DA content decreased significantly in STH treated ($p<0.05$) old rats whereas in INS treated old rats DA content increased significantly ($p<0.001$) compared to young rats.

5-HT content decreased significantly in both STH treated ($p<0.001$) and INS treated young rats ($p<0.001$) when compared to saline treated young rats. However, both STH treated and INS treated old rats showed significant increase ($p<0.001$) in 5-HT content when compared to saline treated old rats. 5-HT content decreased significantly ($p<0.001$) in saline treated old rats compared to young rats. STH treated and Insulin treated old rats showed no significant change in 5-HT content compared to young rats (Table-64)

ACETYLCHOLINE ESTERASE ACTIVITY IN CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

Acetylcholine esterase activity in the cerebral cortex of STH treated rats increased significantly ($p<0.001$) with a significant decrease in the K_m ($p<0.01$) when compared to saline treated young rats. STH treated and INS treated old rats showed significant increase ($p<0.001$) in the enzyme activity with a significant decrease in the K_m ($p<0.01$) when compared to saline treated old rats. There was significant decrease ($p<0.001$) in AChE activity of saline treated old rats compared to saline treated young rats. Both STH treated INS treated old rats showed significant decrease ($p<0.001$) in AChE activity with a significant decrease in the K_m ($p<0.01$) when compared to young rats respectively (Table-65; Fig-87).

GENE EXPRESSION STUDIES

MUSCARINIC M1 RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

The Real-Time PCR analysis in the cerebral cortex showed a significant increase in the expression of muscarinic M1 receptor mRNA in both STH treated ($p<0.001$) and INS treated young rats ($p<0.001$) when compared to saline treated young rats. However, both STH treated and INS treated old rats showed significant decrease ($p<0.001$) in expression when compared to saline treated old rats. Also, STH treated and Insulin treated old rats showed significant decrease ($p<0.001$) in mRNA expression compared to young rats. INS treated rats showed increased ($p<0.001$) expressional status for M1 receptor mRNA with that of STH treated rats (Table-66 & Fig-88a,b).

MUSCARINIC M3 RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

Muscarinic M3 receptor mRNA expression increased significantly in the cerebral cortex of both STH treated ($p<0.001$) and INS treated young rats ($p<0.001$) when compared to saline treated young rats. However, both STH treated and INS treated old rats showed decreased ($p<0.001$) expression when compared to saline treated old rats. STH treated and Insulin treated old rats showed significant decrease ($p<0.001$) in mRNA expression compared to young rats (Table-67 & Fig-89a,b).

GLUTAMATE RECEPTOR (NMDAR1) GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

Glutamate receptor (NMDAR1) gene expression in the cerebral cortex increased significantly in both STH treated ($p<0.001$) and INS treated young rats ($p<0.001$) when compared to saline treated young rats. However, both STH treated

and INS treated old rats showed significant decrease ($p<0.001$) in NMDAR1 expression when compared to saline treated old rats. STH treated and Insulin treated old rats showed significant decrease ($p<0.001$) in mRNA expression compared to young rats. INS treated rats showed increased ($p<0.001$) expressional status for NMDAR1 receptor mRNA with that of STH treated rats (Table-68 & Fig-90a,b).

GLUTAMATE RECEPTOR (mGlu-5) GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

Glutamate receptor (mGlu-5) gene expression in the cerebral cortex increased significantly in both STH treated ($p<0.001$) and INS treated young rats ($p<0.001$) when compared to saline treated young rats. However, mGlu-5 gene expression decreased significantly ($p<0.001$)in both STH treated and INS treated old rats when compared to saline treated old rats. STH treated and Insulin treated old rats showed significant decrease ($p<0.001$) in mRNA expression compared to young rats. INS treated rats showed increased expressional status for mGlu-5 receptor mRNA with that of STH treated rats (Table-69 & Fig-91a,b).

DA D2 RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

DA D2 receptor gene expression in the cerebral cortex increased significantly in both STH treated ($p<0.001$) and INS treated young rats ($p<0.001$) when compared to saline treated young rats. However, both STH treated and INS treated old rats showed significant decrease ($p<0.001$) in D2 expression when compared to saline treated old rats. Also, STH treated and INS treated old rats showed significant decrease ($p<0.001$) in D2 expression compared to young rats (Table-70 & Fig-92a,b).

α_{2A}-ADRENERGIC RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

α_{2A}-adrenergic receptor gene expression in the cerebral cortex increased significantly in both STH treated ($p<0.001$) and INS treated young rats ($p<0.001$) compared to saline treated young rats. However, the gene expression decreased significantly ($p<0.001$) in both STH treated and INS treated old rats when compared to saline treated old rats. STH treated and INS treated old rats showed significant decrease ($p<0.001$) in mRNA expression compared to young rats (Table-71 & Fig-93a, b).

β_2-ADRENERGIC RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

β_2-adrenergic receptor gene expression in the cerebral cortex increased significantly in STH treated young rats ($p<0.05$) whereas INS treated young rats showed decreased expression ($p<0.001$) compared to saline treated young rats. However, the gene expression decreased significantly ($p<0.001$) in STH treated old rats whereas in INS treated old rats β_2-adrenergic receptor gene expression increased significantly ($p<0.001$) when compared to saline treated old rats. STH treated old rats showed significant decrease ($p<0.001$) in mRNA expression while in INS treated old rats, the expression increased significantly ($p<0.001$) compared to young rats (Table-72 & Fig-94a,b).

$GABA_{A\alpha1}$ RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

$GABA_{A\alpha1}$ receptor gene expression in the cerebral cortex increased significantly in STH treated young rats ($p<0.05$) whereas INS treated young rats showed decreased expression ($p<0.001$) compared to saline treated young rats. However, the gene expression decreased significantly in both STH treated ($p<0.001$)

and INS treated old rats ($p<0.05$) when compared to saline treated old rats. STH treated old rats showed significant decrease ($p<0.001$) in mRNA expression while INS treated old rats showed increased expression ($p<0.001$) compared to young rats (Table-73 & Fig-95a,b).

$GABA_B$ RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

$GABA_B$ receptor gene expression in the cerebral cortex decreased significantly in STH treated young rats ($p<0.001$) whereas INS treated young rats showed increased expression ($p<0.01$) compared to saline treated young rats. However, the gene expression decreased significantly in both STH treated ($p<0.001$) and INS treated old rats ($p<0.01$) when compared to saline treated old rats. Both STH treated INS treated old rats showed decreased ($p<0.001$) $GABA_B$ receptor gene expression compared to young rats (Table-74 & Fig-96a,b).

$5\text{-}HT_{2C}$ RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

$5\text{-}HT_{2C}$ receptor gene expression in the cerebral cortex decreased significantly in STH treated young rats ($p<0.001$) whereas INS treated young rats showed increased expression ($p<0.05$) compared to saline treated young rats. However, $5\text{-}HT_{2C}$ receptor gene expression increased significantly in both STH treated ($p<0.01$) and INS treated old rats ($p<0.001$) when compared to saline treated old rats. In STH treated INS treated old rats, the mRNA expression increased significantly ($p<0.001$) compared to STH treated INS treated young rats (Table-75 & Fig-97a,b).

IP3 CONTENT IN DIFFERENT BRAIN REGIONS OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

Cerebral cortex

IP3 content decreased significantly in the cerebral cortex of both STH treated ($p<0.001$) and INS treated ($p<0.001$) young rats when compared to saline treated young rats. However, in both STH treated and INS treated old rats, increased IP3 content ($p<0.001$) was observed when compared to saline treated old rats. There was significant increase ($p<0.01$) in IP3 content in the cerebral cortex of saline treated old rats compared to saline treated young rats. Both STH treated INS treated old rats show increased ($p<0.001$) IP3 level when compared to young rats (Table-76 & Fig-98).

Brainstem

IP3 content increased significantly in the brainstem of both STH treated ($p<0.001$) and INS treated ($p<0.001$) young rats when compared to saline treated young rats. However, in STH treated ($p<0.01$) and INS treated old rats ($p<0.05$), decreased IP3 content was observed when compared to saline treated old rats. There was significant increase ($p<0.01$) in IP3 content in the brainstem of saline treated old rats compared to saline treated young rats. Both STH treated INS treated old rats showed significant decrease ($p<0.001$) in IP3 level when compared to young rats (Table-77& Fig- 99).

Corpus striatum

In the corpus striatum of both STH treated and INS treated young rats, IP3 content decreased significantly ($p<0.001$) when compared to saline treated young rats. Also, in STH treated and INS treated old rats, IP3 content decreased significantly ($p<0.001$) when compared to saline treated old rats. There was significant increase ($p<0.01$) in IP3 content in the corpus striatum of saline treated old rats compared to

saline treated young rats. Both STH treated INS treated old rats show decreased ($p<0.001$) IP3 content when compared to young rats (Table-78 & Fig-100).

cGMP CONTENT IN DIFFERENT BRAIN REGIONS OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

Cerebral cortex

cGMP content increased significantly in the cerebral cortex of both STH treated ($p<0.05$) and INS treated ($p<0.05$) young rats when compared to saline treated young rats. Also, STH treated ($p<0.001$) and INS treated ($p<0.001$) old rats show increased cGMP content when compared to saline treated old rats. There was significant increase ($p<0.01$) in cGMP content of saline treated old rats compared to saline treated young rats. Both STH and INS treated old rats show increased ($p<0.001$) cGMP level when compared to young rats (Table-79 & Fig-101).

Brainstem

cGMP content decreased significantly in the brainstem of both STH treated ($p<0.001$) and INS treated ($p<0.01$) young rats when compared to saline treated young rats. STH treated old rats showed no significant change in the cGMP content whereas INS treated old rats showed increased ($p<0.001$) cGMP content when compared to saline treated old rats. There was significant increase ($p<0.01$) in cGMP content in the brainstem of saline treated old rats compared to saline treated young rats. STH treated and INS treated old rats show increased ($p<0.001$) cGMP content when compared to young rats (Table-80 & Fig-102).

Corpus striatum

cGMP content decreased significantly in the corpus striatum of both STH treated ($p<0.001$) and INS treated ($p<0.01$) young rats when compared to saline

treated young rats. STH treated old rats showed no significant change in the cGMP content whereas INS treated old rats show decreased ($p<0.001$) cGMP content when compared to saline treated old rats. There was significant decrease ($p<0.01$) in cGMP content in the corpus striatum of saline treated old rats compared to saline treated young rats. STH treated old rats show increased ($p<0.001$) cGMP content whereas INS treated old rats showed decreased ($p<0.01$) cGMP content when compared to young rats (Table-81 & Fig-103).

TRIIODOTHYRONINE (T3) CONTENT IN SERUM OF CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

T3 content increased significantly in the cerebral cortex of STH treated young rats ($p<0.001$) whereas in INS treated young rats, T3 content decreased significantly ($p<0.01$) when compared to saline treated rats. T3 content decreased significantly in the cerebral cortex of both STH treated ($p<0.001$) and INS treated ($p<0.001$) old rats when compared to saline treated old rats. There was significant increase ($p<0.001$) in T3 content in saline treated old rats compared to saline treated young rats. STH treated old rats show decreased ($p<0.001$) T3 content whereas INS treated old rats show no significant change when compared to young rats (Table-82 & Fig-104).

CALCIUM IMAGING

The isolated islets from 7 weeks and 90 weeks old rats were incubated for 4 hours at room temperature in 1ml of RPMI medium containing 5μM of Ca^{2+} fluorescent dye, fluo 4-AM to monitor the changes in the intracellular Ca^{2+}. The images were continuously acquired before and after addition of 10^{-7}M carbachol, 10^{-6}M pirenzepine (muscarinic M1 receptor antagonist) and 10^{-6}M 4-DAMP mustard (M3 receptor antagonist) for a total of 600 seconds. Carbachol at 10^{-7} M significantly increased Ca^{2+} release from the pancreatic islet cells in both 7 weeks and 90 weeks old rats *in vitro*. Stimulatory effect of carbachol on Ca^{2+} release was inhibited by the

addition of muscarinic M1 and M3 receptor antagonists such as pirenzepine and 4-DAMP mustard respectively. Pirenzepine at 10^{-6}M, significantly inhibited carbachol induced Ca^{2+} release in both 7 weeks and 90 weeks old rats islets when compared to 4-DAMP mustard at 10^{-6}M concentration (Table-83-86 & Fig-105-108).

EEG ANALYSIS OF THE BRAIN ACTIVITY IN CONTROL AND EXPERIMENTAL YOUNG AND OLD RATS

Electroencephalogram analysis showed that there is a change in the brain activity of frontal, parietal, occipital and temporal areas of young and old diabetic rats when compared to control rats. The brain activity differences observed clearly shows that in aged brain the damage is much more than in young brain during hyperglycaemic state. Insulin treatment significantly alters the change in brain wave activity near to control rats. Long term low dose insulin and somatotropin treatment was found to be effective in altering the EEG pattern in both young and old rats. (Fig. 109-110).

Discussion

Neurotransmitters show significant alterations during hyperglycaemia and causes degenerative changes in neurons of the central nervous system (Garris, 1990; Lackovic *et al.*, 1990, Bhardwaj, *et al.*, 1999). Streptozotocin (STZ) is a toxic agent selective to pancreatic β-cells that induces IDDM by causing the β-cell destruction (Like & Rossini, 1976; Paik *et al.*, 1980). Studies on STZ-induced diabetic rat models have shown morphological, behavioural and electrophysiological alterations on diabetes (Jakobsen *et al.*, 1987; Biessels *et al.*, 1996; Chabot *et al.*, 1997). Increased blood glucose and decreased body weight during diabetes is similar with previous reports as a result of the marked destruction of insulin secreting pancreatic islet β-cells by streptozotocin (Junod *et al.*, 1969). Hyperglycaemic state during diabetes is due to the increased gluconeogenic pathway, which is physiologically less sensitive to the inhibition by insulin (Girard *et al.*, 1995). 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 *via* acetyl-CoA. During diabetes there is decrease in body weight as a result of altered metabolic function. Insulin treatment normalised the increased blood glucose level and decreased body weight to control values.

ACETYLCHOLINE ESTERASE ACTIVITY IN BRAIN REGIONS

Acetylcholine is the primary neurotransmitter of the cholinergic system, and its activity is regulated by acetylcholine esterase (AChE). AChE activity has been used as a marker for cholinergic activity (Goodman & Soliman, 1991; Ellman *et al.*, 1961). The termination of nerve impulse transmission is accomplished through STH the degradation of acetycholine into choline and acetyl CoA by AChE (Weihua Xie *et*

al., 2000). It is well recognized that diabetes mellitus results in altered membrane functions in several tissues (Alberti *et al.*, 1982; Osterby., 1988 & Striker *et al.*, 1993). Membrane alterations have been recognized as the underlying primary biochemical defect (Alberti *et al.*, 1982). It has been well established that there is a marked change in the AChE in diabetic condition. Akmayev *et al.*,(1978) showed that there is difference in distribution of the enzyme in the neurons of the central vagal nuclei and medulla oblongata in normal and diabetic adult male rats. Acute hyperglycaemia caused an increase in AChE activity of brain areas like septum, medial preoptic area, median eminence-arcuate region, amygdala, thalamus, hippocampus, pons and medulla (Wahba & Soliman, 1988; Lakhman & Kaur, 1994) It is suggested that the changes in the plasma glucose or insulin levels may be the stimuli that influence the activity of cholinergic neurons. By this mechanism brain cholinergic activity will be implicated in the insulin secretion on ageing.

The cholinergic activity was studied in brain regions of experimental rats using AChE as marker. The activity of AChE, the degradative enzyme of cholinergic system, in the cerebral cortex, brainstem and corpus striatum of 90 weeks old control group decreased significantly compared to 7 weeks old control. An age-related decline in AChE activity was reported in all brain regions (Michalek *et al.*, 1989; Bisso *et al.*, 1991). Impairment in the ability to synthesise and release ACh has been consistently reported in aged rodent cortex (Vannuchi *et al.*, 1990). Hyperglycaemia caused significant increase in AChE activity of both 7 weeks and 90 weeks old rat groups in cerebral cortex and corpus striatum when compared to control group, whereas insulin administration reversed this effect. This may possibly relate to the reported delayed nerve transmission and impaired brain functions (Bartus *et al.*, 1982; Davis *et al.*, 1983; Clements, 1979; Carrington *et al.*, 1991). In brainstem, there was a decrease in activity of 7 weeks old diabetic group when compared to control rats. Decreased V_{max} without alteration in K_m for erythrocyte AChE from diabetic patients has been reported (Suhail & Rizvi, 1989). Decreased AChE activity observed in the brain

regions of 90 weeks old rats suggests a loss of postsynaptic enzyme activity during ageing (Sirviö *et al.*, 1988, Yufu *et al.*, 1994, Michalek *et al.*, 1989). The decrease in AChE activity have a negative correlation with the blood glucose level suggested to be due to impaired glucose oxidation and glucose transport with insulin deficiency.

The activation of central cholinergic system by administration of cholinergic agonist into the third cerebral ventricle reported to produce hyperglycaemia in rats (Iguchi *et al.*, 1985). When carbachol, muscarine, bethanechol, methacholine, or neostigmine was injected into the third cerebral ventricle, it caused a dose-dependent increase in the hepatic venous plasma glucose concentration. Our results showed that diabetic state clearly influenced the kinetic properties of AChE enzyme and the reversal of AChE activity to near control value found in the insulin treated diabetic rats brain regions is a compensatory mechanism to maintain the normoglycaemic level. Also, cholinergic neurons of the basal forebrain complex undergo moderate degenerative changes during ageing, resulting in cholinergic hypofunction that has been related to the progressing memory deficits with ageing. These findings may be relevant to the central cholinergic deficit reported to be associated with cognitive impairment in ageing process. This causes specific alteration in ACh levels and mAChRs, leading to cholinergic dysfunction on diabetes as a function of age.

ACETYLCHOLINE ESTERASE ACTIVITY IN PANCREAS

Acetylcholine esterase positive nerves were distributed in the interacinar regions of the pancreas lying close to the exocrine cells. There was no difference between the cholinergic innervation of the pancreas in normal and diabetic rat (Singh & Adeghate, 1998). The intensity of antimuscarinic acetylcholine receptor antibody M35-immunoreactivity at the periphery and central core of the islets paralleled the density of cholinergic innervation, suggesting a positive correlation between the intensity of cholinergic transmission and the number of muscarinic acetylcholine receptors at the target structures (Van Der Zee *et al.*, 1992). The activity of AChE in

the pancreas of 90 weeks old control group decreased significantly compared to 7 weeks old control. Hyperglycaemia caused significant decrease in the AChE activity in the pancreas of both 7 weeks and 90 weeks old rat groups. Insulin administration showed further decrease in the enzyme activity in both groups. Binding studies using specific M1 and M3 receptor antagonists have supported the existence of both M1 and M3 receptors on pancreatic acinar cells, which was confirmed by the demonstration of specific mRNA for both receptor subtypes in rat pancreatic acinar cells (Elke Niebergall-Roth & Manfred V. Singer, 2003).

MUSCARINIC RECEPTOR ALTERATIONS IN BRAIN REGIONS

Over the past decade, the role of muscarinic receptors in health and disease and the potential therapeutic value of various cholinergic agonists and antagonists have received increasing attention (van Zwieten & Doods , 1995; van Zwieten *et al.*, 1995). Muscarinic acetylcholine receptors are a family of G protein-coupled receptors and subserve numerous vital functions in both the brain and autonomic nervous system (Hassal *et al.*, 1993). The M_1 muscarinic receptor is one of five known muscarinic subtypes in the cholinergic nervous system (Bonner *et al.*, 1987; Hulme *et al.*, 1990; van Zwieten & Doods, 1995) have a primary role in central cholinergic neurotransmission. Specific agonists, which activate postsynaptic muscarinic receptors, stimulate cholinergic signalling (Valentin *et al.*, 2006). It is known that different parts of the brain, particularly the hypothalamus and the brainstem, are important centers involved in the monitoring of glucose status. The effect of the cholinergic agonist blocked by the muscarinic antagonist atropine shows the involvement of muscarinic receptors in the central cholinergic glucose homeostasis. Muscarinic receptor density was found to decrease significantly with ageing in certain brain regions, depending on the ligand used (Surichamorn *et al.*, 1988; Michalek *et al.*, 1989; Schwarz *et al.*, 1990; Norbury *et al.*, 2005).

Cerebral cortex

The RT-PCR and HPLC studies revealed that the M1 receptor was present in a relatively high density in the cerebral cortex (Jian *et al.*, 1994; Oki *et al.*, 2005). Cholinergic agonist carbachol normalized glucose-stimulated insulin secretion and glucose tolerance in mice subjected to a high-fat diet. Intraventricular administration of carbachol produces hyperglycaemia in rats, while the subcutaneous administration is ineffective. This effect is suppressed by intraventricular administration of atropine suggesting that the effect of carbachol is due to its action on central cholinergic receptors (Korner & Ramu, 1976). Central cholinergic neurons participate in the complex neural events responsible for the hyperglycemic response to neurocytoglucopenia and to stressful situations. The hyperglycaemia induced by intracerebroventricular 2-deoxyglucose (2-DG) was significantly reduced by previous intracerebroventricular injection of atropine (Brito *et al.*, 2001). Atropine injected into the third cerebral ventricle suppressed epinephrine secretion and dose-dependently inhibited hepatic venous hyperglycaemia induced by neostigmine in intact rats (Iguchi *et al.*, 1999).

Muscarinic M1 receptor changes during diabetes were studied using [^{3}H]QNB and subtype specific antagonist, pirenzepine. Muscarinic M1 receptors are decreased in the cerebral cortex of control old rats compared to the young rats, with a decrease in K_d indicating an increase in the affinity of receptors during ageing. Total specific binding sites for the muscarinic antagonist, L-QNB showed a decline with age (Briggs *et al.*, 1982) and age-related increase in the affinity of mAChRs was observed in the cortical regions of rats (Yufu *et al.*, 1994). Studies using selective M_1 antagonist, [^{3}H]-pirenzepine ([^{3}H]PZ) reported decreased receptor density in the cortex of aged rats compared to young rats (Schwarz *et al.*, 1990; Ehlert & Tran., 1990). However, Scatchard analysis of [^{3}H]DAMP against 4-DAMP mustard showed no significant change in the muscarinic M_3 receptor number and affinity of control old rats compared to the young rats. These findings indicate differential sensitivity of

muscarinic M_1 and M_3 receptor subtypes in the cortical region to ageing. STZ-induced diabetes results in structural alterations of mAChRs in the brain (Latifpour *et al.*, 1991) which in turn alters cholinergic nerve components (Akria, *et al.*, 1994) with decrease in the Na^+, K^+-ATPase activity (Gurcharan & Sukwinder, 1994). Our studies showed that in diabetic condition, M_1 receptor numbers decreased in 7 weeks old rat group whereas 90 weeks old rat group showed an increase when compared to control. Muscarinic M_3 receptor numbers increased in both 7 weeks and 90 weeks old rat group when compared to control. Studies of Latifpour and McNeill (1984) on long-term STZ-induced diabetes reported large reduction in muscarinic receptor densities as compared with their age-matched controls. In insulin treated diabetic rats binding parameters are reversed back to near control values. Real Time-PCR analysis revealed a down regulation of the muscarinic M1 receptor mRNA in 7 weeks old rats whereas 90 weeks old rats showed an up regulation of muscarinic M1 receptor mRNA level during diabetic condition. Muscarinic M3 receptor gene expression increased in 7 weeks old rats and the expression was decreased in 90 weeks old rat groups. The functional regulation of muscarinic receptors during diabetes is a compensatory mechanism to facilitate insulin secretion and maintenance of normoglycaemia in diabetic rats.

Corpus striatum

Cholinergic terminals within the striatum contain presynaptic muscarinic receptors that inhibit neurotransmitter release (Chesselet, 1984). 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 (Homykiewicz, 1981; Jabbari *et al.*, 1989). 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 (Lehmann & Langer, 1982, 1983; Wainer *et al.*, 1984; Phelps *et*

highest in the corpus striatum (Oki *et al.*, 2005). The structures within the basal ganglia observed to express muscarinic receptor mRNAs were the striatum, the substantia nigra, pars compacta, and the subthalamic nucleus. The M1 mRNA was observed throughout STH the caudate-putamen, nucleus accumbens and olfactory tubercle. Within the caudate-putamen, M1 mRNA was expressed in a lateral-to-medial gradient. The cholinergic innervation of the striatum is intrinsic and originates from a small number (<3%) of the large neurons (Bolam *et al.*, 1984; Moon & Graybiel, 1983).

Muscarinic M1 receptors are increased in the corpus striatum of control old rats compared to the young rats, with an increase in K_d indicating a decrease in the affinity of receptors during ageing. Scatchard analysis of [^{3}H]DAMP against 4-DAMP mustard show decreased B_{max} and K_d of control old rats compared to the young rats indicating increased affinity of muscarinic M_3 receptors on ageing. In diabetic condition, M_1 receptor numbers increased whereas M_3 receptor numbers decreased in 7 weeks old rat group. 90 weeks old rat group showed decreased M_1 receptor numbers and increased muscarinic M_3 receptor numbers when compared to control. In insulin treated diabetic rats, binding parameters were reversed back to near control values. Real Time-PCR analysis revealed a down regulation of the muscarinic M1 receptor mRNA in 7 weeks old rats whereas 90 weeks old rats showed an up regulation of muscarinic M1 receptor mRNA level during diabetic condition. Muscarinic M3 receptor gene expression decreased in both 7 weeks and 90 weeks old rats groups. These receptors activate a multitude of signalling pathways important for modulating neuronal excitability, synaptic plasticity and feedback regulation of ACh release (Volpivelli *et al.*, 2004).

Brainstem serves as one of the key centres of the central nervous system regulating the body homeostasis. Stimulation of the peripheral vagus nerve leads to an increase in circulating insulin levels. Anatomical studies suggest that the origin of these vagal efferent fibres is nucleus ambiguus and dorsal motor nucleus directly innervating pancreas (Bereiter *et al.,* 1981). Most studies did not report effects of age on the total brainstem volume (Raz, 1996). Age-related shrinkage of the midbrain has been observed previously (Doraiswamy *et al.*, 1992; Weis *et al.*, 1993). Shah *et al.* (1991) stated that the age-related decline in midbrain size could be accounted for in part by neuronal death or degeneration with nuclei and/or tracts of the midbrain. Muscarinic M1 receptors are increased in the brainstem of control old rats compared to the young rats, with an increase in K_d indicating a decrease in the affinity of receptors during ageing. Scatchard analysis of [^{3}H]DAMP against 4-DAMP mustard show decreased B_{max} and K_d of control old rats compared to the young rats indicating increased affinity of muscarinic M_3 receptors on ageing.

The dorsal motor nucleus of the vagus nerve is located in the brainstem. It is connected to the endocrine pancreas exclusively *via* vagal fibres and has a role in neurally mediated insulin release. Nucleus ambiguus stimulation reported to increase plasma insulin levels in rats (Bereiter *et al.,* 1981). The insulin was reported to be mitogenic and stimulated pancreatic β-cell proliferation *in vitro.* The muscarinic M1 receptors of the brainstem are found to be decreased whereas M_3 receptor numbers increased in 7 weeks old rat group during diabetic condition. 90 weeks old rat group showed decreased M_1 receptor numbers and increased muscarinic M_3 receptor numbers when compared to control. In insulin treated diabetic rats, binding parameters were reversed back to near control values. Real Time-PCR analysis revealed a down regulation of the muscarinic M1 receptor mRNA in 7 weeks old rats whereas 90 weeks old rats showed an up regulation of muscarinic M1 receptor mRNA

7 weeks old rats and the expression was increased in 90 weeks old rats.

MUSCARINIC RECEPTORS ALTERATIONS IN THE PANCREAS

The autonomic system plays an important role in the insulin release. Physiological insulin secretion is initiated by glucose and augmented by nervous and humoral systems (Ahren *et al.*, 1986). The pancreatic islets are richly innervated by parasympathetic, sympathetic and sensory nerves. Several different neurotransmitters are stored within the terminals of these nerves, both acetylcholine and noradrenaline, and several neuropeptides. Stimulation of the autonomic nerves and treatment with neurotransmitters affect islet hormone secretion. Insulin secretion is stimulated by parasympathetic nerves and inhibited by sympathetic nerves (Ahren, 2000). Acetylcholine mediates insulin release through vagal stimulation. Acetylcholine acts through the activation of Gq-phospholipase C. It stimulates Ca^{2+} influx through the voltage dependent L-type Ca^{2+} channel that is primarily activated by glucose. Studies showed that M1 and M3 are the major muscarinic receptors present in the pancreas (Iismaa *et al.*, 2000). Expression of muscarinic receptors in rat islets, RINm5F cells, and INS-1 cells was established by reverse transcriptase-polymerase chain reaction (RT-PCR) and quantified by RNase protection. Both methods indicated that M1and M3 receptors were expressed approximately equally in the various cellular preparations (Lismaa *et al.*, 2000).

Muscarinic M3 receptor changes were studied in experimental rats using subtype specific antagonist 4-DAMP mustard. Muscarinic M3 receptors are decreased in diabetic rats while K_d was increased when compared to control group. In insulin treated diabetic rats, binding parameters were reversed back to near control values. Administration of choline to rats elevates serum insulin. Pretreatment with atropine methylnitrate, a peripheral muscarinic acetylcholine receptor antagonist, blocked the choline-induced increase in blood insulin. The increase in serum insulin

pirenzepine, or the M1 + M3 antagonist, 4-DAMP. Pretreatment with hexamethonium, an antagonist of ganglionic nicotinic acetylcholine receptors prevented the choline-induced increase in serum insulin. Choline increased the acetylcholine content of the pancreas, and enhanced acetylcholine release from minced pancreas, which suggests that choline stimulates insulin secretion indirectly by enhancing acetylcholine synthesis and release (Ilcol *et al.*, 2003). Muscarinic M3 receptors are involved in the glucose induced insulin secretion. In insulin treated diabetic rats, muscarinic M3 receptor status reversed to near control level. It helps to increase the insulin secretion from remaining β-cells to maintain the normal glucose level. RT-PCR analysis also revealed a down regulation of the muscarinic M3 receptor mRNA level during diabetic condition. This is in concordant with our receptor binding studies.

GLUTAMATE RECEPTOR, NMDAR1 AND mGlu-5 GENE EXPRESSION IN DIFFERENT BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

The functional and biochemical studies show that long-term exposure to hyperglycaemia in STZ-induced diabetic rats is associated with glutamate receptor abnormalities (Gange *et al.*, 1997; Di Luca *et al.*, 1997). Increased glutamate content is reported to cause neuronal degeneration (Atlante *et al.*, 1997; Berman & Murray, 1996; Budd & Nicholas, 1996). Glutamate which causes excitotoxic neuronal damage, increases calcium influx through N-methyl-D-aspartate receptors in post synaptic neurons, leading to phospholipase A_2 mediated arachidonic acid release (Miriam *et al.*, 1996). The increase in arachidonic acid in brain mediate neuronal injury by inhibiting the sodium ion channels (Fraser *et al.*, 1993).

The progression of diabetes is associated with an impaired ability of the neurons in the CNS to release neurotransmitters resulting in behavioural changes (Broderick & Jacoby, 1989). Among them, the neurotransmitter receptor NMDA

expression of glutamate receptor is also involved in the development of diabetic neuropathy (Tomiyama *et al.*, 2005). Previous studies reported that changes in the protein expression of the NMDA receptor subunits occur during the ageing process and it was greater than the changes seen in mRNA expression (Magnusson *et al.*, 2002). Ageing does not affect all brain regions equally. Some regions seem to be more sensitive to ageing than others (Horiuchi & Saitoe, 2005; Lu *et al.*, 2004). The brain regions - cerebral cortex and hippocampus of diabetic rats is suggested to be more vulnerable to glutamate toxicity *via* NMDA receptor activation. An age-related increase in mGlu1 receptor mRNA levels was found in thalamic nuclei, hippocampal CA3 with parallel increases in mGlu1a receptor protein expression (Simonyi *et al.*, 2005).

Diabetes mellitus induces cognitive impairment and defects of long-term potentiation (LTP) in the hippocampus. From the gene expression studies, it is clear that in cerebral cortex, mGlu-5 mRNA increased siginficantly in 7 weeks old diabetic rats compared to control groups. Ageing process affects NMDA receptors more in the intermediate hippocampus than the dorsal (Magnusson *et al.*, 2006). The dysfunction in hippocampal LTP, an electrophysiological model of synaptic plasticity thought to subserve learning and memory processes is associated with diabetic conditions (Biessels *et al.*, 1996; Di Mario *et al.*, 1995). Also, L-[^{3}H]glutamate-labeled NMDA receptors were found to be down regulated in primary sensory cortical regions (Bean *et al.*, 2006).

Brainstem is an important part of the brain in monitoring the glucose status and the regulation of feeding (Pénicaud *et al.*, 2002). In brainstem, corpus striatum and hippocampus, we observed a decreased expression of mGlu-5 mRNA when compared to control groups. 90 weeks old diabetic rats showed decreased expression in cerebral cortex, corpus striatum and hippocampus whereas in the brainstem the expression increased significantly. Deficits in long-term potentiation during chronic

diabetes arise from dysfunction of the NMDA subtype of glutamate receptors in early stages of the disease (Trudeau *et al.*, 2004).

An imbalance between Gs -proteins and Gi/Go protein mediated efficacy of Gs activity as a result of the loss of Gi/Go inhibitory functions has been found in the striatum and other tissues of diabetic animals (Salkovic & Lackovic, 1992). Studies show an age-dependent reduction in the functional response of striatal group I mGlu receptors, which is one of the factors underlying the reduced ability of aged striatum to integrate information (Pintor *et al.*, 2000). Our results suggest that glutamate receptor alterations found in the brain regions contributes to cognitive and memory deficits during diabetes as a function of age.

Studies have shown that regulation of glutamate receptor properties can contribute to learning and memory (Massicotte, 2000). Activation of this neurotransmitter system is also involved in neurodegeneration following a wide range of neurological insults, including ischemia, trauma and epileptic seizures (Hollmann & Heinnemann, 1994; Lipton & Rosenberg, 1994). In rodents, NMDA and non-NMDA (kainate and α-amino-3-hydroxy-5-methylisoxazole-4-propionate, AMPA) receptors are two families of ionotropic receptors stimulated by glutamate that have been implicated in neurodegeneration (Dingledine *et al.*, 1999). Overactivation of these receptors can cause cell damage by increasing intracellular calcium concentration in neurons, thereby leading to the generation of free radicals and activation of proteases, phospholipases and endonucleases (Coyle & Puttfarcken, 1993; Siesjo, 1988; Siesjo *et al.*, 1995) as well as transcriptional activation of specific cell death programme (Schreiber & Baudry, 1995).

Excess activation of NMDA receptors by glutamate increases cytoplasmic concentrations of sodium and calcium to levels that exceed the capacity of neuronal homeostatic mechanisms, thereby altering transmembrane ion gradients. Neurons impaired of energy metabolism are highly sensitive to excitotoxicity (Simon *et al.*, 1984; Wieloch, 1985; Monyer *et al.*, 1989; Cebers *et al*, 1998). NMDA receptor

activation is also important in long-term potentiation and the formation of synaptic networks (Tsien & Malinow, 1993; Worley, 1990). NMDA receptor activity is also involved in regulating the process of apoptosis or programmed cell death *via* changes in cytoplasmic and nuclear calcium concentrations (Jane, 1999).

In 7 weeks old D+I group, mGlu-5 mRNA expression was significantly decreased in cerebral cortex and corpus striatum whereas the expression increased significantly in brainstem and hippocampus. 90 weeks old D+I group showed an increased expression in cerebral cortex, while it was decreased significantly in corpus striatum, brainstem and hippocampus compared to their respective controls. Insulin is reported to regulate the reuptake of catecholamine transporters. Intracerebroventricular injection of insulin is reported to cause an increased mRNA expression of dopamine transporters (Figlewicz, *et al.*, 1998). Short-term insulin treatment was found to alter NMDA receptor activation (Liu *et al.*, 1995) as well as to interact with AMPA receptor trafficking between the plasma membrane and the intracellular compartment in neuronal cell culture (Man *et al.*, 2000) indicating that mechanisms underlying diabetic neuropathies could be initiated in the early stages of the disease, as a consequence of abnormal glutamate receptor properties. This is relevant to the clinical situation because excessive activation of glutamate receptors is a characteristic feature of brain damage during stroke and ischaemia (McCall, 1992), conditions that are exacerbated by hyperglycaemic states (Mandrup-Poulsen, 1998).

According to earlier studies, changes in glutamate receptors account for modifications of long-term potentiation in various models of diabetes mellitus. In diabetic rats, we observed an increased expression of NMDA receptors in the hippocampus and brainstem whereas the expression showed a significant decrease in the cerebral cortex and corpus striatum. The brain regions - hippocampus and brainstem of diabetic rats is suggested to be more vulnerable to glutamate toxicity *via* NMDA receptor activation. Diabetes mellitus induces cognitive impairment and defects of long-term potentiation in the hippocampus. Deficits in long-term

potentiation during chronic diabetes arise from dysfunction of the NMDA subtype of glutamate receptors in early stages of the disease (Trudeau *et al.*, 2004). Recent studies reported that abnormal expression of NMDA receptor is involved in the development of diabetic neuropathy (Tomiyama *et al.*, 2005). Our results suggest that NMDA receptor alterations found in the brain regions particularly hippocampus and brainstem during diabetes could contribute to cognitive and memory deficits during diabetes.

DOPAMINE RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL RATS

DA receptors are reported to be increased in diabetes causing significant alterations in central dopaminergic system (Lozovsky *et al.*, 1981). DA D_2 receptor density has been reported to be increased in the striatum of diabetic rats (Lozovsky *et al.*, 1981; Trulson & Hummel, 1983; Serri *et al.*, 1985). Dopamine D2 receptor gene expression in the cerebral cortex was increased significantly in both young and old diabetic rats compared to control. Intracerebroventricular application of alloxan and streptozotocin in rat striatum is reported to have an alteration in DA receptors and increased DA content which had a similar effect to peripheral, diabetogenic administration of these drugs (Salkovic *et al.*, 1992). The affinity of striatal DA D_1 receptors was significantly increased without changes in the number of binding sites, while the binding of DA D_2 receptors was significantly increased without affecting its affinity in the diabetic rats (Hio *et al.*, 1994). DA D_1 receptors are reported to decrease in hyporesponsiveness (Kamei *et al.*, 1994). The increase in the central dopaminergic postsynaptic receptors has been related to decrease the locomotor and ambulatory activity in STZ-induced diabetic rats (Kobayashi *et al.*, 1990; Shimomura *et al.*, 1990). Age-related alterations in the dopaminergic system were observed in the gene expression studies. Recent studies from our laboratory reported DA D_2 receptor

alterations in the brain and pancreas of STZ- induced diabetic rats (Eswar *et al.*, 2007).

ADRENERGIC RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL RATS

Diabetes mellitus has been reported to cause degenerative changes in neurons of the central nervous system (Bhattacharya & Saraswathi, 1991; Garris, 1990; Lackovic *et al.*, 1990). Our previous studies demonstrated adrenergic receptor function alterations in the brain of diabetic rats (Abraham & Paulose, 1999; Padayatti & Paulose, 1999; Paulose *et al.*, 1999). The concentration of 5-HT, DA and NE increased in the brain regions of diabetic rats and accumulation of these monoamines is produced by inhibition of monoamine oxidase activity (Salkovic *et al.*, 1992). Norepinephrine has been reported to increase in several brain regions during diabetes. Ohtani *et al.*, (1997) have reported a significant decrease in extracellular concentrations of NE, 5HT and their metabolites in the ventro medial hypothalamus (VMH). Epinephrine (EPI) levels were significantly increased in the striatum, hippocampus and hypothalamus of diabetic rats and these changes were reversed to normal by insulin treatment (Ramakrishan & Namasivayam, 1995). Diabetes is reported to cause a high level of degeneration in neurons in different regions of the brain. Streptozotocin induced diabetes and acute deficiency of insulin is reported to result in increased concentrations of EPI in the supra chiasmatic nucleus. It is also reported that β-adrenergic receptor populations were decreased in diabetes (Garris, 1995). 5-HT content in the brain is reported to be decreased during diabetes (Jackson & Paulose, 1999; Chu *et al.*, 1986; Sumiyoshi *et al.*, 1997). Garris (1995) reported chronically elevated levels of NE in the brain regions of amydgala, hypothalamus and medulla of diabetic mice. This was proposed to be associated with the expression of the gene causing diabetes mellitus. Hyperglycaemia is reported to alter the noradrenergic and cholinergic nerve components (Akria *et al.*, 1994) with decrease in

the Na^+ K^+ ATPase activity in different brain regions (Gurcharan *et al.*, 1994). NE, DA and 5-HIAA are reported to be increased in the heart and adrenal gland in STZ rats. In the heart, the initial changes in short-term diabetes included an increase in NE concentration but did not persist in the long term diabetic animals. In the adrenal gland, there was an initial reduction followed by a steady increase in the concentration of NE and EPI (Morrison *et al.*, 2001).

α_{2A}- adrenergic receptor gene expression in the cerebral cortex was decreased whereas β_2-adrenergic receptor gene expression increased significantly in 7 weeks old diabetic rat groups when compared to control. In 90 weeks old rat groups, α_{2A}-adrenergic receptor gene expression was increased whereas β_2-adrenergic receptor gene expression decreased significantly when compared to control. Previous studies have shown that in diabetic condition α_{2A}-receptors are more activated which brought out the insulin inhibition and in turn hyperglycaemia (Lacey *et al.*, 1993). Rat islet cell membrane is equipped with α_{2A} (Filipponi *et al.*, 1986) which are linked to adenylate cyclase and inhibits insulin secretion. Studies conducted in C57BL/KsJ mice revealed that all of the α_1- and α_2-adrenergic receptor population was elevated in the regional brain samples of diabetic compared with controls. However, β-adrenergic receptor populations were depressed in diabetes compared with age-matched controls (Garris, 1990). Studies from our lab have shown that α_1-adrenoceptors expressed altered affinity in hypothalamus and brainstem of STZ induced diabetic rats (Pius, 1996).

GABA RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL RATS

GABA is the main inhibitory neurotransmitter in central nervous system. GABA is reported to present in the endocrine pancreas at concentrations comparable with those found in central nervous system. The highest concentration of GABA within the pancreatic islet is confined to β-cells (Sorenson *et al.*, 1991). Glutamate

decarboxylase, the primary enzyme that is involved in the synthesis of GABA, has been identified as an early target antigen of the T-lymphocyte mediated destruction of pancreatic β-cells causing insulin-dependent diabetes mellitus (Baekkeskov *et al.*, 1990). $GABA_A$ and $GABA_B$ receptor gene expression decreased significantly in young and old diabetic rat groups when compared to control. GABA through its receptors has been demonstrated to attenuate the glucagon and somatostatin secretion from pancreatic α-cells and δ-cells respectively (Gaskins *et al.*, 1995). GABA which is present in the cytoplasm and in synaptic-like microvesicles (Reetz *et al.*, 1991) is co-released with insulin from β-cells in response to glucose. The released GABA inhibits islet α-and δ-cell hormonal secretion in a paracrine manner. During diabetes the destruction of β-cells will lead to decrease in GABA release resulting in the enhancement of glucagon secretion from α-cells leading to hyperglycaemia. The brain GABAergic mechanisms also play an important role in glucose homeostasis. Inhibition of central $GABA_A$ receptors increases plasma glucose concentration (Lang, 1995). Thus, any impairment in the GABAergic mechanism in central nervous system and/or in the pancreatic islets is important in the pathogenesis of diabetes.

$5\text{-}HT_{2c}$ RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL RATS

$5\text{-}HT_{2C}$ receptor gene expression in the cerebral cortex was decreased significantly in young rats whereas the expression increased significantly in old rat groups compared to control. 5-HT content is increased in the brain regions and hypothalamic nuclei (Chen & Yang, 1991); (Lackovic *et al.*, 1990), but there are reports suggesting a decrease in brain 5-HT content during diabetes (Jackson & Paulose, 1999; Sumiyoshi *et al.*, 1997; Sandrini *et al.*, 1997). Ohtani *et al.*, (1997) have reported a significant decrease in extracellular concentrations of NE, 5-HT and their metabolites in the ventro medial hypothalamus (VMH). The ratio of 5-HIAA/5-HT was increased. A similar observation was reported by (Ding *et al.*, 1992) with a

decrease in 5-HT in cortex (19%) and 5-HT turnover (5-HIAA/5-HT) that increased by 48%. Chu *et al.*, (1986) has reported lower 5-HT levels in both hypothalamus and brainstem but not in corpus striatum. Insulin treatment brought about an increase in the cerebral concentration of 5-HIAA and accelerated the cerebral 5-HT turnover (Juszkiewicz, 1985). The 5-HIAA concentration was reported to be approximately twice as high as the controls regardless of duration of treatment. Brain tryptophan, the precursor of 5-HT, was also reduced in brain regions during diabetes (Jamnicky *et al.*, 1991). Insulin treatment was reported to reverse this reduced tryptophan content to normal (Jamnicky *et al.*, 1993). There was a significant increase in 5-HIAA observed at 2-6 hours after insulin administration (Kwok & Juorio, 1987).

IP3 CONTENT IN DIFFERENT BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

The mechanisms by which NMDA receptor activation can modulate muscarinic receptor-stimulated phosphoinositide turnover have been studied in neonatal rat cerebral cortex slices (Challis *et al.*, 1994). IP3 content in the cerebral cortex, corpus striatum and brainstem was increased in young diabetic rat groups whereas it was decreased in old diabetic groups compared to control. Insulin treatment significantly reversed the altered IP3 content near to control. Phosphoinositide turnover in the rat frontal cortex *in vivo* is stimulated by 5-HT_2 receptor activation (Hide *et al.*, 1989). Noradrenaline-induced accumulation of ^{3}H-labelled inositol mono-, bis-, and trisphosphate (IP1, IP2, and IP3, respectively) in lithium-treated slices of rat cerebral cortex preincubated with [^{3}H]inositol was potentiated by GABA (Crawford & Young, 1990). IP3 have been measured in tissues and medium in their response to the effect of α-MSH alone or in the presence of the peptide plus pilocarpine (selective muscarinic agonist) or atropine (selective muscarinic antagonist). Pilocarpine by itself brought about an increase of IP3 only when the highest doses were used. Atropine did not modify the IP3 content. The blockage of the

muscarinic receptor with atropine blocked the IP3 increase induced by α-MSH as well. α-MSH does not induce changes in cGMP but it does change the IP3 levels, probably acting at the muscarinic receptor level (Lezcano & Celis, 1996). The exocrine dysfunction in IP3R2 and IP3R3 double knock-out mice caused difficulties in nutrient digestion. Severely impaired calcium signalling in acinar cells of the salivary glands and the pancreas in the double mutants ascribed the secretion deficits to a lack of intracellular calcium release (Akira *et al.*, 2005). IP3-dependent increase in nuclear Ca^{2+} influx with increasing cerebral tissue hypoxia, suggesting a hypoxia-induced modification of the nuclear membrane IP3 receptors. Increase in intranuclear Ca^{2+} that leads to altered transcription of apoptotic genes and activation of nuclear endonucleases resulting in hypoxia-induced programmed neuronal death (Mishra & Delivoria-Papadopoulos, 2004).

cGMP CONTENT IN DIFFERENT BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

cGMP mediates physiological effects in the cardiovascular, endocrinological, and immunological systems as well as in CNS. In the CNS, activation of the NMDA receptor induces Ca^{2+}-dependent NOS and NO release, which then activates soluble guanylate cyclase for the synthesis of cGMP. Both compounds appear to be important mediators in long-term potentiation and long-term depression, and thus play an important role in the mechanisms of learning and memory. Altered modulation of cGMP levels in brain seems to be responsible for the impairment of cognitive function (Erceg *et al.*, 2005). cGMP has been implicated in the regulation of many essential functions in the brain, such as synaptic plasticity, phototransduction, olfaction and behavioural state. cGMP content in the cerebral cortex and brainstem was increased in young diabetic rat groups whereas it was decreased in old diabetic groups compared to control. Insulin treatment significantly reversed the altered cGMP content near to control. The cGMP content of the cerebellar cortex is altered by drugs that change

either the excitatory input of climbing or mossy fibers or the inhibitory input mediated by the activation of GABA receptors. Diazepam and muscimol lowered the cGMP content by activating GABA receptors. In contrast, morphine and haloperidol lowered the cerebellar cortex cGMP by decreasing the excitation of mossy fibers whereas harmaline increased the cGMP by increasing the excitation of the climbing fibers (Biggio *et al.*, 1977).

cGMP modulates phosphorylation in cerebellum by changing the relationship between cGMP-dependent protein kinase and type 2 inhibitor content (Biggio *et al.*, 1977). The ability of rats to learn a Y-maze conditional discrimination task depends on the function of the glutamate–nitric oxide–cGMP pathway in brain (Blanca Piedrafita *et al.*, 2007). cGMP content and NMDA receptor-Nitric Oxide (iNOS) activity was increased significantly in hippocampus, striatum and cerebral cortex during ischemic injury, indicating an important role of NMDA receptor-NO-cGMP in ischemic injury of the brain regions (Qiang *et al.*, 1999). In addition to the decrease of cGMP levels in neuronal structures induced by diazepam, increased cGMP immunoreactivity was observed in glial cells in the cerebellum, hippocampus and cerebral cortex (Aerden *et al.*, 2004). Infusion of D-serine (1 mM) enhanced (150-200%) extracellular cGMP in the cerebellum with no age-related differences (Vallebuona & Raiteri, 1995).

Ageing coincided with a decrease in the basal level of cGMP as a consequence of a more active degradation of cGMP by a phosphodiesterase in the aged brain as compared to the adult brain. Moreover, a loss of the NMDA receptor-stimulated enhancement of the cGMP level determined in the presence of cGMP-phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) was observed in hippocampus and cerebellum of aged rats. cGMP-dependent signal transduction in hippocampus and cerebellum may become insufficient in senescent brain and may have functional consequences in disturbances of learning and memory processes (Chalimoniuk & Strosznajder, 1998). Activation of NMDA receptor stimulates more significantly NO/cGMP production in hippocampus than in brain cortex suggesting

the role of NO in neuronal form of NOS and inhibitor of guanylate cyclase protect the brain against excessive production of nitric oxide and cGMP during ischemia-reperfusion. These compounds offer a new strategy in the therapy of brain ischemia (Chalimoniuk *et al.*, 1996).

MUSCARINIC STIMULATION OF INSULIN SYNTHESIS AND SECRETION FROM PANCREATIC β-CELL *IN VITRO*

Acetylcholine agonist, carbachol, at low concentration (10^{-7}M) stimulated insulin secretion in both 7 weeks and 90 weeks old rats at both concentrations (4 and 20 mM) of glucose. Stimulatory effect of carbachol on glucose-induced insulin secretion was reversed by the addition of muscarinic M1 and M3 receptor antagonists such as pirenzepine and 4-DAMP mustard respectively. Pirenzepine at 10^{-6}M concentration, inhibited carbachol induced insulin secretion in 7 weeks old rats whereas 4-DAMP mustard at 10^{-6}M inhibited the insulin secretion in 90 weeks old rats at 4mM glucose concentration. In the presence of 20mM glucose concentration, carbachol induced insulin secretion was inhibited by pirenzepine in both 7 weeks and 90 weeks old rats, confirming the role of muscarinic receptors in cholinergic involvement in insulin secretion. PKC plays an important role in mediating insulin secretion in response to cholinergic stimulation (Persaud *et al.*, 1989; Wollheim & Regazzi *et al.*, 1990). PKC also mediates densensitisation in many cell types. Activation of PKC by carbamylcholine leads to densensitisation and TPA (phorbol 12-myristate 13-acetate) treatment inactivates PKC leading to the inhibition of the densitisation process in islets (Verspohl & Wienecke, 1998). It is also reported that the desensitisation of PLC –coupled muscarinic receptors is mediated by PKC (Haga *et al.*, 1990). The inhibition of insulin secretion by the addition of hiSTH concentration of carbamylcholine is the result of the receptor desensitisation by PKC. Muscarinic M1 and M3 receptor subtype antagonists, pirenzepine and 4-DAMP mustard, inhibited cholinergic mediated insulin secretion confirming the role of these

two subtypes of receptors (Iismaa *et al.*, 2000) in insulin synthesis/secretion. Our *in vitro* studies confirmed the stimulatory role of acetylcholine in the insulin secretion from pancreatic islets. *In vitro* culture studies showed stimulatory role of muscarinic M1 and M3 receptors in insulin secretion. The M1 receptors are functionally prominent at basal glucose level, 4mM and 20mM in 7 weeks old rats. In 90 weeks old rats, M3 receptors were found to be pronounced at 4mM whereas M1 receptors were pronounced at 20mM glucose concentration.

EFFECT OF CARBACHOL AND MUSCARINIC ANTAGONISTS ON IP3 LEVELS IN ONE HOUR PANCREATIC ISLETS *IN VITRO*

Cholinergic stimulation of pancreatic β-cells increases insulin secretion. This effect is mediated by muscarinic receptors. Acetylcholine stimulation-insulin secretion coupling is mediated by complex mechanisms of signal transduction and several factors are involved. ACh is released from cholinergic synapses on β-cells during the cephalic phase of digestion causing a transient increase in insulin secretion. It has been proposed that ACh activates phospholipid turn over and thereby increases the intracellular calcium levels. IP3 mediates Ca^{2+} mobilization from intracellular Ca^{2+} stores and plays an important role in insulin secretion from pancreatic β-cells (Laychock, 1990). Carbachol, the cholinergic agonist, at 10^{-7}M significantly increased glucose induced IP3 release in both 7 weeks and 90 weeks old rats at 4mM and 20mM glucose concentrations. Pirenzepine, the M1 receptor antagonist at 10^{-6}M concentration inhibited carbachol induced IP3 release in both 7 weeks and 90 weeks old rats at 4mM glucose concentration. In the presence of 20mM glucose concentration, carbachol induced IP3 release was inhibited by pirenzepine in 7 weeks old rats whereas 4-DAMP mustard at 10^{-6}M inhibited the IP3 release in 90 weeks old rats. IP_3 exerts its action through receptors that are ligand-activated, Ca^{2+} selective channels. IP_3 receptors have been localized to the endoplasmic reticulum, nucleus and insulin granules (Yoo *et al.*, 1990). Calcium waves have been shown to be mediated

by either metabotropic glutamate receptor (mGluR) or muscarinic receptor (mAChR) activation. Metabotropic receptor activation evoked an IP3-receptor mediated calcium-induced calcium-release, raising nuclear calcium into the micro-molar range (John & Pankaj, 2007). Carbachol addition resulted in maintained increases in Ins(1,4,5)P3 and Ins(1,3,4,5)P4 mass levels in hippocampus and cerebral cortex, whereas in striatal slices these responses declined significantly over a 30-min incubation period (Jenkinson *et al.*, 1994). *In vivo* microdialysis studies have demonstrated the presence of extracellular levels of IP3 that can be increased in a concentration-dependent manner by muscarinic receptor activation (Roberts *et al.*, 1997). The muscarine effect was mimicked by oxotremorine-M; pirenzepine prevented the muscarine-induced IP3 increase, whereas 4-DAMP was ineffective. Expression of Ml muscarinic receptors coupled to phospholipase C and to internal calcium stores in cultured skeletal muscle is proposed; nicotinic receptors could be acting *via* ion fluxes and membrane depolarization (Reyes & Jaimovich, 1996).

EFFECT OF CARBACHOL AND MUSCARINIC ANTAGONISTS ON cGMP LEVELS IN ONE HOUR PANCREATIC ISLETS *IN VITRO*

cGMP modulation by neurotransmitters has functional relevance in living brain and peripheral tissues. Microdialysis studies have reported that the brain NO synthase/guanylyl cyclase pathway is mainly controlled by NMDA, AMPA and metabotropic glutamate receptors. It can also be influenced by other neurotransmitters like GABA, acetylcholine and neuropeptides through polysynaptic circuits interacting with glutamatergic system. Acetylcholine agonist, carbachol, at 10^{-7}M concentration significantly stimulated cGMP release in the pancreatic islets of young rats. Stimulatory effect of carbachol in cGMP release was reversed by pirenzepine at 10^{-6}M concentration in the presence of both 4 mM and 20 mM glucose in young rats. In old rats, carbachol induced cGMP release was inhibited by pirenzepine in 4mM glucose whereas it was inhibited by 4-DAMP mustard in 20mM glucose concentration. These

studies could be useful to get a better insight into the functional role of cGMP in physiological and pathological situations like diabetes, learning, memory formation, and age related neurodegenerative diseases (Fedele & Raiteri, 1999).

TRIIODOTHYRONINE (T3) CONTENT IN SERUM OF CONTROL AND EXPERIMENTAL RATS

Thyroid hormone is essential for maintaining normal neurological functions both during development and in adult life. Type III-iodothyronine deiodinase (D3) degrades thyroid hormones by converting thyroxine and 3,3′,5-triiodo-L-thyroinine (T3) to inactive metabolites. A regional expression of D3 activity has been observed in the human CNS, and a critical role for D3 has been suggested in the regulation of local T3 content in concert with other enzymes. The serum T3 levels, basal TSH levels and TSH response to thyrotropin releasing hormone (TRH) is influenced by the glycemic status (Schlienger *et al.*, 1982). T3 content in the serum was increased significantly in 7 weeks and 90 weeks old diabetic groups compared to control. Long term thyrotoxicosis has been shown to cause β cell dysfunction resulting in reduced pancreatic insulin content, poor insulin response to glucose and decreased rate of insulin secretion (Bech *et al.*, 1996). Insulin treatment significantly reversed the increased T3 content near to control. In control old rats, T3 content decreased significantly compared to the young rats. Also, 90 weeks old diabetic and D+I groups showed decreased T3 content compared to 7 weeks old diabetic and D+I groups. A reduced secretion of thyroid hormones with age has been documented in humans and animals with no substantial increase in TSH secretion, which is indicative of an age-related impairment of the pituitary sensitivity to the negative control exerted by thyroid hormones (Schlienger *et al.*, 1982).

NE, EPI, DA AND 5-HT CONTENT IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

Cerebral cortex

α and β-adrenergic neurotransmission have important roles in regulating STH secretion. NE content in the cerebral cortex decreased significantly in saline treated old rats compared to young rats. STH treated and INS treated young rats showed decreased NE content when compared to saline treated rats. In old rats, NE content showed no change in STH treated rats whereas INS treated rats showed significant decrease when compared to saline treated rats. Insulin signalling has been implicated as an important factor in invertebrate and vertebrate development, nutrient signalling, growth and ageing (Hafen, 2004; Bartke, 2006). EPI content in the cerebral cortex decreased significantly in saline treated old rats compared to young rats. EPI content showed significant decrease in both STH treated rats and INS treated young rats when compared to saline treated young rats. In old rats, EPI content decreased significantly in both STH treated and INS treated rats when compared to saline treated rats. α_2 adrenergic neuron appears to be dominant since co-administration of clonidine (α_2 adrenergic antagonist) and atropine stimulate STH release. Furthermore, treatment with yohimbine (α_2 adrenergic antagonist) can completely block the stimulatory effects on STH secretion of enhancing cholinergic tone with pyridostigmine, a cholinesterase inhibitor (Devassa *et al.*, 1991). Blockade of β-adrenergic receptors enhances the STH response to STHRH and other provocative stimuli appear to have no effect on spontaneous STH secretion (Blizzard & Rogol, 1988).

Although the mechanism responsible for cognitive deficits in stress-related neuropsychiatric disorders has been obscure, prefrontal cortical (PFC) dopaminergic dysfunction is thought to be involved. In animals, the mesoprefrontal dopaminergic system is particularly vulnerable to stress. PFC is a cortical area involved in selecting and retaining information to produce complex behaviours (Arianna *et al.*, 2007). DA

is known to influence human STH release both *in vivo* and *in vitro*. DA content decreased significantly in saline treated old rats compared to young rats. DA content in the cerebral cortex of young rats decreased significantly in both STH treated and INS treated rats compared to saline treated rats. STH treated and INS treated old rats showed significant decrease in DA content, when compared to saline treated rats. Various studies have reported that stress reduces DA transmission in the PFC (Mizoguchi *et al.*, 2000). There is an optimal DA receptor stimulation for proper PFC function (Zahrt *et al.*, 1997; Arnsten & Goldman-Rakic, 1998), which indicates an important role for DA modulation of the neural processes within the PFC in working memory. Dopamine can stimulate basal STH release at the pituitary level and that this stimulation is mediated through D1 receptors (Bluet-Pajot, 1990). Administration of STHRH antagonist significantly suppresses the stimulatory effect of L-dopa on STH release (Jaffe *et al.*, 1996). 5-HT content decreased significantly in saline treated old rats compared to young rats. 5-HT content decreased significantly in both STH treated and INS treated young rats compared to saline treated rats. In old rats, 5-HT content increased in STH treated rats whereas INS treated rats showed significant decrease in the 5-HT content when compared to saline treated rats. Specific 5-HT_{1D} receptor agonist, sumatriptan stimulates STH secretion in humans (Rolandi *et al.*, 1992; Herdman *et al.*, 1994). Our studies showed that in cerebral cortex low dose long-term treatment of INS and STH functionally improved the adrenergic neurotransmission that will influence emotion, motivation, cognition and many other aspects of behaviour as a function of age.

Corpus striatum

NE is reported to amplify the mitogenic signals of both EGF and HGF by acting through STH α_1 adrenergic receptors. It induces the production of EGF and HGF at distal sites and also enhances the response to HGF at target tissues (Broten, *et al* 1999). NE content in the corpus striatum decreased significantly in saline treated

old rats compared to young rats. NE content showed no significant change in both STH treated and INS treated young rats when compared to saline treated rats. In old rats, NE content increased significantly in both STH treated and INS treated rats when compared to saline treated rats. EPI content decreased significantly in saline treated, STH treated and INS treated old rats compared to young rats. EPI content increased significantly in both STH treated and in INS treated young rats compared to saline treated rats. STH treated old rats showed significant decrease in the EPI content whereas INS treated rats showed significant increase when compared to saline treated rats. Adrenergic neurotransmission changes in synaptic connectivity might affect plasticity and gene expression, resulting in altered dynamics of neuronal circuits in ageing brain (Geinisman *et al.*, 1992; Nicholson *et al.*, 2004).

Dopamine containing neurons arise mainly from DA cell bodies in the substantia nigra and ventral tegmental area in mid-brain region (Tarazi *et al.*, 1997 a, b, 1998 a, b, 2001; Tepper, *et. al.*, 1997; Royh, *et. al.*, 1991; Carlsson, 1993; Lookingland *et al.*, 1995). DA content decreased significantly in saline treated old rats compared to young rats. DA content in the corpus striatum of young rats decreased significantly in both STH treated and INS treated rats compared to saline treated rats. DA D_2 receptors are reported to regulate the release of DA from dopaminergic neurons originating in the ventral tegmental area as well as in the substantia nigra (Plantje *et al.*, 1987). *In vivo* release of DA from mesolimbic and neostriatal DA neurons appears to be modulated by DA D_2 receptors, whereas both receptor types can modulate DA metabolism (Boyar & Altar, 1987). STH treated old rats showed no significant change in DA content whereas INS treated rats showed significant increase when compared to saline treated rats. 5-HT content decreased significantly in saline treated old rats compared to young rats. 5-HT content increased significantly in STH treated whereas INS treated young rats showed no significant change compared to saline treated rats. STH treated old rats showed no significant change in 5-HT content whereas INS treated rats showed significant increase when

compared to saline treated rats. The results suggest that low dose long term INS and STH treatment have functional regulatory role in dopaminergic and serotonergic neurotransmitter functions that is critically involved in many aspects of complex behaviour and cognition beyond reward/reinforcement and motor function as a function of age.

Brainstem

Brainstem is an important part of the brain in monitoring the glucose status and the regulation of feeding (Guillod *et al.*, 2003). It has been established that central nervous system cell groups projecting into the pancreatic vagal motor neurons received inputs from the adrenergic, noradrenergic and serotonergic neurons from the lower brainstem (Lowey *et al.*, 1994). Lesions in these brain regions are reported to affect the neuronal cell population and growth. NE content in the brainstem decreased significantly in saline treated old rats compared to young rats. NE content decreased significantly in STH treated young rats whereas INS treated young rats showed no significant change compared to saline treated rats. In old rats, NE content decreased significantly in both STH treated and INS treated rats compared to saline treated rats. Electrical stimulation of brainstem nuclei, the locus coeruleus, rostral portion of the nucleus tractus solitarius and lateral reticular nucleus suppressed STH secretion and the central gray of the pons showed a tendency for the suppression of STH secretion. Electrical stimulation of brainstem nuclei excites somatostatin neurons in the periventricular nucleus which are responsible for the suppression of STH secretion (Noriyuki *et al.*, 1989). EPI content decreased significantly in saline treated old rats compared to young rats. EPI content decreased significantly in both STH treated and INS treated young rats compared to saline treated young rats respectively. EPI content decreased significantly in STH treated old rats whereas INS treated old rats showed no significant change when compared to saline treated old rats. Alterations of central adrenergic neurotransmission can change the relative contribution of sympathetic

outflow to the pancreas, liver, adrenal medulla and adipose tissues, leading to the modulation of glucose and fat metabolism (Nonogaki, 2000).

Dopaminergic neurotransmission is critically involved in many aspects of complex behaviour and cognition beyond reward/reinforcement and motor function. DA content in the brainstem decreased significantly in saline treated old rats compared to young rats. DA content decreased significantly in both STH treated and INS treated young rats compared to saline treated young rats. STH treated old rats showed significant decrease in DA content whereas INS treated rats showed significant increase when compared to saline treated rats. Saline treated old rats showed no significant change in 5-HT content compared to young rats. 5-HT content decreased significantly in both STH treated and INS treated young rats compared to saline treated rats. STH treated old rats showed significant decrease in 5-HT content whereas INS treated rats showed no significant change when compared to saline treated old rats. Our previous studies demonstrated adrenergic, serotonergic and DA D_2 receptor function alterations in the brainstem of diabetic rats (Abraham & Paulose, 1999; Padayatti & Paulose, 1999; Paulose *et al.*, 1999; Eswar *et al.*, 2007). The significant changes in the DA and 5-HT content showed the regulatory role of these neurotransmitters in rejuvenation of striatal function by regulating neural plasticity and enhancing the repair as a function of age.

Hypothalamus

Noradrenergic systems are integrally involved in the release of STH from the anterior pituitary gland and in regulating the activity of hypothalamic growth hormone-releasing hormone (STHRH) neurones. STH secretagogues act at both the pituitary and the hypothalamus to facilitate the release of STH. NE content decreased significantly in saline treated old rats compared to young rats. NE content in the hypothalamus of both STH treated and INS treated young rats decreased significantly when compared to saline treated young rats. However, in both STH treated and INS

treated old rats, NE content decreased significantly when compared to saline treated old rats. EPI content decreased significantly in saline treated and STH treated old rats whereas in INS treated old rats EPI content increased significantly compared to young rats. EPI content decreased significantly in both STH treated and in INS treated young rats compared to saline treated rats. Both STH treated and INS treated old rats showed significant increase in the EPI content when compared to saline treated rats. Our results suggest that adrenergic receptor alterations found in the hypothalamus during the treatment could contribute to efficient management of cognitive and memory deficits on ageing.

Dopaminergic action is important in the regulation of hypothalamic-pituitary hormone release. Also, dopamine and its receptors are implicated in satiety, hunger, and body weight maintenance. DA content decreased significantly in saline treated old rats compared to young rats. DA content in the hypothalamus of young rats decreased significantly in both STH treated and INS treated rats compared to saline treated rats. However, in both STH treated and INS treated old rats, DA content increased significantly when compared to saline treated old rats. 5-HT content decreased significantly in saline treated old rats compared to young rats. 5-HT content decreased significantly in both STH treated and INS treated young rats when compared to saline treated young rats. However, both STH treated and INS treated old rats showed significant increase in 5-HT content when compared to saline treated old rats. From our studies it is clear that in brain regions like corpus striatum, brainstem and hypothalamus, long term low dose treatment of INS and STH significantly increased the DA content when compared to saline treated old rats. This will have tremendous importance in functions including locomotor activity, cognition, emotion, positive reinforcement, food intake, and endocrine regulation. DA also plays multiple roles in the periphery as a modulator of cardiovascular function, catecholamine release, hormone secretion, vascular tone, renal function, and gastrointestinal motility (Missale *et al.*, 1998) on ageing.

GENE EXPRESSION STUDIES

MUSCARINIC RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL RATS

Cholinergic pathways in the central nervous system positively influence growth hormone (STH) secretion. Pyridostigmine, a cholinesterase inhibitor, enhances both basal and STHRH induced STH secretion. Pirenzepine, an antagonist of muscarinic M1 receptors, inhibits the STH response to STHRH and to other physiological and pharmacological stimuli. The Real-Time PCR analysis in the cerebral cortex showed a significant increase in the expression of muscarinic M1 receptor mRNA in both STH treated and INS treated young rats. However, both STH treated and INS treated old rats showed significant decrease in expression when compared to saline treated old rats. Muscarinic M3 receptor mRNA was highly expressed in STH treated and INS treated young rats. However, both STH treated and INS treated old rats showed decreased expression when compared to saline treated old rats. The effect of the cholinergic system on STH secretion takes place *via* inhibition of the release of endogenous somatostatin (Mazza *et al.*, 1994).

GLUTAMATE RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL RATS

The excitatory amino acids (EAAs), glutamate are pivotal elements in the hypothalamic circuitry involved in the control of pituitary function. The actions of EAAs are mediated by NMDA, kainate, AMPA and metabotropic receptors (Tena-Sempera *et al.*, 2000). NMDAR1 gene expression in the cerebral cortex increased significantly in both STH treated and INS treated young rats when compared to saline treated young rats. However, both STH treated and INS treated old rats showed significant decrease in NMDAR1 expression when compared to saline treated old rats. The analysis of interactions between EAA receptors and other neuronal pathways evidenced the close interactions between different systems involved in the control of

STH secretion. Blockade of glutamate receptors abolished the stimulatory effect of GABA and ghrelin on STH secretion and inversely, blockade of ghrelin or GABA receptors abolished the stimulatory effect of EAAs (Aguilar *et al.*, 2005). mGlu-5 gene expression in the cerebral cortex increased significantly in both STH treated and INS treated young rats when compared to saline treated young rats. However, mGlu-5 gene expression decreased significantly in both STH treated and INS treated old rats when compared to saline treated old rats. STH treated and INS treated old rats showed significant decrease in mRNA expression compared to young rats.

DOPAMINE RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL RATS

The DAD2 is the predominant dopamine-receptor subtype in the anterior pituitary and mediates dopamine's inhibitory actions on lactotropes (Missale *et al.* 1998, Ben-Jonathan & Hnasko 2001, Diaz-Torga *et al.* 2002, Cristina *et al.* 2005). The importance of D2R in normal body growth and STH secretion in the neonatal period has been studied (Diaz-Torga *et al.* 2002). DAD2 receptor gene expression in the cerebral cortex increased significantly in both STH treated and INS treated young rats when compared to saline treated young rats. However, both STH treated and INS treated old rats showed significant decrease in DAD2 expression when compared to saline treated old rats. DAD2R alters the STHRH -STH–insulin-like growth factor-I (IGF-I) axis, and impairs body growth. An anatomical basis for an interaction of the dopaminergic and the STHRH system has been provided by colocalization studies, which indicate that a subset of STHRH neurons in the ventrolateral part of the arcuate nucleus contains tyrosine hydroxylase, the key enzyme of catecholamine biosynthesis in rats (Niimi *et al.* 1992, Zoli *et al.* 1993), and mice (Phelps *et al.* 2003). Although some studies suggest that dopaminergic agonist stimulates the STH release *via* suppression of somatostatin (Guistina & Veldhuis, 1998), administration of STHRH antagonist significantly suppresses the stimulatory effect of L-dopa on STH release

(Jaffe *et al.*, 1996). In contrast, bromocriptine and other dopaminergic agonists inhibit STH release in patients with STH-secreting pituitary tumours (Jaffe & Barkan, 1992).

ADRENERGIC RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL RATS

Adrenergic pathways mediate the effects of physiological factors that regulate STH secretion. Many of the stimuli for growth hormone secretion such as physical and surgical stress, psychic stress, exercise, 2-deoxyglucose hypoglycaemia, histamine, vasopressin and pyrogen result in increased catecholamine concentrations in blood and tissues. α-adrenergic pathways mediate the STH response to insulin-induced hypoglycaemia, exercise and certain stresses since these responses can be blocked by the administration of phentolamine (Martin, 1973). α_{2A}-adrenergic receptor gene expression in the cerebral cortex increased significantly in both STH treated and INS treated young rats compared to saline treated young rats. However, the gene expression decreased significantly in both STH treated and INS treated old rats when compared to saline treated old rats. β_2-adrenergic receptor gene expression in the cerebral cortex increased significantly in STH treated young rats whereas INS treated young rats showed decreased expression compared to saline treated young rats. However, the gene expression decreased significantly in STH treated old rats whereas in INS treated old rats β_2-adrenergic receptor gene expression increased significantly when compared to saline treated old rats. The STH response to stress involves adrenergic pathways in the limbic system because blockade of catecholamine synthesis in the rat inhibits STH release induced by electrical stimulation of the hippocampus and the baso-lateral amygdala (Martin, 1973).

GABA RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL RATS

GABA is the main inhibitory neurotransmitter in the mammalian brain, and GABAergic transmission modulates a variety of other neurotransmitter and neuropeptide systems (Lloyd *et al.* 1989; Miller & Ferrendelli 1990). This neurotransmitter acts on two main receptors in the brain, $GABA_A$ and $GABA_B$ (Hill & Bowery 1981; Matsumoto 1989). $GABA_{A\alpha1}$ receptor gene expression in the cerebral cortex increased significantly in STH treated young rats whereas INS treated young rats showed decreased expression compared to saline treated young rats. However, the gene expression decreased significantly in both STH treated and INS treated old rats when compared to saline treated old rats. $GABA_B$ receptor sites are reported to be involved in the modulation of STH secretion (Gamse *et al.* 1980; Muller 1987). The administration of baclofen induces a significant increase in STH concentrations in normal healthy humans (Koulu *et al.* 1979). $GABA_B$ receptor gene expression in the cerebral cortex decreased significantly in STH treated young rats whereas INS treated young rats showed increased expression compared to saline treated young rats. However, the gene expression decreased significantly in both STH treated and INS treated old rats when compared to saline treated old rats. A significant reduction of STH response to baclofen in patients with major depression compared with matched healthy controls, supporting the idea that down regulated $GABA_B$ receptor function is associated with depression (Marchesi *et al.* 1991; O'Flynn & Dinan 1993; Monteleone *et al.* 1990a). In STH-producing cells, GABA acts as an autocrine factor *via* $GABA_B$ receptors to control STH levels (Katia Gamel-Didelon *et al.*, 2002).

5-HT_{2C} RECEPTOR GENE EXPRESSION IN THE CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL RATS

5-HT_{2C} receptors play a stimulatory role on STH secretion by acting through a decrease in hypothalamic somatostatin release (Ignacio Valverde *et al.*, 2000). 5-HT_{2C} receptor gene expression in the cerebral cortex decreased significantly in STH treated young rats whereas INS treated young rats showed increased expression compared to saline treated young rats. However, 5-HT_{2C} receptor gene expression increased significantly in both STH treated and INS treated old rats when compared to saline treated old rats. The regulation of STH secretion and STH mRNA content by the serotonergic agonist, quipazine (QUIP) indicate that this neurotransmitter not only regulate STH secretion but also regulate STH mRNA content and thus affect hormone synthesis.

IP3 CONTENT IN DIFFERENT BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

Intracellular Ca^{2+} release channels are key players in the regulation of Ca^{2+} homeostasis. The impairment of Ca^{2+}-dependent protein kinase C phosphorylation of endogeneous substrates regulates the quantitative alterations of PKC and IP3 receptor in phosphoinositide metabolism (Martini *et al.*, 1994). The cerebral cortex of saline treated old rats showed significant increase in IP3 content when compared to saline treated young rats. IP3 content decreased significantly in the cerebral cortex and corpus striatum of both STH treated and INS treated young rats when compared to saline treated young rats. However, the cerebral cortex of both STH treated and INS treated old rats showed increased IP3 content whereas corpus striatum and brainstem showed decreased IP3 content when compared to saline treated old rats. Several studies suggest that alterations in the receptor-mediated phosphoinositide cascade are involved in the pathophysiology of ageing. Neuronal Ca^{2+} signalling through inositol

triphosphate receptors in low dose long term INS and STH treatment regulate cell viability and cellular metabolic pathways.

cGMP CONTENT IN DIFFERENT BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

Altered modulation of cGMP levels in brain seems to be responsible for the impairment in cognitive function (Erceg *et al.*, 2005). cGMP content increased significantly in the cerebral cortex of STH treated and INS treated young rats whereas in the corpus striatum and brainstem, cGMP content decreased significantly when compared to saline treated young rats. STH treated and INS treated old rats showed increased cGMP content in cerebral cortex whereas in corpus striatum and brainstem, STH treated old rats showed no significant change in the cGMP content whereas INS treated old rats show decreased cGMP content when compared to saline treated old rats. An increase in cGMP content was observed in the cerebral cortex and brainstem whereas in corpus striatum, cGMP content was decreased when compared to saline treated young rats. Low dose long term INS and STH treatment differentially regulates cGMP content in brain regions which is a common regulator of ion channel conductance, glycogenolysis and cellular apoptosis (Sharron & Jackie, 1999).

TRIIODOTHYRONINE (T3) CONTENT IN SERUM OF CONTROL AND EXPERIMENTAL RATS

Thyroid hormones are key players in tissue differentiation, growth, and functioning with major effects on metabolism and oxygen consumption. The thyroxine (T4) is important for transport and negative feed-back regulation. T3 is the active thyroid hormone acting at the target cells. T3 content was increased significantly in the serum of STH treated young rats whereas in INS treated young rats, T3 content decreased significantly when compared to saline treated rats. T3 content decreased significantly in the cerebral cortex of both STH treated and INS

treated old rats when compared to saline treated old rats. There was significant increase in T3 content in saline treated old rats compared to saline treated young rats.T3 stimulates mRNA transcription, resulting in protein synthesis and anabolic effects. Also, it stimulates Na+, K+-ATPase at the cell membrane, thus increasing O_2 consumption. Other effects are increased temperature, behavioural activity, weight loss, increased glucose turn-over, cholesterol catabolism, stimulation of growth and maturation (Veerkamp *et al.*, 2003).

CALCIUM IMAGING

Intracellular Ca^{2+} plays a major role in the physiological responses of excitable cells, and excessive accumulation of internal Ca^{2+} is a key determinant of cell injury and death (Yubo *et al.*, 2000). The production and metabolism of IP3 require several calcium-dependent enzymes (Mitsuhiro *et al*, 2007). ACh-induced hyperpolarizing responses were blocked by atropine and pirenzepine (Gulledge & Stuart, 2005). Our studies showed that carbachol, the cholinergic agonist, significantly increased Ca^{2+} release from the pancreatic islet cells of young and old rats *in vitro*. Stimulatory effect of carbachol on Ca^{2+} release was inhibited by the addition of muscarinic M1 and M3 receptor antagonists such as pirenzepine and 4-DAMP mustard respectively. Pirenzepine, significantly inhibited carbachol induced Ca^{2+} release in both young and old rats islets when compared to 4-DAMP mustard. Transient activation muscarinic M1 receptors induced calcium release from IP3-sensitive intracellular Ca^{2+} stores and subsequent activation of an apamin-sensitive, SK-type calcium-activated potassium conductance (Gulledge & Stuart, 2005). Recent studies have shown that IP3 production was increased by a combination of acetylcholine receptor and Ca^{2+} dependent components, rather than by Ca^{2+} alone. Increases in Ca^{2+} levels prior to receptor activation did not affect the subsequent receptor induced IP3 increase, indicating that Ca^{2+} does not influence IP3 production without receptor activation. Removal of both the receptor agonists and Ca^{2+} rapidly

restored calcium and IP3 levels, whereas removal of Ca^{2+} alone restored Ca^{2+} to its basal concentration (Mitsuhiro *et al*, 2007).

ELECTROPHYSIOLOGICAL CHANGES IN HYPERGLYCAEMIC BRAIN

Evidences suggest that the neurological symptoms characteristic of hypoglycaemic encephalopathy prior to isoelectric EEG stages result from neurotransmission failure (Butterworth, 1999). There was a significant change in the brain wave activity of diabetic rats compared to the control which shows the neurophysiological changes in diabetic condition. Recurrent severe hypoglycaemia and poor metabolic control are risk factors for EEG abnormalities in adolescents with type 1 diabetes receiving multiple insulin injection therapy treatment (Hyllienmark *et al.*, 2005). EEG at the time of diagnosis of IDDM is reported to be useful in identifying those patients at increased risk for coma and/or convulsion (Tupola *et al.*, 1998). Learning and memory deficits are associated with Type 1 and Type II diabetes mellitus (Gispen & Biessels, 2000) and brain morphological abnormalities have been found in diabetic patients, mainly in the cortex area (Dejgaard *et al.*, 1991). Previous studies also showed similar changes in electrical activity in the brain of diabetic rats (Gireesh, 2007). It is reported that metabolic control influences the EEG and improvement of glucose metabolism is an important factor in avoiding EEG abnormalities in young diabetic patients (Hauser *et al.*, 1995). Our results showed significant changes in the brain activity in the frontal region of 7 weeks and 90 weeks old diabetic rat groups. The brain activity differences observed clearly shows that in aged brain the damage is much more than in young brain during hyperglycaemic state. Insulin treatment significantly alters the change in brain wave activity near to control rats. Long term low dose insulin and somatotropin treatment was found to be pronounced in altering the EEG pattern in both young and old rats. In conclusion, low dose long-term treatment of INS and STH functionally improved the neurotransmitter receptor subtypes status and brain wave signalling pattern in brain regions. Thus the

results highlight the rejuvenation of brain function by regulating neural plasticity, reducing the damage and enhancing the repair as a function of age.

Our results showed a prominent cholinergic functional disturbance in the diabetic brain as a function of age. The receptor mediated functional studies and *in vitro* studies using antagonists for the receptor subtypes confirmed the specific receptor mediated neurotransmitter changes during diabetes. These findings have important implications for understanding the molecular mechanisms underlying cognitive impairment due to diabetes on ageing. Electrophysiological studies using EEG recording in young and old rats showed a prominent brain activity difference during diabetes. Long term low dose STH and INS administration found to be pronounced in improving memory, cognitive impairment and rejuvenating brain functions on ageing. Studies on neurotransmitter receptor interaction pathways and gene expression regulation by second messengers like IP3 and cGMP in turn lead to the development of therapeutic agents to manage diabetes and efficient brain function during ageing.

Our results suggests that muscarinic M1, M3, glutamate NMDAR1, mGlu-5, α_{2A}, β_2, $GABA_{A\alpha1}$ and $GABA_B$, DAD2 and $5\text{-}HT_{2C}$ receptors functional balance mediated through IP3 and cGMP will lead to therapeutic applications in the management of diabetes. Also, long term low dose STH and INS treatment rejuvenated the brain function which has clinical significance in improved life during ageing.

Summary

1. Streptozotocin induced diabetic rats were used as model to study the alterations of Muscarinic M1 and M3 receptors and their regulation by insulin treatment.

2. Acetylcholine esterase (AChE) activity was used as a marker for cholinergic function. AChE activity was decreased in the cerebral cortex, brainstem and corpus striatum of old rats compared to young rats. During diabetic stage it was increased in both young and old rats in cerebral cortex, and corpus striatum while in brainstem it was decreased. In insulin treated rats the activity of the enzyme was reversed to near control.

3. Muscarinic receptor functional status was analysed by Scatchard and displacement analysis using specific [^{3}H] ligands. Receptor analysis was confirmed by studying the mRNA status of the corresponding receptor using Real-Time PCR.

4. Muscarinic M1 receptors of old rats were down regulated in cerebral cortex while in corpus striatum and brainstem it was up regulated. Muscarinic M3 receptors of old rats showed no significant change in cerebral cortex while in corpus striatum and brainstem, muscarinic receptors were down regulated.

5. During diabetes, muscarinic M1 receptors were down regulated in cerebral cortex and brainstem of young rats while in corpus striatum they were up regulated. In old rats, M1 receptors were up regulated in cerebral cortex, corpus striatum and in brainstem they were down regulated.

Muscarinic M3 receptors were up regulated in cerebral cortex and brainstem of young rats while in corpus striatum they were down regulated. In old rats, muscarinic M1 receptors were up regulated in cerebral cortex, corpus striatum and brainstem. In insulin treated diabetic rats the activity of the receptors were reversed to near control. Muscarinic M3 receptors were up regulated in the pancreas of both young and old rats during diabetes.

6. Gene expression studies using DA D2 and β_2 receptor mRNA showed an increased expressional status whereas α_{2A} adrenergic, $GABA_{A\alpha I}$, $GABA_B$ and 5-HT_{2C} receptor gene expression were decreased in the cerebral cortex of young diabetic rats. Also, an increased DA D2, α_{2A} adrenergic and 5-HT_{2C} expressional status was observed in old diabetic rats whereas β_2 adrenergic, $GABA_{A\alpha I}$ and $GABA_B$ expression were decreased in old diabetic rats.

7. Gene expression studies using glutamate receptor NMDAR1 showed decreased expressional status in the cerebral cortex, corpus striatum and hippocampus whereas it was increased in brainstem of young diabetic rats. mGlu-5 receptor mRNA showed increased expressional status in the cerebral cortex and brainstem whereas it was increased in corpus striatum and hippocampus of old diabetic rats.

8. *In vitro* studies in pancreatic islets using carbachol and muscarinic M1 and M3 antagonists showed that glucose stimulated insulin secretion were mediated through muscarinic M1 receptors in young rats whereas it was mediated through muscarinic M3 receptors in old rats at normal glucose concentration. In diabetic condition, glucose induced insulin secretion was mediated through muscarinic M1 receptors in young and old rats.

9. IP3 and cGMP content increased in young diabetic rat brain regions whereas both second messengers decreased significantly in old diabetic rat brain regions.

10. *In vitro* studies in pancreatic islets using carbachol and muscarinic M1 and M3 antagonists showed that IP3 and cGMP release were mediated through Muscarinic M1 and M3 receptors in pancreatic islets. In diabetic condition, IP3 and cGMP release were mediated through muscarinic M1 receptors in young rats whereas IP3 and cGMP release were mediated through muscarinic M3 receptors in old rats.

11. *In vitro* studies in pancreatic islets using dopamine and carbachol showed that M1 and M3 mediated IP3 and cGMP release were inhibited at lower dopamine concentration whereas IP3 and cGMP release were stimulated at higher dopamine concentration in young and old rats.

12. Serum T3 concentration was decreased in old rats compared to the young rats. During diabetes, serum T3 concentration was increased in both young and old rats. In insulin treated diabetic rats the concentration was reversed to near control.

13. NE, EPI and 5-HT content decreased in the cerebral cortex, corpus striatum, brainstem and hypothalamus of long time low dose somatotropin and insulin treated old rats compared to treated young rats. The AChE activity was increased in the cerebral cortex of long term low dose insulin and somatotropin treated young and old rats compared to saline treated young and old rats. An increased DA content was observed in corpus striatum, brainstem and hypothalamus of somatotropin and insulin treated old rats compared to saline treated old rats. No significant change in DA content was observed in somatotropin and insulin treated young rats compared to saline treated young rats.

14. Gene expression studies using muscarinic M1, M3, glutamate receptor NMDAR1, mGlu-5, DAD2, α_{2A}, β_2 adrenergic, $GABA_{A\alpha1}$ and $GABA_B$ showed an increased expressional status in the cerebral cortex of long term low dose insulin and somatotropin treated young rats whereas 5-HT_{2C} gene expression was decreased in insulin and somatotropin treated young rats.

    Somatotropin and insulin treated old rats showed decreased muscarinic M1, M3, glutamate receptor NMDAR1, mGlu-5, α_{2A}, $GABA_{A\alpha1}$ and $GABA_B$ expressional status whereas DAD2 and 5-HT_{2C} mRNA showed an increased expressional status in the cerebral cortex when compared to saline treated old rats. Our studies on long term low dose treatment of INS and STH improved the cholinergic, glutamergic, adrenergic, dopaminergic, GABAergic and serotonergic receptor function in the cerebral cortex. Thus the results suggest the rejuvenation of brain function by increased neural plasticity and activity as a function of age.

15. Serum T3 concentration was decreased in STH treated young and old rats compared to saline treated young and old rats. In insulin treated young and old rats the content was reversed to near control. Our results suggest that the balanced metabolic hormones make the cellular function more efficient.

16. Calcium imaging results showed that carbachol, the cholinergic agonist, increased the Ca^{2+} release from the pancreatic islet cells of young and old rats *in vitro*. Stimulatory effect of carbachol on Ca^{2+} release was mediated through muscarinic M1 receptors in young and old rats.

17. A prominent brain activity difference was observed in diabetic rats when compared to control rats by EEG analysis. Long term low dose insulin and STH treatment found to be pronounced in brain wave signaling pattern in old rats. This

will have tremendous importance in improving memory, cognitive impairment and rejuvenating brain functions on ageing.

It is evident from our results that brain muscarinic M1 and M3 receptor functional balance plays a major role in cholinergic functional regulation during diabetes as a function of age. Gene expression studies of muscarinic M1 and M3 receptors showed a prominent cholinergic functional difference in brain regions of diabetic rats. *In vitro* studies confirmed the regulatory role of acetylcholine, muscarinic M1 and M3 receptor subtypes in IP3 and cGMP release by pancreatic islets. These findings have important implications for understanding the molecular mechanisms underlying memory and cognitive impairment by second messengers due to diabetes and ageing. A reduced secretion of thyroid hormones with age was observed, which is an indicative of an age-related impairment in metabolic and neurological functions. Calcium imaging studies revealed a prominent role of muscarinic M1 mediated Ca^{2+} release from the pancreatic islet cells of young and old rats. Electrophysiological studies using EEG recording showed disturbed brain activity during diabetes in old rats compared to young. Long term low dose STH and INS administration found to be effective in neurotransmitter receptor functional regulation in improving memory, cognitive impairment and rejuvenating brain functions.

The functional improvement of muscarinic M1, M3, glutamate NMDAR1, mGlu-5, α_{2A}, β_2, $GABA_{A\alpha1}$ and $GABA_B$, DAD2 and $5\text{-}HT_{2C}$ receptors mediated through IP3 and cGMP will lead to therapeutic applications in the management of diabetes. Also, long term low dose STH and INS treatment improved brain function which has clinical significance in managing functional deterioration during ageing.

Conclusion

We conclude from our studies that acetylcholine through muscarinic M1 and M3 receptors play an important role in the brain function during diabetes as a function of age. Cholinergic activity as indicated by acetylcholine esterase, a marker for cholinergic function, decreased in the brain regions - the cerebral cortex, brainstem and corpus striatum of old rats compared to young rats. In diabetic condition, it was increased in both young and old rats in cerebral cortex, and corpus striatum while in brainstem it was decreased. The functional changes in the muscarinic receptors were studied in the brain regions and it showed that muscarinic M1 receptors of old rats were down regulated in cerebral cortex while in corpus striatum and brainstem it was up regulated. Muscarinic M3 receptors of old rats showed no significant change in cerebral cortex while in corpus striatum and brainstem muscarinic receptors were down regulated. During diabetes, muscarinic M1 receptors were down regulated in cerebral cortex and brainstem of young rats while in corpus striatum they were up regulated. In old rats, M1 receptors were up regulated in cerebral cortex, corpus striatum and in brainstem they were down regulated. Muscarinic M3 receptors were up regulated in cerebral cortex and brainstem of young rats while in corpus striatum they were down regulated. In old rats, muscarinic M1 receptors were up regulated in cerebral cortex, corpus striatum and brainstem. In insulin treated diabetic rats the activity of the receptors were reversed to near control. Pancreatic muscarinic M3 receptor activity increased in the pancreas of both young and old rats during diabetes. *In vitro* studies using carbachol and antagonists for muscarinic M1 and M3 receptor subtypes confirmed the specific receptor mediated neurotransmitter changes during diabetes. Calcium imaging studies revealed muscarinic M1 mediated Ca^{2+} release from the pancreatic islet cells of young and old rats. Electrophysiological studies using EEG recording in young and old rats showed a brain activity difference during

diabetes. Long term low dose STH and INS treated rat brain tissues were used for gene expression of muscarinic M1, M3, glutamate NMDAR1, mGlu-5, α_{2A}, β_2, $GABA_{A\alpha1}$ and $GABA_B$, DAD2 and 5-HT_{2C} receptors to observe the neurotransmitter receptor functional interrelationship for integrating memory, cognition and rejuvenating brain functions in young and old. Studies on neurotransmitter receptor interaction pathways and gene expression regulation by second messengers like IP3 and cGMP in turn will lead to the development of therapeutic agents to manage diabetes and brain activity.

Thus from our results it is suggested that functional improvement of muscarinic M1, M3, glutamate NMDAR1, mGlu-5, α_{2A}, β_2, $GABA_{A\alpha1}$ and $GABA_B$, DAD2 and 5-HT_{2C} receptors mediated through IP3 and cGMP will lead to therapeutic applications in the management of diabetes. Also, our results from long term low dose STH and INS treatment showed rejuvenation of the brain function which has clinical significance in maintaining healthy period of life as a function of age.

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Papers Published

1. T .R. Renuka, **B. Savitha** and C. S. Paulose **(2005).** Muscarinic M1 and M3 receptor binding alterations in pancreas during pancreatic regeneration of young rats, ***Endocrine Research***: 31(4), 259-270.

2. V. Ani Das, **B. Savitha** and C. S. Paulose **(2006).** Decreased alpha1-adrenergic receptor binding in the cerebral cortex and brain stem during pancreatic regeneration in rats. ***Neurochemical Research***, 31 (6), 727-734.

Awards/Achievements

1. **University Senior Research Fellow**, Cochin University of Science and Technology, Cochin, Kerala, INDIA. October 6th, 2006- to date.

2. **University Junior Research Fellow**, Cochin University of Science and Technology, Cochin, Kerala, INDIA. October 6th, 2004- October 5th, 2006.

3. Selected for "**4th Congress of the Federation of Asian-Oceanian Neuroscience Societies (FAONS)**" conducted in Hong Kong during November 30th - December 2nd, 2006.

4. Received "**THE APRC-IBRO & FAONS Award** for travel assistance to attend the **4th FAONS Congress**" conducted in Hong Kong during November 30th - December 2nd, 2006.

5. Selected for **6th IBRO School of Neuroscience**, Asia Pacific Region, organised by National Centre for Biological Sciences (NCBS), Bangalore, INDIA, August 8th - 20th, 2005.

6. **1st Rank** in Ph.D. Entrance Examination, Department of Biotechnology, Cochin University of Science and Technology, Cochin, Kerala, INDIA, 2004.

7. **3rd Rank** in MSc Biotechnology, Cochin University of Science and Technology, Cochin, Kerala, INDIA, 2003.

8. **Sri M. P. Pathrose Memorial Endowment** for securing highest marks in B. Sc. Zoology in the University Examination held in March-April 2000-2001.

9. Secured **1st position** for BSc Zoology, Christ College, Irinjalakuda, Thrissur (Dist), Kerala, INDIA, 2001.

10. Secured **3rd prize** in Inter Collegeate Zoo-Quiz competition, St. Joseph's College, Irinjalakuda, Thrissur (Dist), Kerala, INDIA, 2000.

Abstracts Presented

1. C. S. Paulose and **Savitha Balakrishnan**, Adrenergic, dopaminergic and serotonergic gene expression in low dose long time insulin and somatotropin treatment to ageing rats: rejuvenation of brain function. International Symposium on Regenerative Neuroscience, National Institute of Mental Health & Neuro Sciences (NIMHANS) Bangalore, **January 17th -18th, 2008.**

2. Pretty Mary Abraham and **Savitha Balakrishnan,** Kinetic parameters of glutamate dehydrogenase in the kidney of streptozotocin induced and insulin treated diabetic rats as a function of age. 76th Annual meeting SBC(I), Tirupati, **November 24th - 26th, 2007.**

3. **Savitha Balakrishnan** and C. S. Paulose, Muscarinic M3 receptors functional regulation in the corpus striatum of streptozotocin induced diabetic rats: its regulatory role in insulin secretion as a function of age. "National Conference on Molecular Medicine", organised by Society for Biotechnologists (INDIA) and Amrita Institute of Medical Sciences, Kochi, **January 13th -14th, 2007.**

4. **Savitha Balakrishnan** and C. S. Paulose, Cholinergic functional regulation and muscarinic M3 receptors gene expression in brain stem of streptozotocin induced diabetic rats as a function of age. Oral presentation in "4th Congress of the Federation of Asian-Oceanian Neuroscience Societies (FAONS)" conducted in Hong Kong during **November 30th - December 2nd, 2006.**

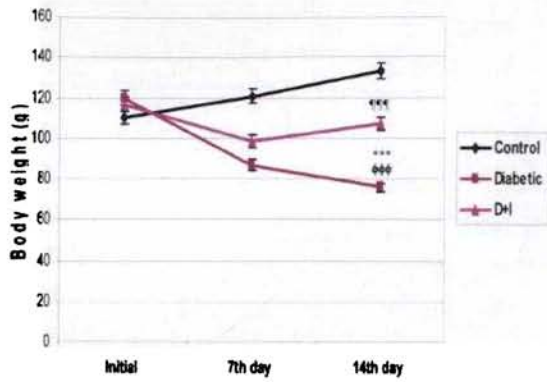
5. **Savitha Balakrishnan**, Up-regulation of cholinergic receptors in the cerebral cortex of Hepatectomised rats. Abstract in proceedings, XVIII Kerala Science Congress, pp no. 199-200, organised by Centre for Earth Science Studies, Thiruvananthapuram, **January 29th –31st, 2006.**

6. **Savitha Balakrishnan** and C. S. Paulose, Up-regulation of cholinergic receptors in the cerebral cortex of Hepatectomised rats. Poster presentation in 6th IBRO School of Neuroscience, Asia Pacific Region, organised by National Centre for Biological Sciences (NCBS), Bangalore, **August 8th - 20th, 2005.**

7. **Savitha Balakrishnan,** Ani Das V and C. S. Paulose, Decreased α_1 and α_2 Adrenergic receptors in the pancreatic islets during pancreatic regeneration in young rats. Abstract in proceedings, International Conference on Biotechnology and Neuroscience, pp no. 322-325, organised by Centre for Neuroscience, Cochin University of Science and Technology, Cochin, **December. 29th –31st, 2004.**

8. Akash. K. George, Remya Robinson, **Savitha Balakrishnan** and C. S. Paulose, Decreased 5-HT content and increased ALDH activity in the liver of Alcoholic rats. Abstract presented in IAN, SNCI, International Conference, University of Hyderabad, **May 2004**.

Figure-1
Body weight (g) of 7 weeks (Young) Experimental rats



Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats.
***p<0.001 when compared with control, ♦♦♦p<0.001 when compared with initial weight, ¶¶¶p<0.001 when compared with diabetic group.
D + I- Insulin treated diabetic

Table-1
Body weight (g) of 7 weeks (Young) Experimental rats

Animal status	Initial	7th day	14th day
Control	110.0 ± 10.0	120.5 ± 12.0	133.3 ± 14.5
Diabetic	120.0 ± 5.7	86.6 ± 4.6	75.7 ± 3.6*** ♦♦♦
D + I	116.6 ± 3.4	98.2 ± 2.0	106.6 ± 5.3¶¶¶

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats.
***p<0.001 when compared with control, ♦♦♦p<0.001 when compared with initial weight, ¶¶¶p<0.001 when compared with diabetic group.
D + I- Insulin treated diabetic

Figure-2
Body weight (g) of 90 weeks (Old) Experimental rats

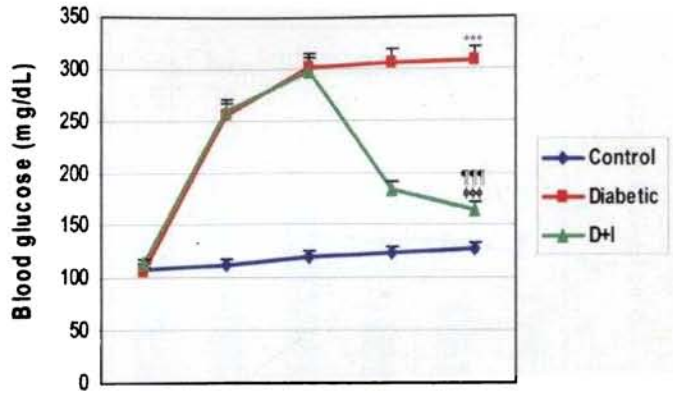
Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats.
***p<0.001 when compared with control, ϕϕϕp<0.001 when compared with initial weight, ¶¶¶ p<0.001 when compared with diabetic group.
D + I- Insulin treated diabetic

Table-2
Body weight (g) of 90 weeks (Old) Experimental rats

Animal status	Initial	7th day	14th day
Control	320.0 ± 12.0	325.0 ± 14.3	330.0 ± 15.0
Diabetic	360.0 ± 16.5	280.2 ± 3.3	250.7 ± 2.3*** ϕϕϕ
D + I	350.0 ± 13.3	300.6 ± 5.0	310.0 ± 5.8¶¶¶

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats.
***p<0.001 when compared with control, ϕϕϕp<0.001 when compared with initial weight, ¶¶¶ p<0.001 when compared with diabetic group.
D + I- Insulin treated diabetic

Figure-3
Blood glucose (mg/dL) level in 7 weeks (Young) Experimental rats



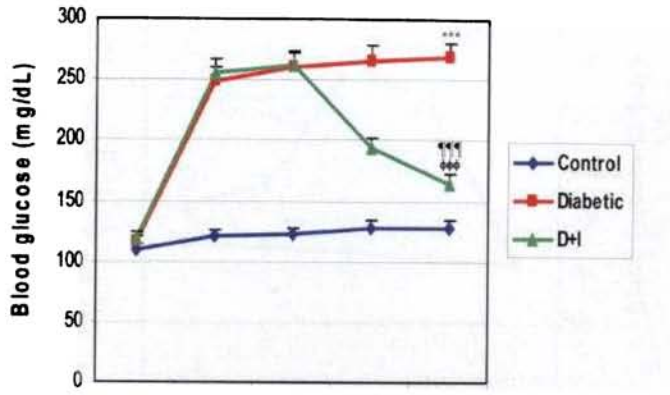
Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats.
*** p<0.001 when compared to control, ¶¶¶ p<0.001 when compared to diabetic group, ♦♦♦ p<0.001 when compared with initial reading.
D + I- Insulin treated diabetic

Table-3
Blood glucose (mg/dL) level in 7 weeks (Young) Experimental rats

Animal status	0 day (Before STZ injection)	3rd day (Initial)	6th day	10th day	14th day (Final)
Control	108.6± 1.7	113.1± 0.8	120.5± 1.5	123.7± 0.7	127.5± 0.9
Diabetic	105.9± 0.7	256.2± 0.4	301.2± 0.5	305.7± 0.7	306.9± 1.3***
D + I	113.5± 1.2	258.8± 0.3	298.4± 0.9	182.8± 1.3	130.0± 1.1¶¶¶ ♦♦♦

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats.
*** p<0.001 when compared to control, ¶¶¶ p<0.001 when compared to diabetic group, ♦♦♦ p<0.001 when compared with initial reading.
D + I- Insulin treated diabetic

Figure-4
Blood glucose (mg/dL) level in 90 weeks (Old) Experimental rats



Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats.
***P<0.001 when compared to control, ¶¶¶ P<0.001 when compared to diabetic group,
♦♦♦ p<0.001 when compared with initial reading.
D + I- Insulin treated diabetic

Table-4
Blood glucose (mg/dL) level in 90 weeks (Old) Experimental rats

Animal status	0 day (Before STZ injection)	3rd day (Initial)	6th day	10th day	14th day (Final)
Control	110.1± 1.5	120.2± 1.7	122.5± 0.6	127.9± 2.1	128.3± 1.21
Diabetic	115.7± 1.2	248.9± 1.5	260.5± 0.7	265.7± 0.4	267.9±1.2***
D + I	119.2± 0.7	254.8± 0.6	262.4± 0.9	193.8± 1.2	164.6 ± 0.9¶¶¶ ♦♦♦

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats.
***P<0.001 when compared to control, ¶¶¶ P<0.001 when compared to diabetic group,
♦♦♦ p<0.001 when compared with initial reading.
D + I- Insulin treated diabetic

Figure-5
Acetylcholine esterase activity in the cerebral cortex of Control, Diabetic and D+I in 7 weeks (Young) and 90 weeks (Old) rats

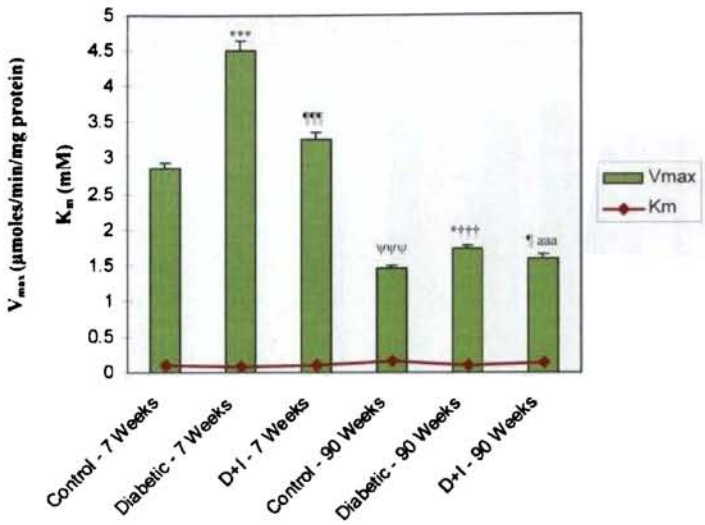


Table-5
Acetylcholine esterase activity in the cerebral cortex of Control, Diabetic and D+I in 7 weeks (Young) and 90 weeks (Old) rats

Animal status	V_{max} (µmoles/min/mg protein)		K_m (mM)	
	7 weeks	90 weeks	7 weeks	90 weeks
Control	2.84± 0.13	1.46±0.08$^{\Psi\Psi\Psi}$	0.100±0.004	0.150±0.007
Diabetic	4.50±0.02***	1.73±0.05*†††	0.080±0.002	0.100±0.003
D+I	3.25±0.04¶¶¶	1.60±0.04$^{\P aaa}$	0.090±0.007	0.130±0.014

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats.
$^{*}p<0.05$, $^{***}p<0.001$ when compared to respective controls, $^{\P}p<0.05$, $^{\P\P\P}p<0.001$ when compared to diabetic, $^{\Psi\Psi\Psi}p<0.01$ when compared to 7 weeks control, $^{\dagger\dagger\dagger}p<0.001$ when compared to 7 weeks diabetic, $^{aaa}p<0.001$ when compared to 7 weeks insulin treated diabetic
D + I- Insulin treated diabetic

Figure-6
Acetylcholine esterase activity in the brainstem of Control, Diabetic and D+I in 7 weeks (Young) and 90 weeks (Old) rats

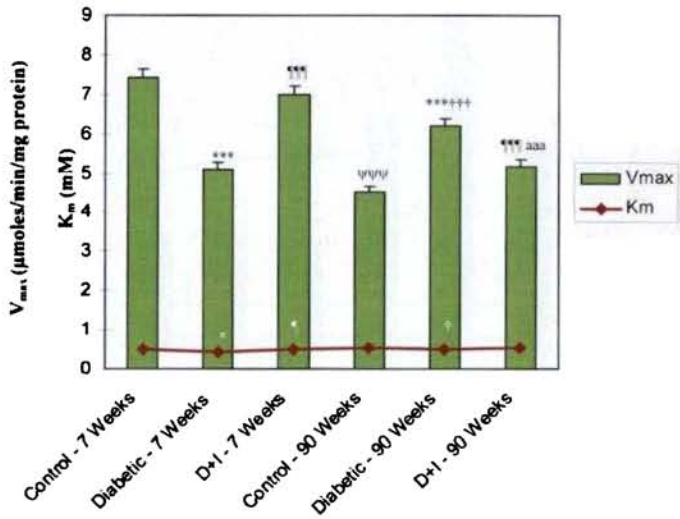


Table-6
Acetylcholine esterase activity in the brainstem of Control, Diabetic and D+I in 7 weeks (Young) and 90 weeks (Old) rats

Animal status	V_{max} (µmoles/min/mg protein)		K_m (mM)	
	7 weeks	90 weeks	7 weeks	90 weeks
Control	7.43± 0.39	4.53± 0.13$^{\psi\psi\psi}$	0.50± 0.16	0.50± 0.07
Diabetic	5.10± 0.14***	6.19± 0.04***†††	0.42± 0.02*	0.50± 0.04†
D+I	7.00± 0.42¶¶¶	5.17± 0.06$^{\P\P\P\ aaa}$	0.49± 0.05¶	0.50± 0.06

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats.
*p<0.05, ***p<0.001 when compared to respective controls, ¶p<0.05, ¶¶¶p<0.001 when compared to diabetic, $^{\psi\psi\psi}$p<0.01 when compared to 7 weeks control, †p<0.05, †††p<0.001 when compared to 7 weeks diabetic, ^{aaa}p<0.001 when compared to 7 weeks insulin treated diabetic
D + I- Insulin treated diabetic

Figure-7
Acetylcholine esterase activity in the corpus striatum of Control, Diabetic and D+I in 7 weeks (Young) and 90 weeks (Old) rats

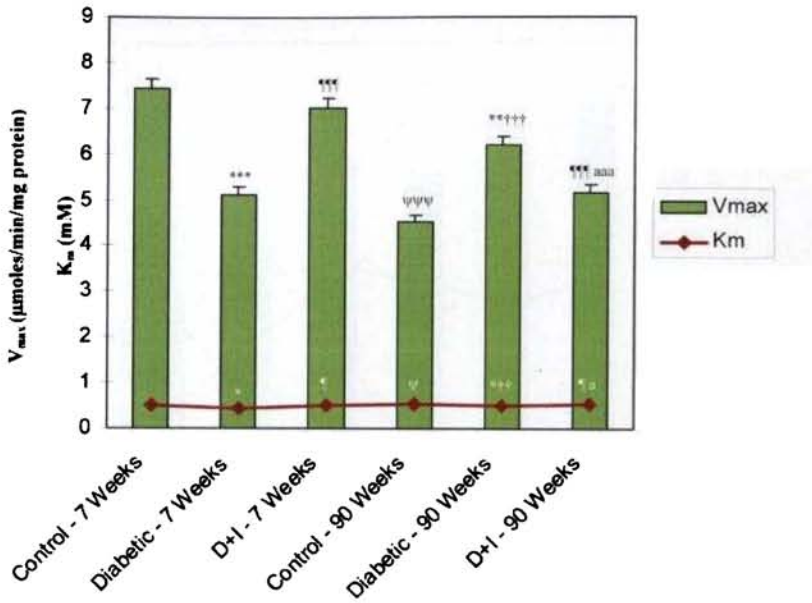


Table-7
Acetylcholine esterase activity in the corpus striatum of Control, Diabetic and D+I in 7 weeks (Young) and 90 weeks (Old) rats

Animal status	V_{max} (µmoles/min/mg protein)		K_m (mM)	
	7 weeks	90 weeks	7 weeks	90 weeks
Control	3.14±0.04	2.71± 0.08$^{\Psi\Psi\Psi}$	0.50± 0.02	0.42± 0.13$^{\Psi}$
Diabetic	4.68±0.13***	3.70± 0.12**†††	0.70±0.05*	0.30± 0.05*††
D+I	3.21±0.02¶¶¶	2.93± 0.04$^{\P\P\P\ aaa}$	0.50±0.03¶	0.40± 0.09$^{\P\ a}$

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats.
$^{*}p<0.05$, $^{***}p<0.001$ when compared to respective controls, $^{\P}p<0.05$, $^{\P\P\P}p<0.001$ when compared to diabetic, $^{\Psi}p<0.05$, $^{\Psi\Psi\Psi}p<0.01$ when compared to 7 weeks control, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ when compared to 7 weeks diabetic, $^{a}p<0.05$, $^{aaa}p<0.001$ when compared to 7 weeks insulin treated diabetic.
D + I- Insulin treated diabetic

Figure-8
Acetylcholine esterase activity in the pancreas of Control, Diabetic and D+I in 7 weeks (Young) and 90 weeks (Old) rats

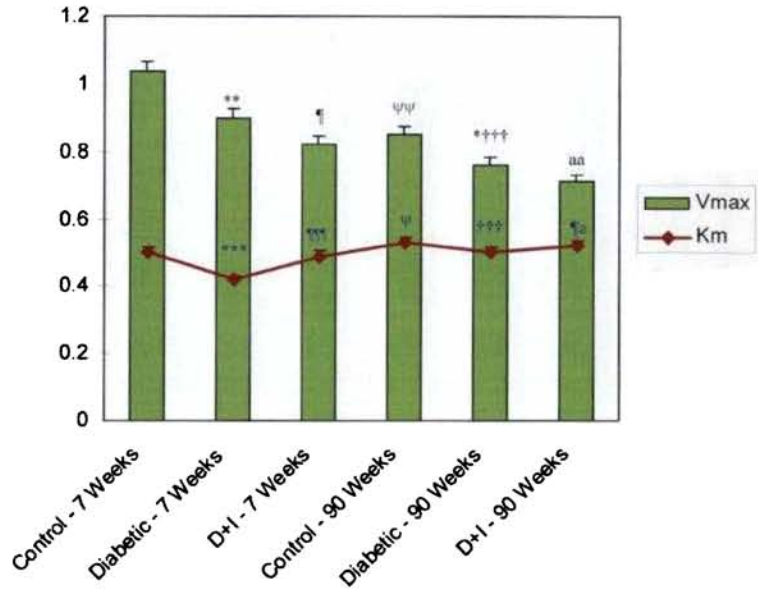


Table-8
Acetylcholine esterase activity in the pancreas of Control, Diabetic and D+I in 7 weeks (Young) and 90 weeks (Old) rats

Animal status	V_{max} (µmoles/min/mg protein)		K_m (mM)	
	7 weeks	90 weeks	7 weeks	90 weeks
Control	1.04± 0.06	$0.85\pm0.05^{\Psi\Psi}$	0.49± 0.03	$0.43\pm0.05^{\Psi}$
Diabetic	$0.90\pm0.02^{**}$	$0.76\pm0.08^{****}$	$0.72\pm0.02^{***}$	$0.28\pm0.07^{******}$
D+I	$0.82\pm0.05^{\P}$	$0.71\pm0.04^{\P aa}$	$0.50\pm0.05^{\P\P\P}$	$0.42\pm0.04^{\P a}$

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats.
*p<0.05, **p<0.01, ***p<0.001 when compared to respective controls, ¶p<0.05, ¶¶¶p<0.001 when compared to diabetic, $^{\Psi}$p<0.05, $^{\Psi\Psi}$p<0.01 when compared to 7 weeks control, $^{+++}$p<0.001 when compared to 7 weeks diabetic, ^{a}p<0.05, ^{aa}p<0.01 when compared to 7 weeks insulin treated diabetic
D + I- Insulin treated diabetic

Figure-9

Scatchard analysis of [^{3}H]Acetylcholine iodide binding against Acetylcholine iodide in the cerebral cortex of 7 weeks (Young) Control, Diabetic and Insulin treated diabetic rats

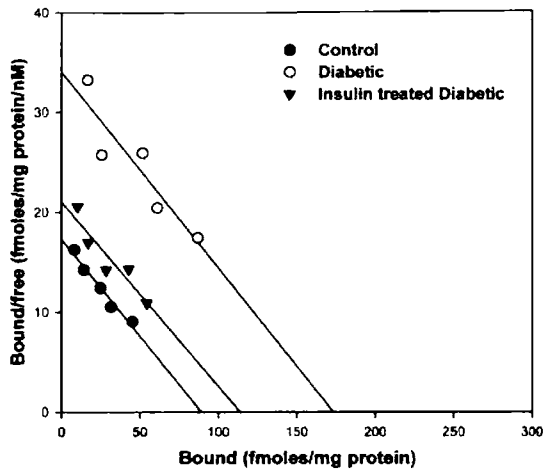


Figure-10

Scatchard analysis of [^{3}H]Acetylcholine iodide binding against Acetylcholine iodide in the cerebral cortex of 90 weeks (Young) Control, Diabetic and Insulin treated diabetic rats

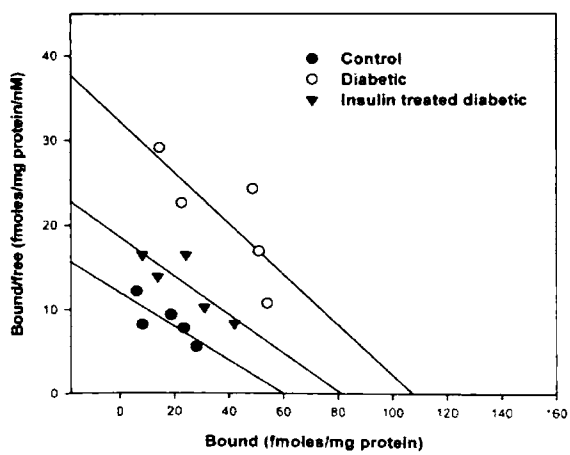


Figure-11

Scatchard analysis of [^{3}H]Acetylcholine iodide binding against Acetylcholine iodide in the cerebral cortex of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

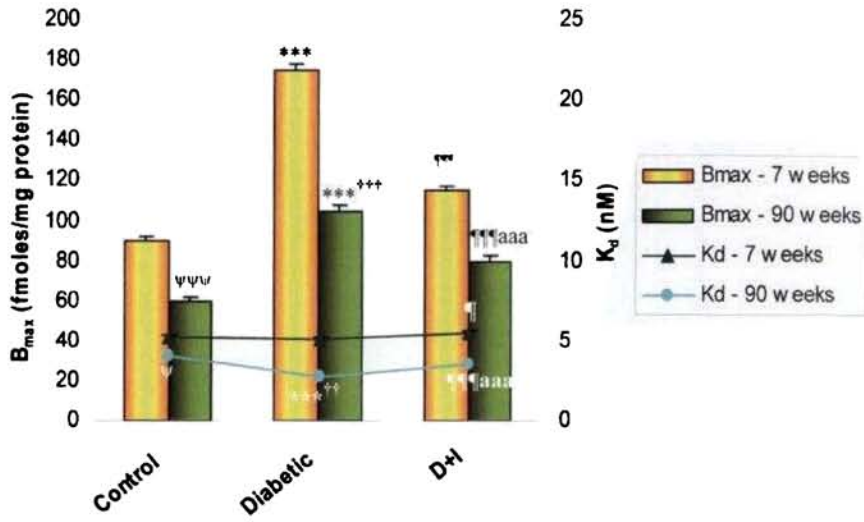


Table -9

Scatchard analysis of [^{3}H]Acetylcholine iodide binding against Acetylcholine iodide in the cerebral cortex of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Animal status	B_{max} (fmoles/mg protein)		K_d (nM)	
	7 weeks	90 weeks	7 weeks	90 weeks
Control	90.0± 4.30	60.0± 2.50$^{\psi\psi\psi}$	5.29±0.09	4.00±0.13$^{\psi}$
Diabetic	175.0± 9.33***	105.0± 6.40^{***+++}	5.14±0.16	2.80±0.08^{***++}
D+I	115.0± 5.79¶¶¶	80.0± 5.03 $^{\P\P\P\ aaa}$	5.48±0.06¶	3.55±0.14$^{\P\P\ aaa}$

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats.

$^{***}p<0.001$ when compared to respective controls, $^{\P}p<0.01$, $^{\P\P\P}p<0.001$ when compared to diabetic, $^{\psi}p<0.05$, $^{\psi\psi\psi}p<0.01$ when compared to 7 weeks control, $^{++}p<0.01$, $^{+++}p<0.001$ when compared to 7 weeks diabetic, $^{a}p<0.05$, $^{aaa}p<0.001$ when compared to 7 weeks insulin treated diabetic

D + I- Insulin treated diabetic

Table-10

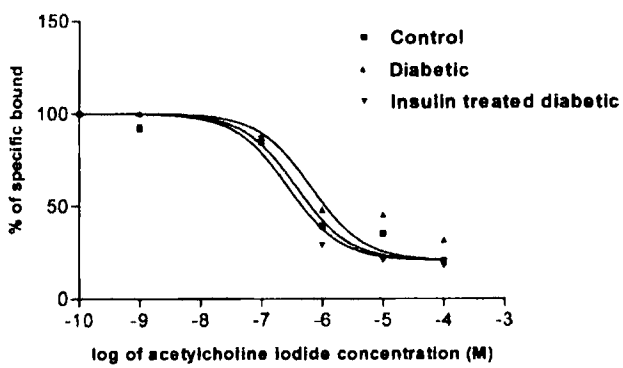
Binding parameters of [^{3}H]Acetylcholine iodide against acetylcholine iodide in the cerebral cortex of 7 weeks (Young) control, diabetic and insulin treated diabetic rats

Animal status	Best fit model	Log (EC_{50})	Ki	Hill slope
Control	One-site	-6.423	$3.778x10^{-7}$	-0.963
Diabetic	One-site	-6.183	$6.555x10^{-7}$	-0.970
Insulin treated diabetic	One-site	-6.563	$2.738x10^{-7}$	-0.974

Values are Mean of 4-6 separate experiments. Each group consists of 6-8 rats.

Figure -12

Displacement of [^{3}H]Acetylcholine Iodide with acetylcholine iodide in the cerebral cortex of 7 weeks experimental rats



Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki - The affinity of the receptor for the competing drug. EC_{50} is the concentration of the competitor that competes for half the specific binding.

Table-11

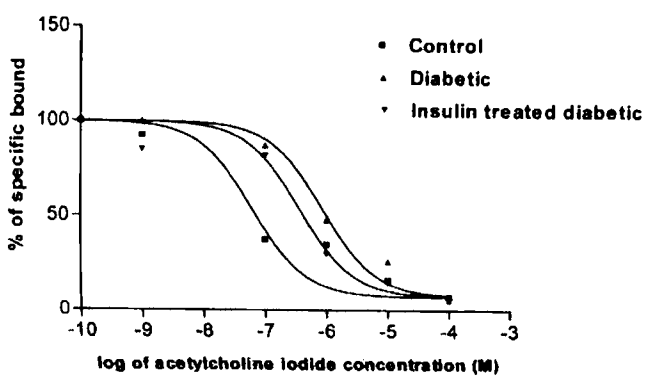
Binding parameters of [^{3}H]Acetylcholine iodide against acetylcholine iodide in the cerebral cortex of 90 weeks (Old) control, diabetic and insulin treated diabetic rats

Animal status	Best fit model	Log (EC_{50})	Ki	Hill slope
Control	One-site	-7.210	6.162×10^{-8}	-0.917
Diabetic	One-site	-6.065	8.620×10^{-7}*	-0.981
Insulin treated diabetic	One-site	-6.439	3.642×10^{-7}*	-0.970

Values are Mean of 4-6 separate experiments. Each group consists of 6-8 rats.
*$p<0.05$ when compared to control

Figure -13

Displacement of [^{3}H]Acetylcholine Iodide with acetylcholine iodide in the cerebral cortex of 90 weeks experimental rats



Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki - The affinity of the receptor for the competing drug. EC_{50} is the concentration of the competitor that competes for half the specific binding.

Figure-14
Scatchard analysis of [^{3}H]QNB receptors binding against pirenzepine in the cerebral cortex of 7 weeks (Young) Control, Diabetic and Insulin treated diabetic rats

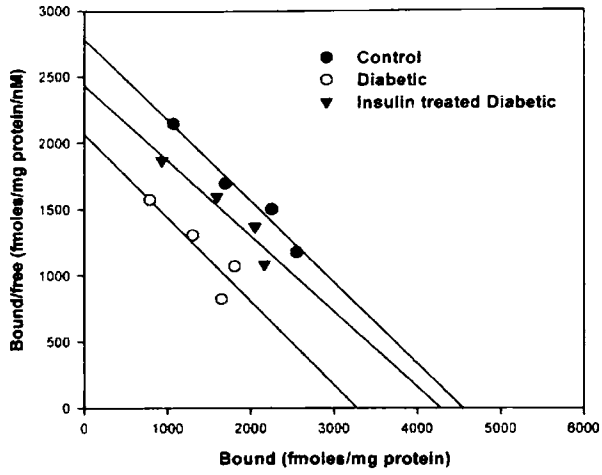


Figure-15
Scatchard analysis of [^{3}H]QNB receptors binding against pirenzepine in the cerebral cortex of 90 weeks (Old) Control, Diabetic and Insulin treated diabetic rats

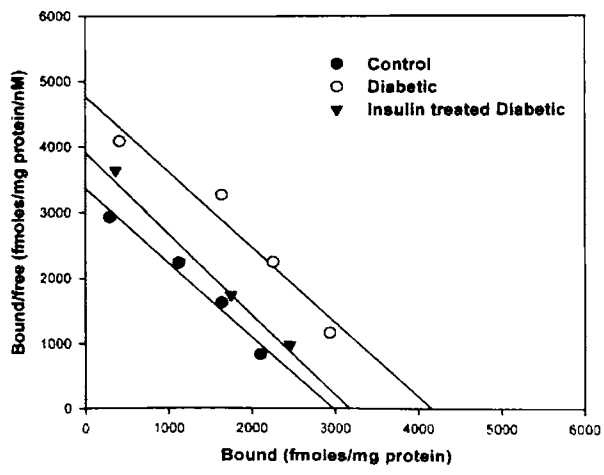


Figure-16
Scatchard analysis of [^{3}H]QNB receptors binding against pirenzepine in the cerebral cortex of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

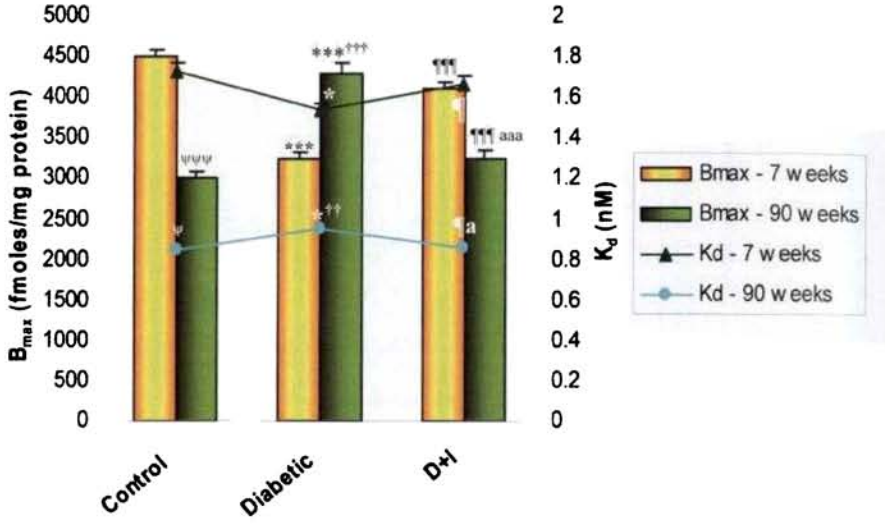


Table-12
Scatchard analysis of [^{3}H]QNB receptors binding against pirenzepine in the cerebral cortex of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Animal status	B_{max} (fmoles/mg protein)		K_d (nM)	
	7 weeks	90 weeks	7 weeks	90 weeks
Control	4500.0± 33.30	3000.0± 14.50$^{\Psi\Psi\Psi}$	1.73± 0.16	0.85± 0.06$^{\Psi}$
Diabetic	3250.0± 12.33***	4300.0± 28.40***†††	1.54±0.09*	0.95±0.14*††
D+I	4100.0± 31.79¶¶¶	3250.0± 18.00 ¶¶¶aaa	1.67± 0.13 ¶	0.86± 0.08$^{\P a}$

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats.
*p<0.05, ***p<0.001 when compared to respective controls, ¶p<0.05, ¶¶¶p<0.001 when compared to diabetic, $^{\Psi}$p<0.05, $^{\Psi\Psi\Psi}$p<0.01 when compared to 7 weeks control, ††p<0.01, †††p<0.001 when compared to 7 weeks diabetic, ^{a}p<0.05, ^{aaa}p<0.001 when compared to 7 weeks insulin treated diabetic
D + I- Insulin treated diabetic

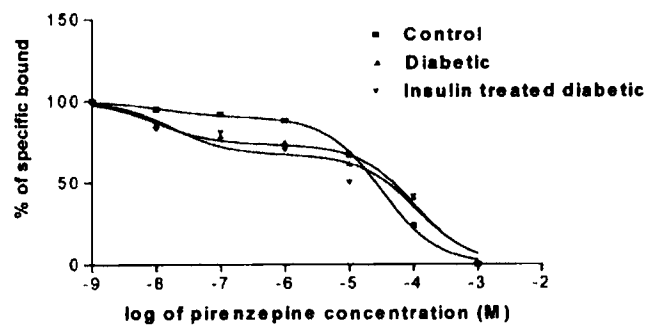
Table-13
Binding parameters of [^{3}H]QNB against pirenzepine in the cerebral cortex of 7 weeks (Young) control, diabetic and insulin treated diabetic rats

Animal status	Best fit model	Log (EC_{50})-1	Log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control	One-site	-4.456	-	$3.4x10^{-5}$	-	-0.993
Diabetic	Two-site	-7.013	-3.958	$9.7x10^{-8}$***	$1.10x10^{-4}$	-0.879
Insulin treated diabetic	Two-site	-6.095	-3.723	$8.3x10^{-7}$**	$1.89x10^{-4}$	-0.874

Values are Mean of 4-6 separate experiments. Each group consists of 6-8 rats
***p<0.001 when compared to control, **p<0.01 when compared to control

Figure -17

Displacement of [^{3}H]QNB with pirenzepine in the cerebral cortex of 7 weeks experimental rats



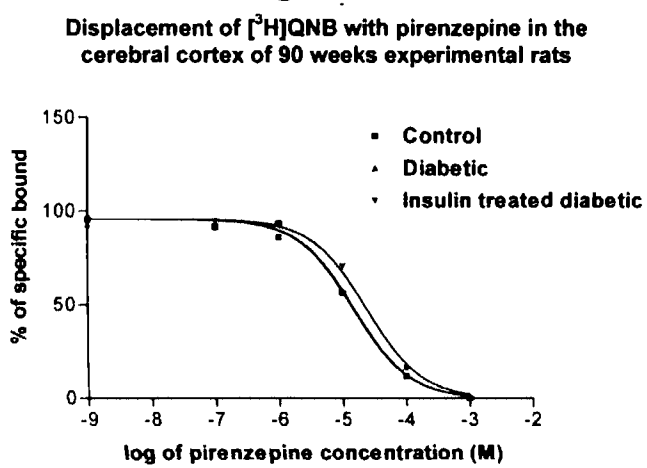
Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki - The affinity of the receptor for the competing drug. EC_{50} is the concentration of the competitor that competes for half the specific binding.

Table-14
Binding parameters of [^{3}H]QNB against pirenzepine in the cerebral cortex of 90 weeks (Old) control, diabetic and insulin treated diabetic rats

Animal status	Best fit model	Log (EC_{50})	Ki	Hill slope
Control	One-site	-4.829	$1.48x10^{-5}$	-0.997
Diabetic	One-site	-4.816	$1.52x10^{-5}$	-0.992
Insulin treated diabetic	One-site	-4.619	$2.40x10^{-5}$	-0.992

Values are Mean of 4-6 separate experiments. Each group consists of 6-8 rats

Figure -18



Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki - The affinity of the receptor for the competing drug. EC_{50} is the concentration of the competitor that competes for half the specific binding.

Figure-19

Real-Time PCR amplification of Muscarinic M1 receptor mRNA from the cerebral cortex of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Fig-19a (7 weeks old)

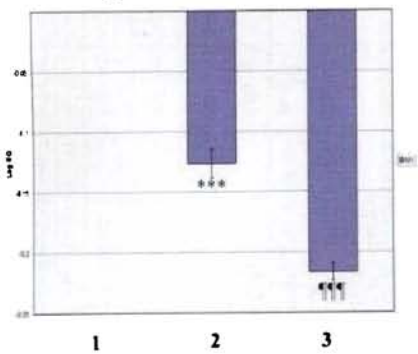


Fig-19b (90 weeks old)

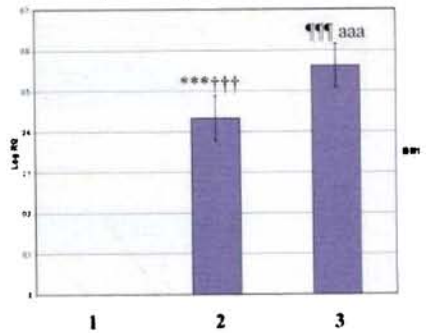


Table-15

Real-Time PCR amplification of Muscarinic M1 receptor mRNA from the cerebral cortex of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Sample No.	Animal status	Log RQ value	
		7 weeks old	90 weeks old
1	Control	0.0	0.0
2	Diabetic	-0.13±0.01***	0.43±0.06***†††
3	Insulin treated Diabetic	-0.22 ±0.01¶¶¶	0.56±0.05¶¶¶ aaa

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats.

***p< 0.001 when compared to respective controls, ¶¶¶p<0.001 when compared to diabetic, †††p<0.001 when compared to 7 weeks old diabetic, aaap<0.001 when compared to 7 weeks old insulin treated diabetic

Figure-20

Scatchard analysis of [^{3}H]DAMP receptors binding against 4-DAMP mustard in the cerebral cortex of 7 weeks (Young) Control, Diabetic and Insulin treated diabetic rats

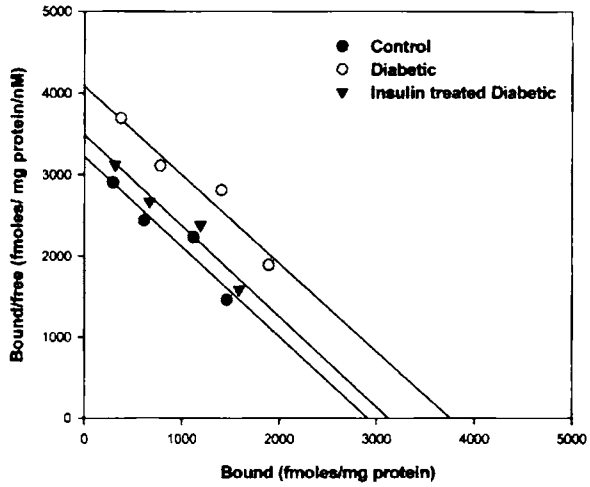


Figure-21

Scatchard analysis of [^{3}H]DAMP receptors binding against 4-DAMP mustard in the cerebral cortex of 90 weeks (Old) Control, Diabetic and Insulin treated diabetic rats

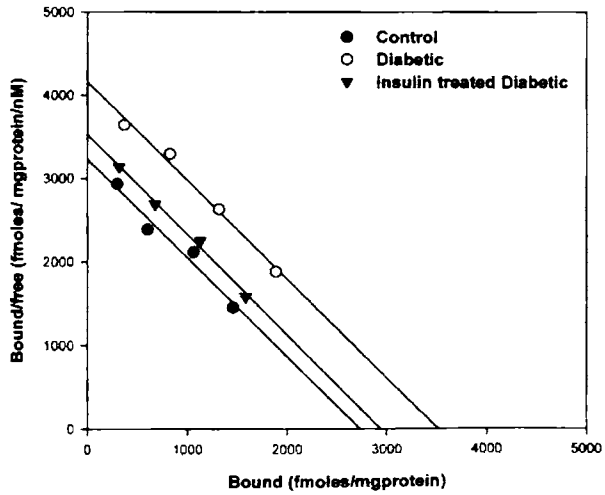


Figure-22

Scatchard analysis of [^{3}H]DAMP receptors binding against 4-DAMP mustard in the cerebral cortex of Control and Experimental 7 weeks (Young) and 90 weeks (Old) rats

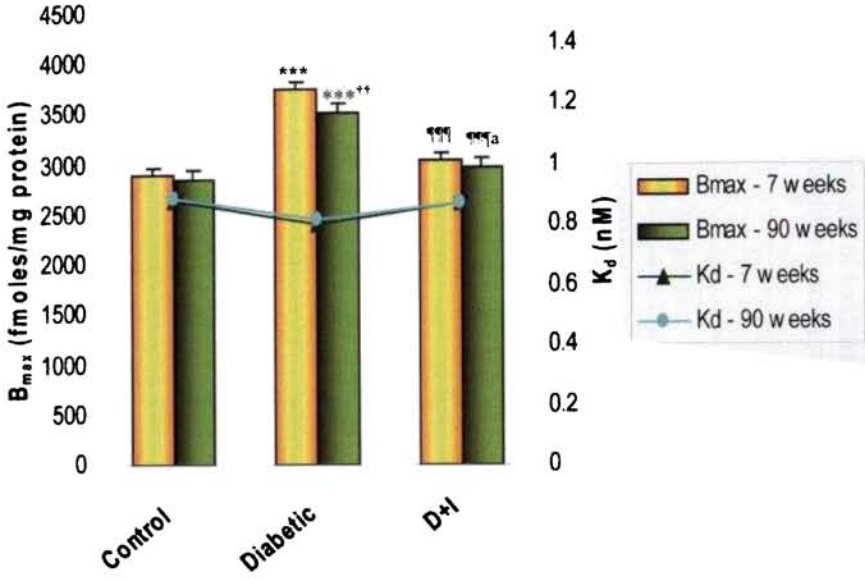


Table-16

Scatchard analysis of [^{3}H]DAMP receptors binding against 4-DAMP mustard in the cerebral cortex of Control and Experimental 7 weeks (Young) and 90 weeks (Old) rats

Animal status	B_{max} (fmoles/mg protein)		K_d (nM)	
	7 weeks	90 weeks	7 weeks	90 weeks
Control	2900.0± 25.00	2850.0± 28.00	0.88± 0.08	0.89± 0.07
Diabetic	3750.0± 33.33***	3500.0± 53.33***††	0.80± 0.03	0.82± 0.02
D+I	3050.0± 16.62¶¶¶	2975.0 ± 13.56¶¶¶ a	0.87± 0.06	0.87± 0.04

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats.
***p<0.001 when compared to respective controls, ¶¶¶p<0.001 when compared to diabetic, ††p<0.01 when compared to 7 weeks diabetic, [a] p<0.05 when compared to 7 weeks insulin treated diabetic
D + I- Insulin treated diabetic

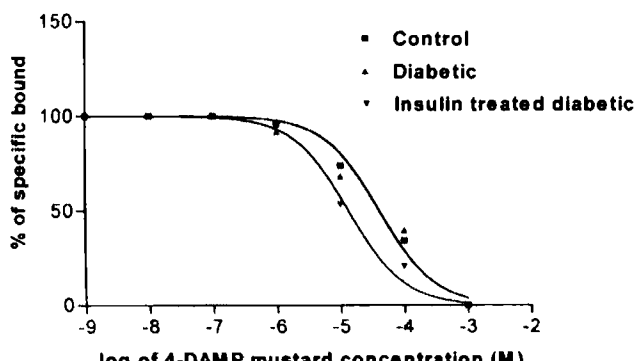
Table-17
Binding parameters of [^{3}H]DAMP against 4-DAMP mustard in the cerebral cortex of 7 weeks (Young) control, diabetic and insulin treated diabetic rats

Animal status	Best fit model	Log (EC_{50})	Ki	Hill slope
Control	One-site	-4.394	$4.038x10^{-5}$	-0.991
Diabetic	One-site	-4.385	$4.121x10^{-5}$	-0.965
Insulin treated diabetic	One-site	-4.872	$1.342x10^{-5}$	-0.991

Values are Mean of 4-6 separate experiments. Each group consists of 6-8 rats

Figure -23

Displacement of [^{3}H]DAMP with 4-DAMP mustard in the cerebral cortex of 7 weeks experimental rats



Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki - The affinity of the receptor for the competing drug. EC_{50} is the concentration of the competitor that competes for half the specific binding.

Table-18

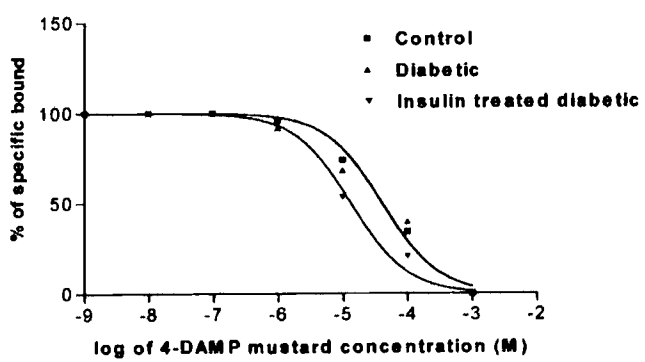
Binding parameters of [^{3}H]DAMP against 4-DAMP mustard in the cerebral cortex of 90 weeks (Old) control, diabetic and insulin treated diabetic rats

Animal status	Best fit model	Log (EC_{50})	Ki	Hill slope
Control	One-site	-5.870	$1.35x10^{-6}$	-0.996
Diabetic	One-site	-5.777	$1.67x10^{-6}$	-0.997
Insulin treated diabetic	One-site	-5.694	$2.02x10^{-6}$	-0.996

Values are Mean of 4-6 separate experiments. Each group consists of 6-8 rats

Figure -24

Displacement of [^{3}H]DAMP with 4-D AMP mustard in the cerebral cortex of 90 weeks experimental rats



Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki - The affinity of the receptor for the competing drug. EC_{50} is the concentration of the competitor that competes for half the specific binding.

Figure-25

Real-Time PCR amplification of Muscarinic M3 receptor mRNA from the cerebral cortex of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Fig-25a (7 weeks old)

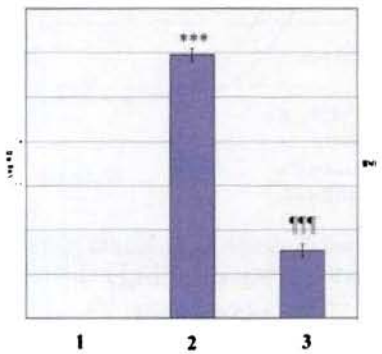


Fig-25b (90 weeks old)

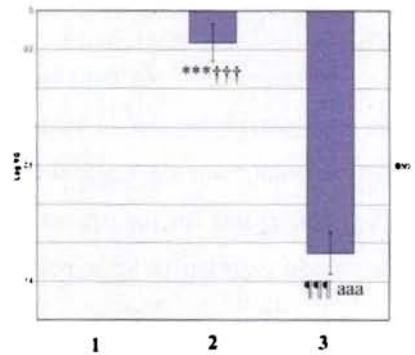


Table-19

Real-Time PCR amplification of Muscarinic M3 receptor mRNA from the cerebral cortex of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Sample No.	Animal status	Log RQ value	
		7 weeks old	90 weeks old
1	Control	0.0	0.0
2	Diabetic	$0.59\pm0.02^{***}$	$-0.16\pm0.09^{***}\dagger\dagger\dagger$
3	Insulin treated Diabetic	$0.15\pm0.02^{¶¶¶}$	$-1.25\pm0.11^{¶¶¶\ aaa}$

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats.

***p<0.001 when compared to respective controls, ¶¶¶p<0.001 when compared to diabetic, †††p<0.001 when compared to 7 weeks old diabetic, aaap<0.001 when compared to 7 weeks old insulin treated diabetic

Figure-26

Scatchard analysis of [^{3}H]QNB receptors binding against Pirenzepine in the brainstem of 7 weeks (Young) Control, Diabetic and Insulin treated diabetic rats

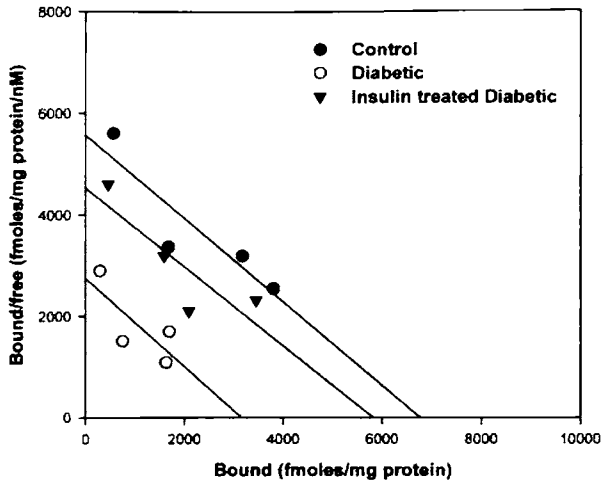


Figure-27

Scatchard analysis of [^{3}H]QNB receptors binding against Pirenzepine in the brainstem of 90 weeks (Old) Control, Diabetic and Insulin treated diabetic rats

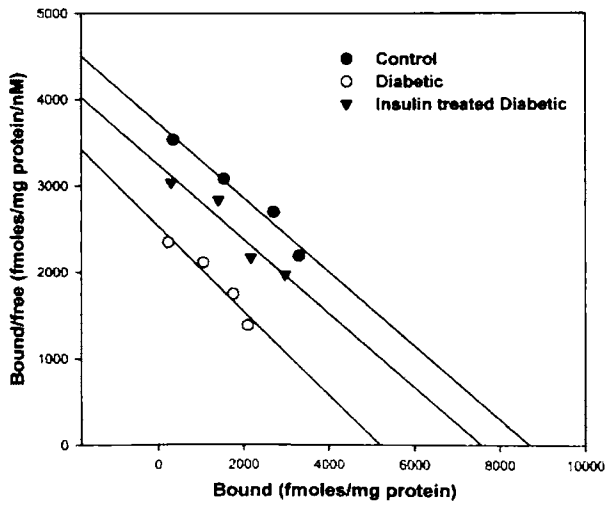


Figure-28
Scatchard analysis of [^{3}H]QNB receptors binding against Pirenzepine in the brainstem of Control and Experimental 7 weeks (Young) and 90 weeks (Old) rats

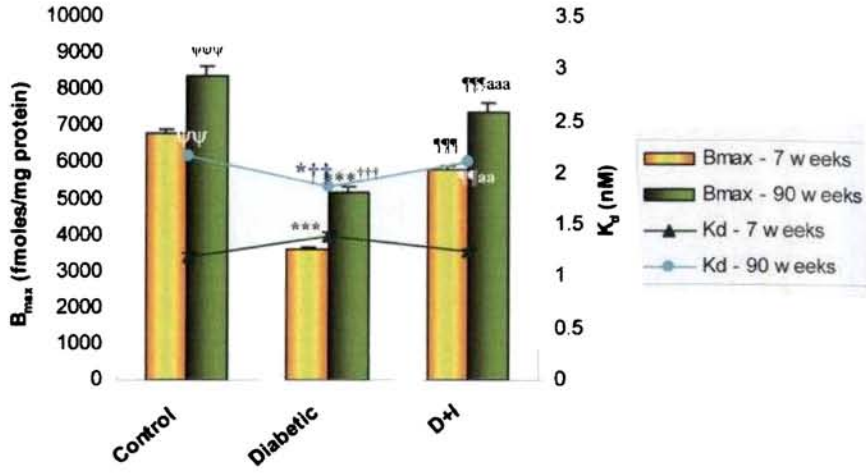


Table-20
Scatchard analysis of [^{3}H]QNB receptors binding against pirenzepine in the brainstem of Control and Experimental 7 weeks (Young) and 90 weeks (Old) rats

Animal status	B_{max} (fmoles/mg protein)		K_d (nM)	
	7 weeks	90 weeks	7 weeks	90 weeks
Control	6800.0± 28.35	8400.0± 58.27$^{\psi\psi\psi}$	1.20± 0.03	2.17±0.05$^{\psi\psi}$
Diabetic	3600.0± 12.50***	5200.0± 15.60***†††	1.40± 0.06	1.86±0.02*††
D+I	5800.0± 47.00¶¶¶	7400.0± 66.00$^{\P\P\P\ aaa}$	1.24± 0.02	2.10± 0.04$^{\P\P\ aa}$

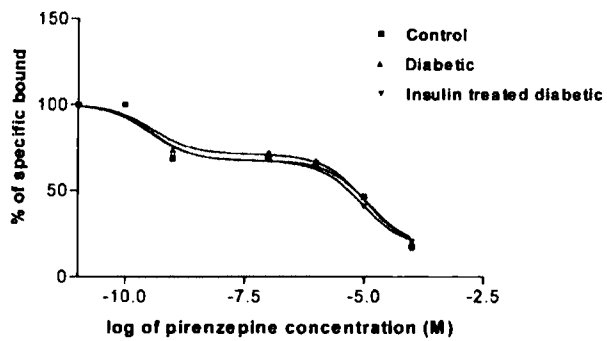
Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats.
**p<0.01, ***p<0.001 when compared to respective controls, ¶¶p<0.01, ¶¶¶p<0.001 when compared to diabetic, $^{\psi\psi}$p<0.01, $^{\psi\psi\psi}$p<0.01 when compared to 7 weeks control, ††p<0.01, †††p<0.001 when compared to 7 weeks diabetic, ^{aa}p<0.01, ^{aaa}p<0.001 when compared to 7 weeks insulin treated diabetic
D+I- Insulin treated diabetic

Table-21
Binding parameters of [^{3}H]QNB against pirenzepine in the brainstem of 7 weeks (Young) control, diabetic and insulin treated diabetic rats

Animal status	Best fit model	Log (EC_{50})-1	Log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control	Two-site	-9.480	-4.913	$3.310x10^{-10}$	$1.22 x10^{-5}$	-0.393
Diabetic	Two-site	-9.435	-4.987	$3.673x10^{-10}$	$1.03x10^{-5}$	-0.349
Insulin treated diabetic	Two-site	-9.451	-5.051	$3.537x10^{-10}$	$8.80x10^{-6}$*	-0.395

Values are Mean of 4-6 separate experiments. Each group consists of 6-8 rats
*$p<0.05$ when compared to control

Figure -29
Displacement of [^{3}H]QNB with pirenzepine in the brainstem of 7 weeks experimental rats



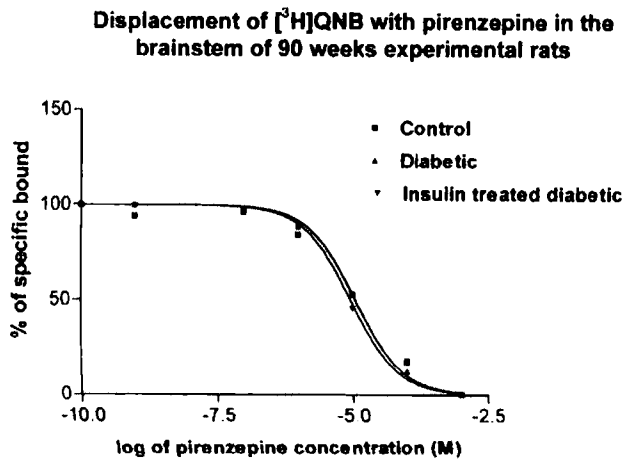
Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki - The affinity of the receptor for the competing drug. EC_{50} is the concentration of the competitor that competes for half the specific binding.

Table-22
Binding parameters of [^{3}H]QNB against pirenzepine in the brainstem of 90 weeks (Old) control, diabetic and insulin treated diabetic rats

Animal status	Best fit model	Log (EC_{50})	Ki	Hill slope
Control	One-site	-4.938	$1.15x10^{-5}$	-0.985
Diabetic	One-site	-4.943	$1.14x10^{-5}$	-0.998
Insulin treated diabetic	One-site	-5.030	$9.32x10^{-6}$*	-0.991

Values are Mean of 4-6 separate experiments. Each group consists of 6-8 rats.
*p<0.05 when compared to control

Figure -30



Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki - The affinity of the receptor for the competing drug. EC_{50} is the concentration of the competitor that competes for half the specific binding.

Figure-31

Real-Time PCR amplification of Muscarinic M1 receptor mRNA from the Brainstem of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Fig-31a (7 weeks old)

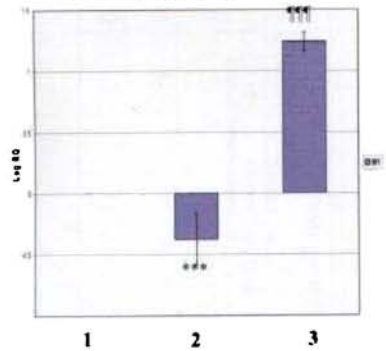


Fig-31b (90 weeks old)

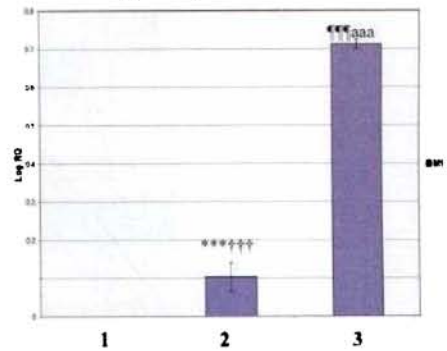


Table-23

Real-Time PCR amplification of Muscarinic M1 receptor mRNA from the Brainstem of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Sample No.	Animal status	Log RQ value	
		7 weeks old	90 weeks old
1	Control	0.0	0.0
2	Diabetic	-0.376±0.211***	0.102±0.037***†††
3	Insulin treated diabetic	1.241±0.076¶¶¶	0.712±0.011¶¶¶ aaa

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats.

*p<0.05, ***p<0.001 when compared to respective controls, ¶¶¶p<0.001 when compared to diabetic, †††p<0.001 when compared to 7 weeks old diabetic, aaap<0.001 when compared to 7 weeks old insulin treated diabetic

Figure-32
Scatchard analysis of [^{3}H]DAMP receptors binding against 4-DAMP mustard in the brainstem of 7 weeks (Young) Control, Diabetic and Insulin treated diabetic rats

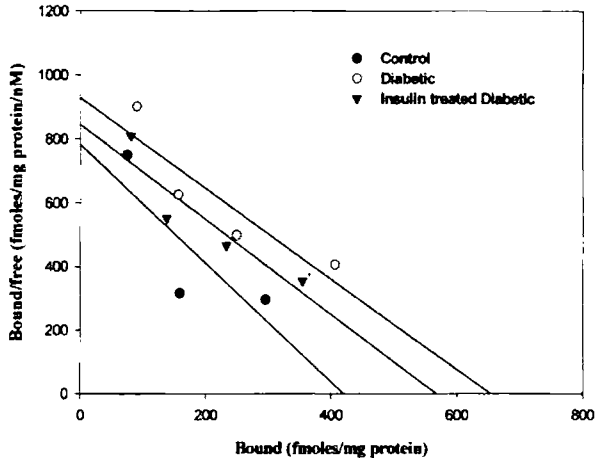


Figure-33
Scatchard analysis of [^{3}H]DAMP receptors binding against 4-DAMP mustard in the brainstem of 90 weeks (Old) Control, Diabetic and Insulin treated diabetic rats

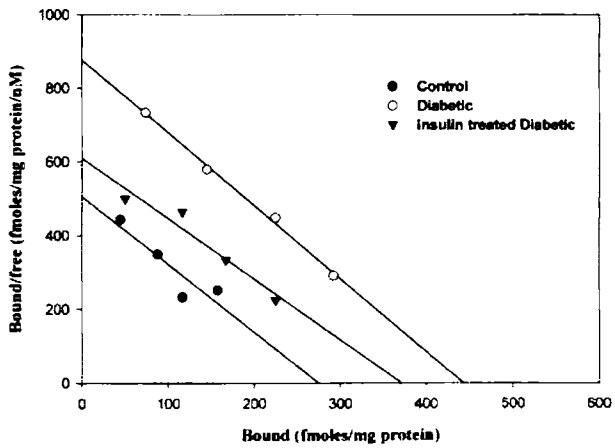


Figure-34
Scatchard analysis of [^{3}H]DAMP receptors binding against 4-DAMP mustard in the brainstem of Control and Experimental 7 weeks (Young) and 90 weeks (Old) rats

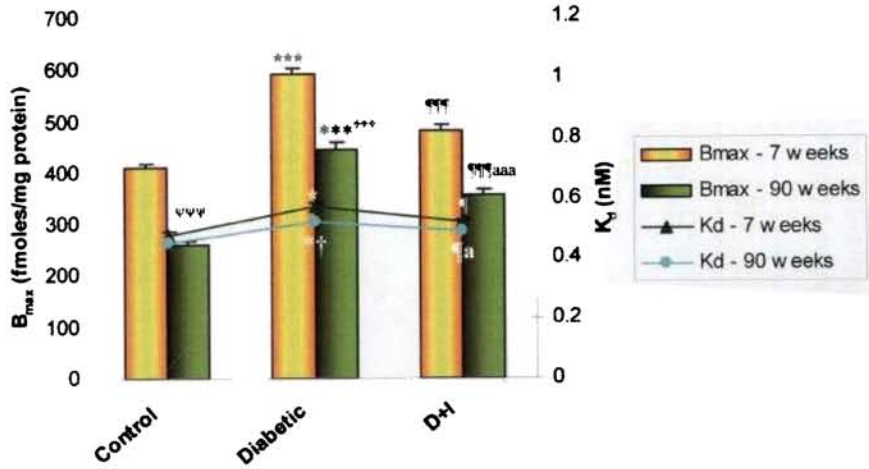


Table-24
Scatchard analysis of [^{3}H] DAMP receptors binding against 4-DAMP mustard in the brainstem of Control and Experimental 7 weeks (Young) and 90 weeks (Old) rats

Animal status	B_{max} (fmoles/mg protein)		K_d (nM)	
	7 weeks	90 weeks	7 weeks	90 weeks
Control	410.0± 25.30	260.0 ± 12.00$^{\Psi\Psi\Psi}$	0.48± 0.02	0.45± 0.02
Diabetic	590.0± 48.50***	445.0± 23.53***†††	0.57± 0.07*	0.52± 0.05*†
D+I	480.0± 15.52¶¶¶	355.0± 8.30$^{\P\P\P aaa}$	0.52± 0.01¶	0.49± 0.03$^{\P a}$

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats.
**p<0.01, ***p<0.001 when compared to respective controls, †p<0.05, †††p<0.001 when compared to diabetic, $^{\Psi\Psi\Psi}$p<0.01 when compared to 7 weeks control, †p<0.05, †††p<0.001 when compared to 7 weeks diabetic, ^{a}p<0.05, ^{aaa}p<0.001 when compared to 7 weeks insulin treated diabetic
D+I- Insulin treated diabetic

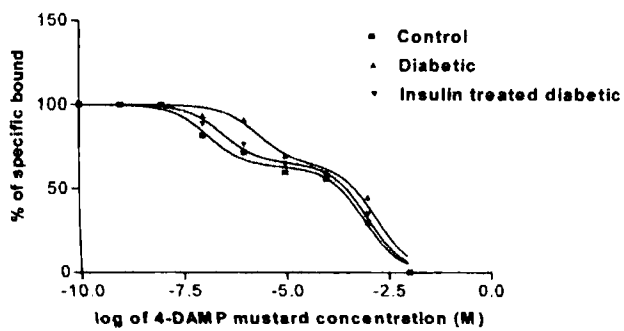
Table-25
Binding parameters of [^{3}H]DAMP against 4-DAMP mustard in the brainstem of 7 weeks (Young) control, diabetic and insulin treated diabetic rats

Animal status	Best fit model	Log (EC_{50})-1	Log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control	Two-site	-6.871	-3.091	$1.34x10^{-7}$	$8.1x10^{-4}$	-0.366
Diabetic	Two-site	-5.649	-2.794	$2.24x10^{-6}$*	$1.6x10^{-3}$*	-0.350
Insulin treated diabetic	Two-site	-6.512	-3.007	$3.07x10^{-7}$	$9.8x10^{-4}$	-0.344

Values are Mean of 4-6 separate experiments. Each group consists of 6-8 rats.
*$p<0.05$ when compared to control

Figure -35

Displacement of [^{3}H]DAMP with 4-DAMP mustard in the brainstem of 7 weeks experimental rats



Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki - The affinity of the receptor for the competing drug. EC_{50} is the concentration of the competitor that competes for half the specific binding.

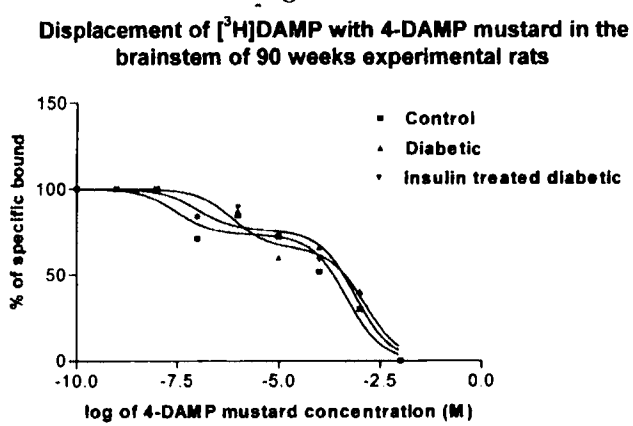
Table-26

Binding parameters of [^{3}H]DAMP against 4-DAMP mustard in the brainstem of 90 weeks (Old) control, diabetic and insulin treated diabetic rats

Animal status	Best fit model	Log (EC_{50})-1	Log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control	Two-site	-7.518	-3.326	$3.036x10^{-8}$	$4.70x10^{-4}$	-0.264
Diabetic	Two-site	-6.137	-2.874	$7.291x10^{-7}$*	$1.33x10^{-3}$*	-0.341
Insulin treated diabetic	Two-site	-7.003	-3.117	$9.932x10^{-8}$	$7.64x10^{-4}$	-0.239

Values are Mean of 4-6 separate experiments. Each group consists of 6-8 rats.
*$p<0.05$ when compared to control

Figure -36



Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki - The affinity of the receptor for the competing drug. EC_{50} is the concentration of the competitor that competes for half the specific binding.

Figure-37

Real-Time PCR amplification of Muscarinic M3 receptor mRNA from the Brainstem of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Fig-37a (7 weeks old)

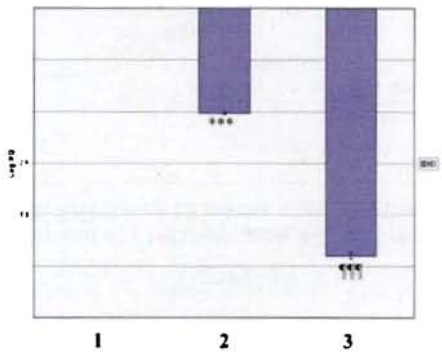


Fig-37b (90 weeks old)

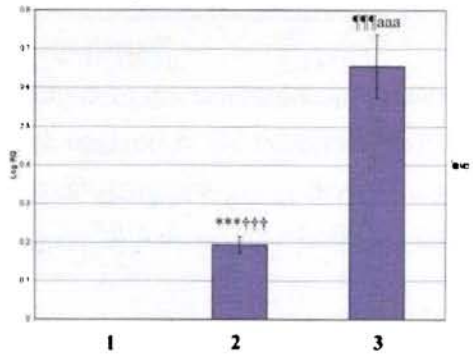


Table-27

Real-Time PCR amplification of Muscarinic M3 receptor mRNA from the Brainstem of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Sample No.	Animal status	Log RQ value	
		7 weeks old	90 weeks old
1	Control	0.0	0.0
2	Diabetic	$-0.405\pm0.003^{***}$	$0.193\pm0.021^{***}\dagger\dagger\dagger$
3	Insulin treated Diabetic	$-0.959\pm0.013^{¶¶¶}$	$0.655\pm0.080^{¶¶¶\ aaa}$

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats.

***p<0.001 when compared to respective controls, ¶¶¶p<0.001 when compared to diabetic, †††p<0.001 when compared to 7 weeks old diabetic, ^{aaa}p<0.001 when compared to 7 weeks old insulin treated diabetic

Figure-38
Scatchard analysis of [^{3}H]QNB receptors binding against Pirenzepine in the corpus striatum of 7 weeks (Young) Control, Diabetic and Insulin treated diabetic rats

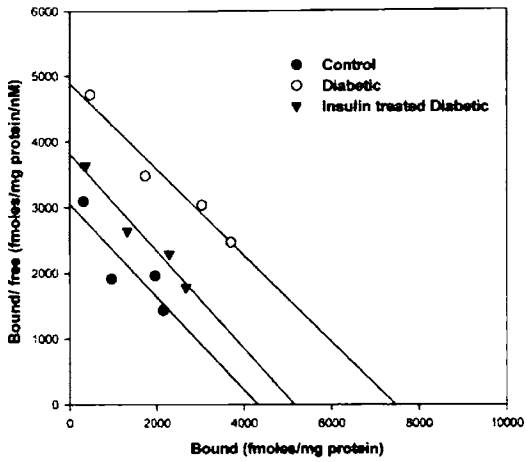


Figure- 39
Scatchard analysis of [^{3}H]QNB receptors binding against Pirenzepine in the corpus striatum of 90 weeks (Old) Control, Diabetic and Insulin treated diabetic rats

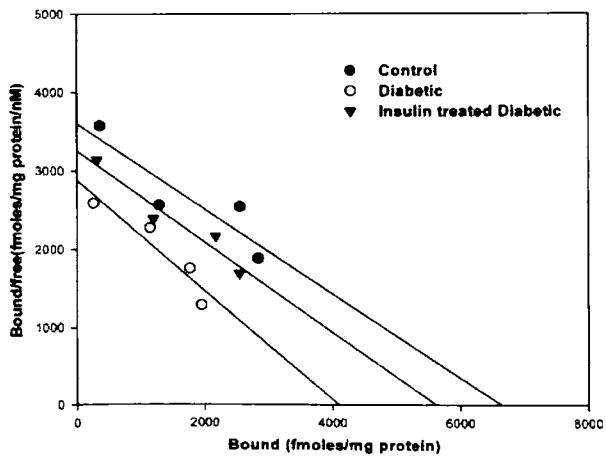


Figure-40
Scatchard analysis of [^{3}H]QNB receptors binding against Pirenzepine in the Corpus striatum of Control and Experimental 7 weeks (Young) and 90 weeks (Old) rats

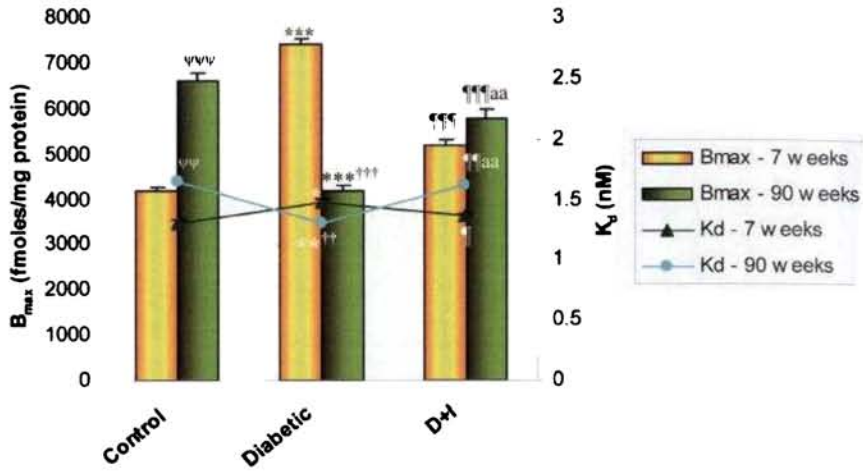


Table-28
Scatchard analysis of [^{3}H]QNB receptors binding against pirenzepine in the corpus striatum of Control and Experimental 7 weeks (Young) and 90 weeks (Old) rats

Animal status	B_{max} (fmoles/mg protein)		K_d (nM)	
	7 weeks	90 weeks	7 weeks	90 weeks
Control	4200.0± 53.33	6600.0± 44.00$^{\psi\psi\psi}$	1.31± 0.02	1.65± 0.04$^{\psi\psi}$
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$^{\P\P\P aa}$	1.36± 0.01¶	1.61± 0.02$^{\P\P aa}$

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats.
**p<0.01, ***p<0.001 when compared to respective controls, ¶p<0.05, ¶¶p<0.01, ¶¶¶p<0.001 when compared to diabetic, $^{\psi\psi}$p<0.01, $^{\psi\psi\psi}$p<0.01 when compared to 7 weeks control, ††p<0.01, †††p<0.001 when compared to 7 weeks diabetic, ^{aa}p<0.01 when compared to 7 weeks insulin treated diabetic
D+I- Insulin treated diabetic

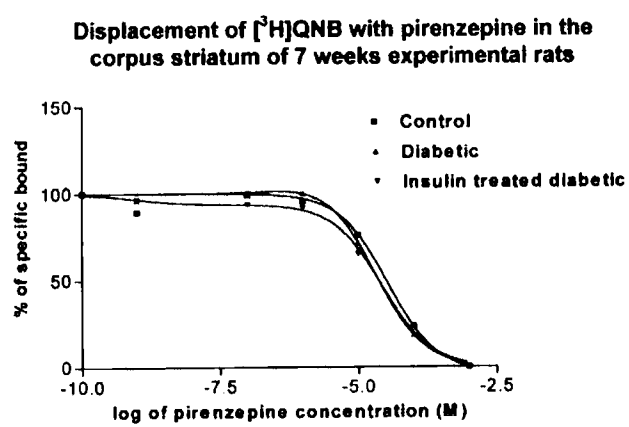
Table-29

Binding parameters of [^{3}H]QNB against pirenzepine in the corpus striatum of 7 weeks (Young) Control, Diabetic and Insulin treated diabetic rats

Animal status	Best fit model	Log (EC_{50})-1	Log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control	Two-site	-9.323	-4.473	$2.1x10^{-9}$	$3.36 x10^{-5}$	-0.540
Diabetic	Two-site	-6.296	-4.690	$5.05x10^{-7**}$	$2.04x10^{-5}$	-0.218
Insulin treated diabetic	Two-site	-9.024	-4.570	$9.47x10^{-10*}$	$2.65x10^{-5}$	-0.269

Values are Mean of 4-6 separate experiments. Each group consists of 6-8 rats.
**p<0.001 when compared to control, *p<0.05 when compared to control

Figure -41



Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki - The affinity of the receptor for the competing drug. EC_{50} is the concentration of the competitor that competes for half the specific binding.

Table-30

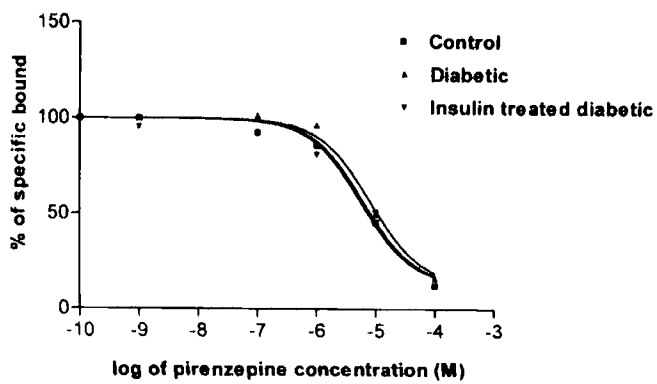
Binding parameters of [^{3}H]QNB against pirenzepine in the corpus striatum of 90 weeks (Old) control, diabetic and insulin treated diabetic rats

Animal status	Best fit model	Log (EC_{50})	Ki	Hill slope
Control	One-site	-5.251	5.60×10^{-6}	-0.990
Diabetic	One-site	-5.091	8.11×10^{-6}	-0.991
Insulin treated diabetic	One-site	-5.209	6.18×10^{-6}	-0.970

Values are Mean of 4-6 separate experiments. Each group consists of 6-8 rats.

Figure -42

Displacement of [^{3}H]QNB with pirenzepine in the corpus striatum of 90 weeks experimental rats



Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki - The affinity of the receptor for the competing drug. EC_{50} is the concentration of the competitor that competes for half the specific binding.

Figure-43

Real-Time PCR amplification of Muscarinic M1 receptor mRNA from the Corpus striatum of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Fig-43a (7 weeks old)

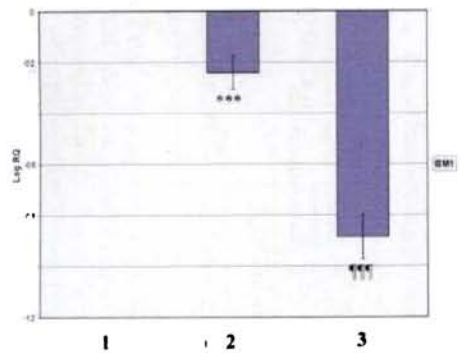


Fig-43b (90 weeks old)

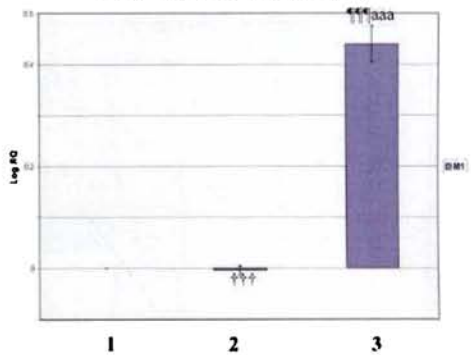


Table-31

Real-Time PCR amplification of Muscarinic M1 receptor mRNA from the Corpus striatum of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Sample No.	Animal status	Log RQ value	
		7 weeks old	90 weeks old
1	Control	0.0	0.0
2	Diabetic	-0.241±0.065***	-0.003±0.008***†††
3	Insulin treated diabetic	-0.886±0.086¶¶¶	0.439±0.035¶¶¶ aaa

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats.

***p<0.001 when compared to respective controls, ¶¶¶p<0.001 when compared to diabetic, †††p<0.001 when compared to 7 weeks old diabetic, aaap<0.001 when compared to 7 weeks old insulin treated diabetic

Figure-44
Scatchard analysis of [^{3}H]DAMP receptors binding against 4-DAMP mustard in the corpus striatum of 7 weeks (Young) Control, Diabetic and Insulin treated diabetic rats

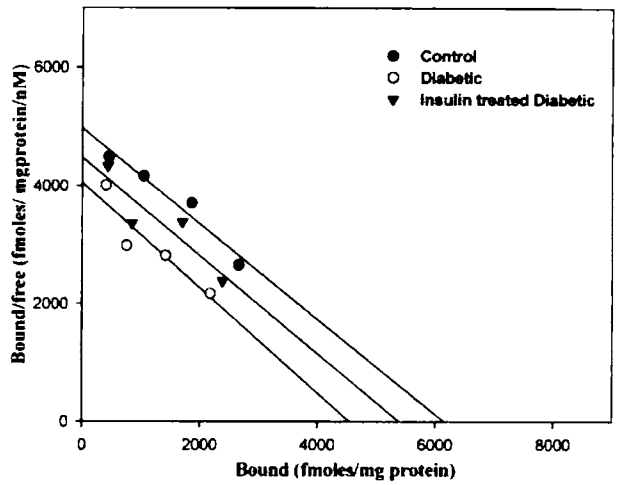


Figure- 45
Scatchard analysis of [^{3}H]DAMP receptors binding against 4-DAMP mustard in the corpus striatum of 90 weeks (Old) Control, Diabetic and Insulin treated diabetic rats

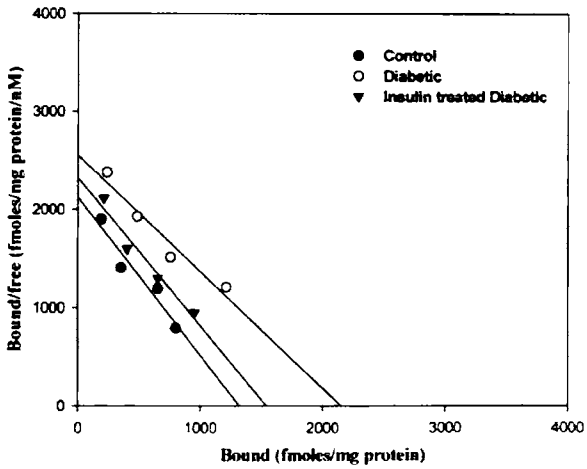


Figure-46
Scatchard analysis of [^{3}H]DAMP receptors binding against 4-DAMP mustard in the Corpus striatum of Control and Experimental 7 weeks (Young) and 90 weeks (Old) rats

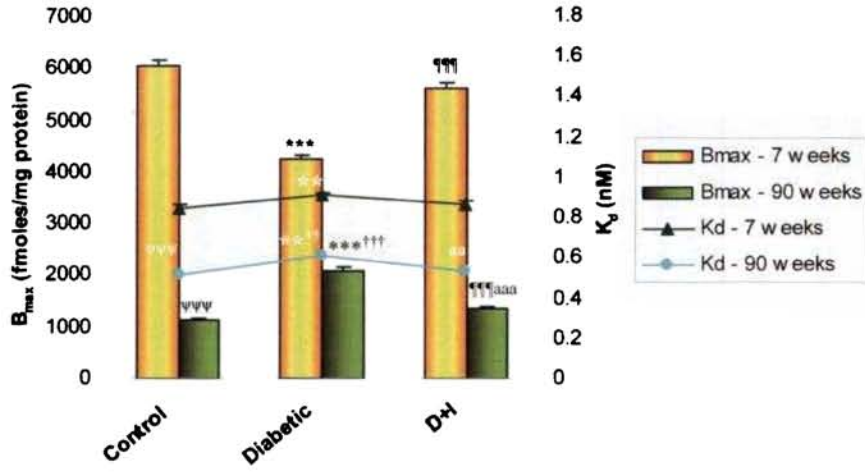


Table-32
Scatchard analysis of [^{3}H] DAMP receptors binding against 4-DAMP mustard in the Corpus striatum of Control and Experimental 7 weeks (Young) and 90 weeks (Old) rats

Animal status	B_{max} (fmoles/mg protein)		K_d (nM)	
	7 weeks	90 weeks	7 weeks	90 weeks
Control	6050.0±28.05	1150.0± 31.79$^{\psi\psi\psi}$	0.85± 0.01	0.52± 0.03$^{\psi\psi\psi}$
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$^{\P\P\P\ aaa}$	0.87± 0.02	0.54± 0.02^{aa}

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats.
$^{**}p<0.01$, $^{***}p<0.001$ when compared to respective controls, $^{\P\P\P}p<0.001$ when compared to diabetic, $^{\psi\psi\psi}p<0.01$ when compared to 7 weeks control, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ when compared to 7 weeks diabetic, $^{aa}p<0.01$, $^{aaa}p<0.001$ when compared to 7 weeks insulin treated diabetic
D + I- Insulin treated diabetic

Table-33

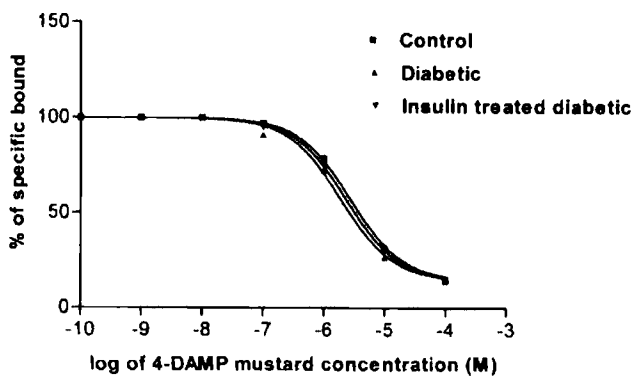
Binding parameters of [^{3}H]DAMP against 4-DAMP mustard in the corpus striatum of 7 weeks (Young) control, diabetic and insulin treated diabetic rats

Animal status	Best fit model	Log (EC_{50})	Ki	Hill slope
Control	One-site	-5.564	$2.73x10^{-6}$	-0.998
Diabetic	One-site	-5.725	$1.88x10^{-6}$	-0.996
Insulin treated diabetic	One-site	-5.638	$2.30x10^{-6}$	-0.999

Values are Mean of 4-6 separate experiments. Each group consists of 6-8 rats.

Figure -47

Displacement of [^{3}H]DAMP with 4-DAMP mustard in the corpus striatum of 7 weeks experimental rats



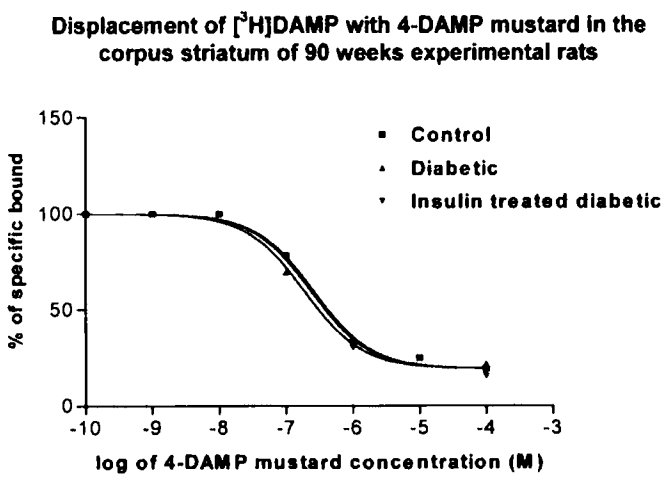
Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki - The affinity of the receptor for the competing drug. EC_{50} is the concentration of the competitor that competes for half the specific binding.

Table-34
Binding parameters of [^{3}H]DAMP against 4-DAMP mustard in the corpus striatum of 90 weeks (Old) control, diabetic and insulin treated diabetic rats

Animal status	Best fit model	Log (EC_{50})	Ki	Hill slope
Control	One-site	-6.587	$2.58x10^{-7}$	-0.996
Diabetic	One-site	-6.725	$1.83x10^{-7}$	-0.993
Insulin treated diabetic	One-site	-6.624	$2.37x10^{-7}$	-0.972

Values are Mean of 4-6 separate experiments. Each group consists of 6-8 rats.

Figure -48



Data were fitted with an iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki - The affinity of the receptor for the competing drug. EC_{50} is the concentration of the competitor that competes for half the specific binding.

Figure-49

Real-Time PCR amplification of Muscarinic M3 receptor mRNA from the Corpus striatum of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Fig-49a (7 weeks old)

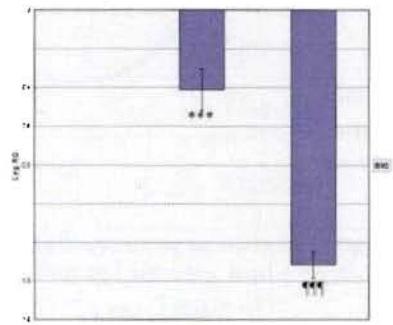


Fig-49b (90 weeks old)

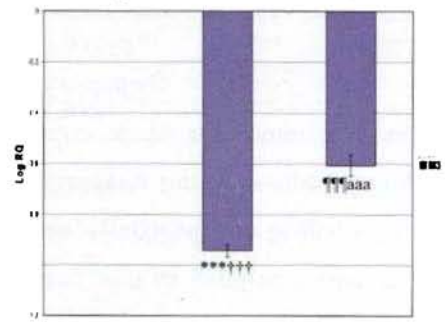


Table-35

Real-Time PCR amplification of Muscarinic M3 receptor mRNA from the Corpus striatum of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Sample No.	Animal status	Log RQ value	
		7 weeks old	90 weeks old
1	Control	0.0	0.0
2	Diabetic	-0.411±0.106***	-0.942±0.023***†††
3	Insulin treated diabetic	-1.316±0.069¶¶¶	-0.604±0.042¶¶¶ aaa

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats.

***p<0.001 when compared to respective controls, ¶¶¶p<0.001 when compared to diabetic, †††p<0.001 when compared to 7 weeks old diabetic, aaap<0.001 when compared to 7 weeks old insulin treated diabetic

Figure- 50
Scatchard analysis of [^{3}H]DAMP receptors binding against 4-DAMP mustard in the Pancreatic islets of 7 weeks (Young) Control, Diabetic and Insulin treated diabetic rats

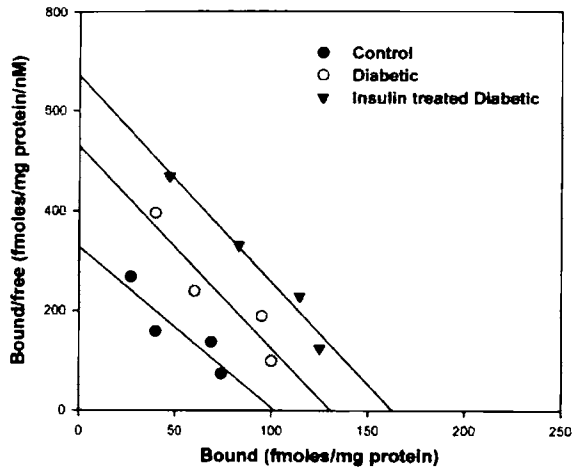


Figure-51
Scatchard analysis of [^{3}H]DAMP receptors binding against 4-DAMP mustard in the Pancreatic islets of 90 weeks (Old) Control, Diabetic and Insulin treated diabetic rats

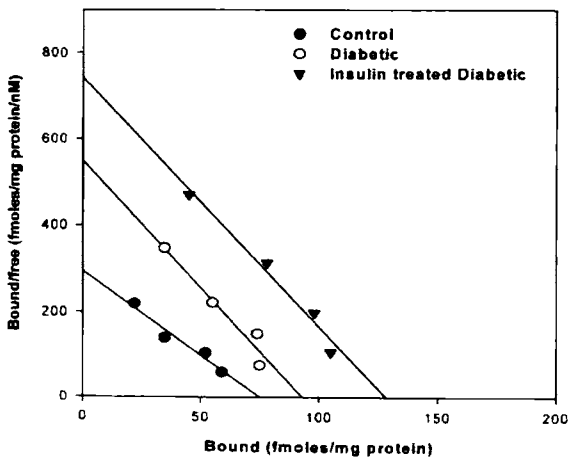


Figure-52
Scatchard analysis of [^{3}H]DAMP receptors binding against 4-DAMP mustard in the pancreas of Control and Experimental 7 weeks (Young) and 90 weeks (Old) rats

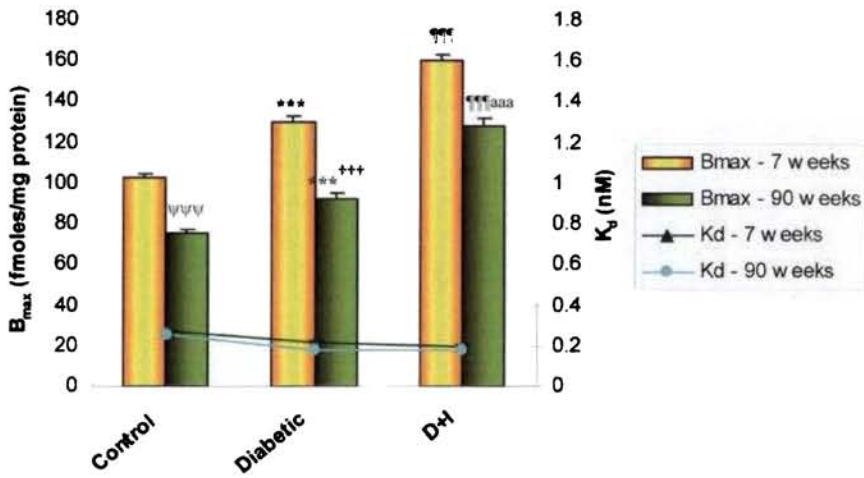


Table-36
Scatchard analysis of [^{3}H] DAMP receptors binding against 4-DAMP mustard in the pancreas of Control and Experimental 7 weeks (Young) and 90 weeks (Old) rats

Animal status	B_{max} (fmoles/mg protein)		K_d (nM)	
	7 weeks	90 weeks	7 weeks	90 weeks
Control	103.0±3.02	75.0± 2.79$^{\Psi\Psi\Psi}$	0.27± 0.01	0.25± 0.02
Diabetic	130.0± 5.33***	92.0± 4.18***†††	0.22± 0.06*	0.17±0.03*††
D+I	160.0± 4.30¶¶¶	128.0± 6.53$^{\P\P\P\,aaa}$	0.20± 0.02	0.17± 0.01^{aa}

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats.
*p<0.05, ***p<0.001 when compared to respective controls, ¶¶¶p<0.001 when compared to diabetic, $^{\Psi\Psi\Psi}$p<0.01 when compared to 7 weeks control, ††p<0.01, †††p<0.001 when compared to 7 weeks diabetic, ^{aa}p<0.01 when compared to 7 weeks insulin treated diabetic
D + I- Insulin treated diabetic

Figure-53

Real-Time PCR amplification of Muscarinic M3 receptor mRNA from the pancreatic islets of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Fig-53a (7 weeks old)

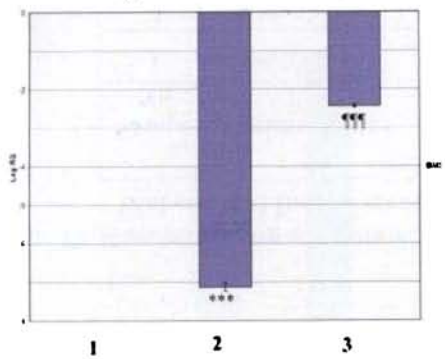


Fig-53b (90 weeks old)

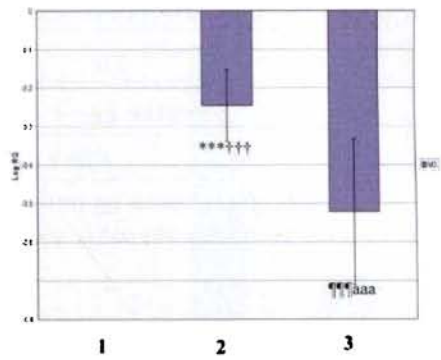


Table-37

Real-Time PCR amplification of Muscarinic M3 receptor mRNA from the pancreatic islets of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Sample No.	Animal status	Log RQ value	
		7 weeks old	90 weeks old
1	Control	0.0	0.0
2	Diabetic	-7.140±0.100***	-0.245±0.093***†††
3	Insulin treated diabetic	-2.438±0.034¶¶¶	-0.521±0.189¶¶¶ aaa

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats.

***p<0.001 when compared to respective controls, ¶¶¶p<0.001 when compared to diabetic, †††p<0.001 when compared to 7 weeks old diabetic, aaap<0.001 when compared to 7 weeks old insulin treated diabetic.

Figure-54

Real-Time PCR amplification of Glutamate receptor (NMDAR1) mRNA from the Cerebral cortex of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Fig-54a (7 weeks old)

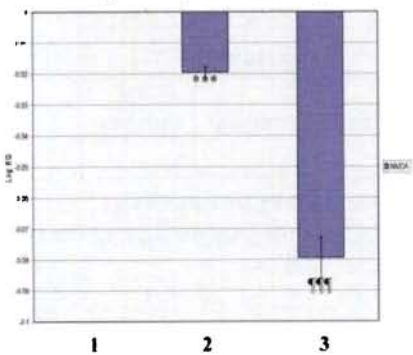


Fig-54b (90 weeks old)

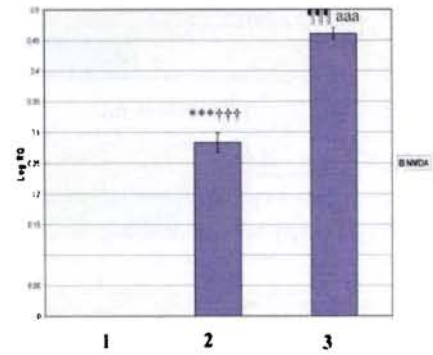


Table-38

Real-Time PCR amplification of Glutamate receptor (NMDAR1) mRNA from the Cerebral cortex of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Sample No.	Animal status	Log RQ value	
		7 weeks old	90 weeks old
1	Control	0.0	0.0
2	Diabetic	-0.019±0.001***	0.238±0.016***†††
3	Insulin treated diabetic	-0.079±0.006¶¶¶	0.461±0.009¶¶¶ aaa

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats.

***p<0.001 when compared to respective controls, ¶¶¶p<0.001 when compared to diabetic, †††p<0.001 when compared to 7 weeks old diabetic ,aaap<0.001 when compared to 7 weeks old insulin treated diabetic

Figure-55

Real-Time PCR amplification of Glutamate receptor (NMDAR1) mRNA from the Brainstem of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Fig-55a (7 weeks old)

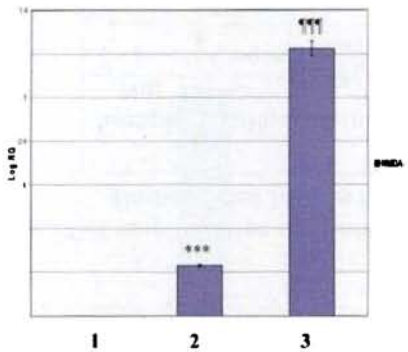


Fig-55b (90 weeks old)

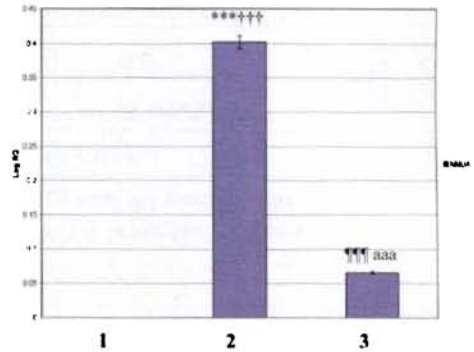


Table-39

Real-Time PCR amplification of Glutamate receptor (NMDAR1) mRNA from the Brainstem of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Sample No.	Animal status	Log RQ value	
		7 weeks old	90 weeks old
1	Control	0.0	0.0
2	Diabetic	$0.229\pm0.005^{***}$	$0.402\pm0.009^{***}\dagger\dagger\dagger$
3	Insulin treated diabetic	$1.225\pm0.033^{¶¶¶}$	$0.065\pm0.002^{¶¶¶\ aaa}$

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats.

***p<0.001 when compared to respective controls, ¶¶¶p<0.001 when compared to diabetic, †††p<0.001 when compared to 7 weeks old diabetic, ^{aaa}p<0.001 when compared to 7 weeks old insulin treated diabetic

Figure-56

Real-Time PCR amplification of Glutamate receptor (NMDAR1) mRNA from the Corpus striatum of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Fig-56a (7 weeks old)

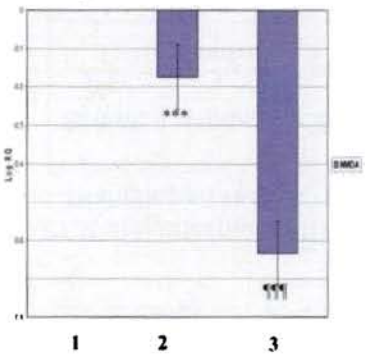


Fig-56b (90 weeks old)

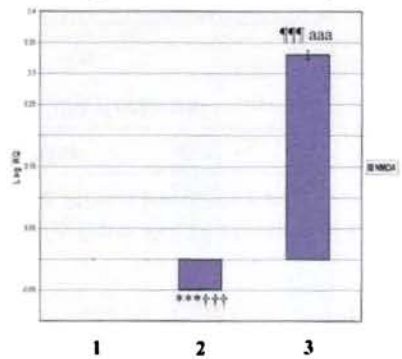


Table-40

Real-Time PCR amplification of Glutamate receptor (NMDAR1) mRNA from the Corpus striatum of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Sample No.	Animal status	Log RQ value	
		7 weeks old	90 weeks old
1	Control	0.0	0.0
2	Diabetic	-0.174±0.100***	-0.049±0.018***†††
3	Insulin treated diabetic	-0.632±0.034¶¶¶	-0.330±0.031¶¶¶ aaa

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats.

***p<0.001 when compared to respective controls, ¶¶¶p<0.001 when compared to diabetic, †††p<0.001 when compared to 7 weeks old diabetic, aaap<0.001 when compared to 7 weeks old insulin treated diabetic

Figure-57

Real-Time PCR amplification of Glutamate receptor (NMDAR1) mRNA from the Hippocampus of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Fig-57a (7 weeks old)

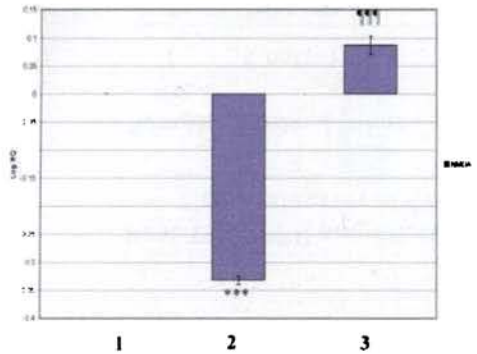


Fig-57b (90 weeks old)

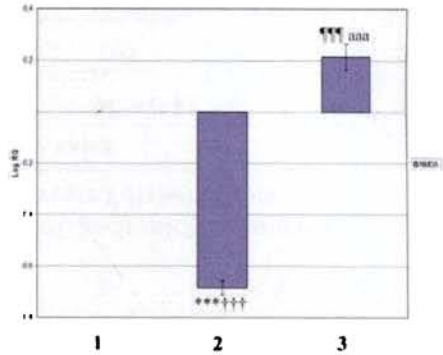


Table-41

Real-Time PCR amplification of Glutamate receptor (NMDAR1) mRNA from the Hippocampus of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Sample No.	Animal status	Log RQ value	
		7 weeks old	90 weeks old
1	Control	0.0	0.0
2	Diabetic	$-0.332\pm0.007^{***}$	$-0.685\pm0.027^{***\dagger\dagger\dagger}$
3	Insulin treated diabetic	$0.087\pm0.015^{\P\P\P}$	$0.215\pm0.051^{\P\P\P\,aaa}$

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats.

***p<0.001 when compared to respective controls, ¶¶¶p<0.001 when compared to diabetic, †††p<0.001 when compared to 7 weeks old diabetic, ^{aaa}p<0.001 when compared to 7 weeks old insulin treated diabetic

Figure-58

Real-Time PCR amplification of Glutamate receptor (mGlu-5) mRNA from the Cerebral cortex of Control, Diabetic and Insulin treated diabetic Rats

Fig-58a (7 weeks old)

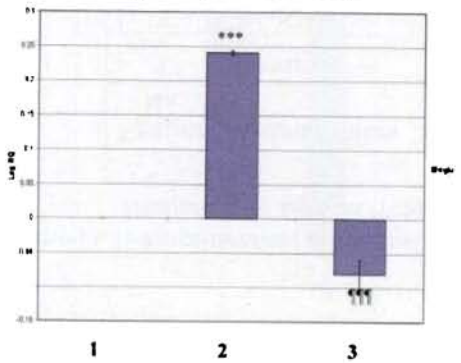


Fig-58b (90 weeks old)

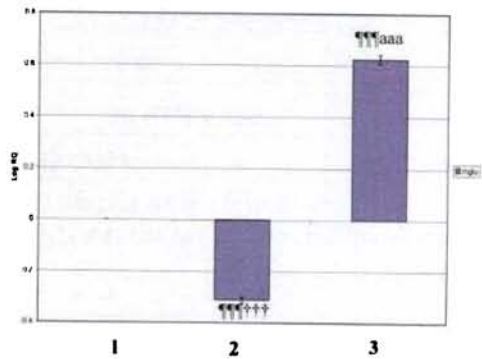


Table-42

Real-Time PCR amplification of Glutamate receptor (mGlu-5) mRNA from the Cerebral cortex of Control, Diabetic and Insulin treated diabetic Rats

Sample No.	Animal status	Log RQ value	
		7 weeks old	90 weeks old
1	Control	0.0	0.0
2	Diabetic	$0.241\pm0.003^{***}$	$-0.311\pm0.011^{***¶¶¶†††}$
3	Insulin treated diabetic	$-0.081\pm0.021^{¶¶¶}$	$0.623\pm0.018^{¶¶¶aaa}$

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats.

$^{***}p<0.001$ when compared to respective controls, $^{¶¶¶}p<0.001$ when compared to diabetic, $^{†††}p<0.001$ when compared to 7 weeks old diabetic, $^{aaa}p<0.001$ when compared to 7 weeks old insulin treated diabetic

Figure-59

Real-Time PCR amplification of Glutamate receptor (mGlu-5) mRNA from the Brainstem of Control, Diabetic and Insulin treated diabetic Rats

Fig-59a (7 weeks old)

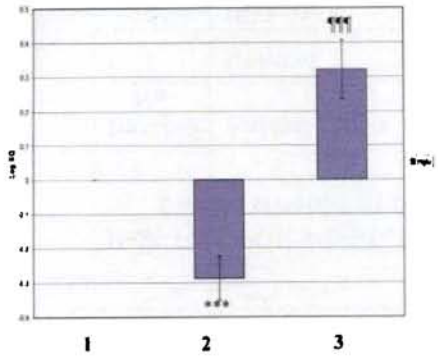


Fig-59b (90 weeks old)

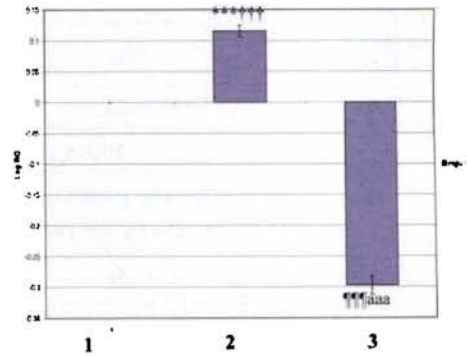


Table-43

Real-Time PCR amplification of Glutamate receptor (mGlu-5) mRNA from the Brainstem of Control, Diabetic and Insulin treated diabetic Rats

Sample No.	Animal status	Log RQ value	
		7 weeks old	90 weeks old
1	Control	0.0	0.0
2	Diabetic	-0.287±0.064***	0.115±0.009***+++
3	Insulin treated diabetic	0.321±0.086¶¶¶	-0.297±0.014¶¶¶aaa

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats.

***p<0.001 when compared to respective controls, ¶¶¶p<0.001 when compared to diabetic, +++p<0.001 when compared to 7 weeks old diabetic, aaap<0.001 when compared to 7 weeks old insulin treated diabetic

Figure-60

Real-Time PCR amplification of Glutamate receptor (mGlu-5) mRNA from the Corpus striatum of Control, Diabetic and Insulin treated diabetic Rats

Fig-60a (7 weeks old)

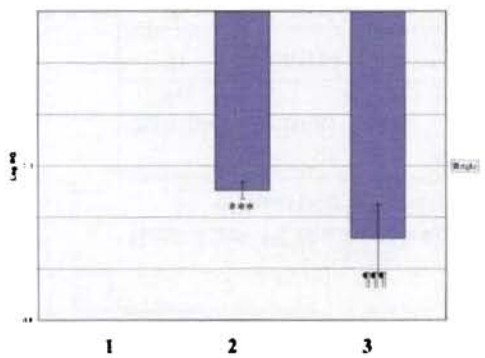


Fig-60b (90 weeks old)

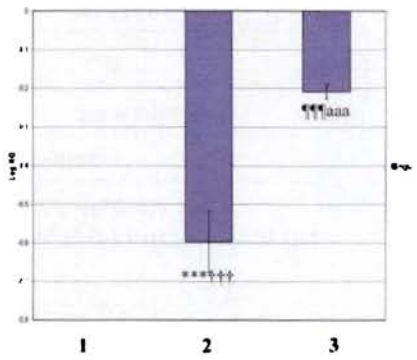


Table-44

Real-Time PCR amplification of Glutamate receptor (mGlu-5) mRNA from the Corpus striatum of Control, Diabetic and Insulin treated diabetic Rats

Sample No.	Animal status	Log RQ value	
		7 weeks old	90 weeks old
1	Control	0.0	0.0
2	Diabetic	$-0.347\pm0.016^{***}$	$-0.596\pm0.078^{***\dagger\dagger\dagger}$
3	Insulin treated diabetic	$-0.441\pm0.066^{\P\P\P}$	$-0.208\pm0.017^{***\P\P\P\,aaa}$

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats.

$^{***}p<0.001$ when compared to respective controls, $^{\P\P\P}p<0.001$ when compared to diabetic, $^{\dagger\dagger\dagger}p<0.001$ when compared to 7 weeks old diabetic.

$^{aaa}p<0.001$ when compared to 7 weeks old insulin treated diabetic

Figure-61

Real-Time PCR amplification of Glutamate receptor (mGlu-5) mRNA from the Hippocampus of Control, Diabetic and Insulin treated diabetic Rats

Fig-61a (7 weeks old)

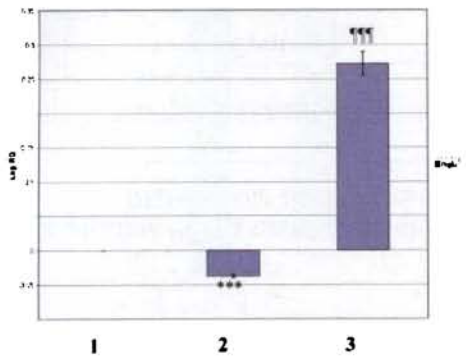


Fig-61b (90 weeks old)

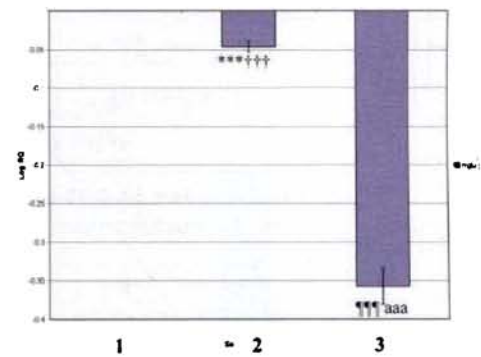


Table-45

Real-Time PCR amplification of Glutamate receptor (mGlu-5) mRNA from the Hippocampus of Control, Diabetic and Insulin treated diabetic Rats

Sample No.	Animal status	Log RQ value	
		7 weeks old	90 weeks old
1	Control	0.0	0.0
2	Diabetic	-0.037±0.002***	-0.045±0.007***†††
3	Insulin treated diabetic	0.272±0.017¶¶¶	-0.357±0.023¶¶¶ aaa

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats.

***p<0.001 when compared to respective controls, ¶¶¶p<0.001 when compared to diabetic, †††p<0.001 when compared to 7 weeks old diabetic, aaap<0.001 when compared to 7 weeks old insulin treated diabetic

Figure-62

Real-Time PCR amplification of DA D2 receptor mRNA from the cerebral cortex of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Fig-62a (7 weeks old)

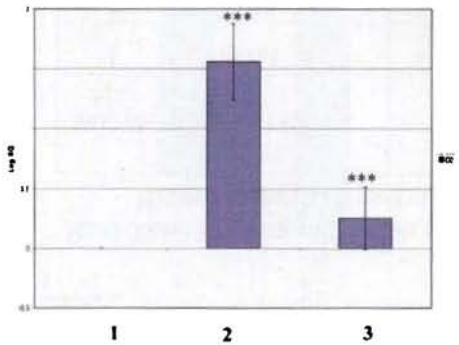


Fig-62b (90 weeks old)

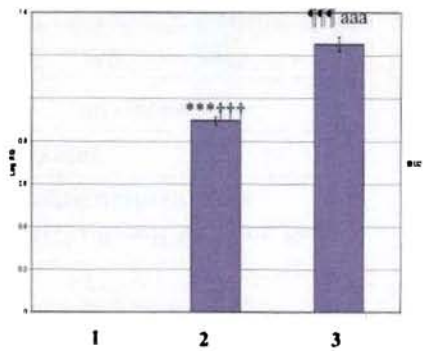


Table-46

Real-Time PCR amplification of DA D2 receptor mRNA from the cerebral cortex of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Sample No.	Animal status	Log RQ value	
		7 weeks old	90 weeks old
1	Control	0.0	0.0
2	Diabetic	$1.56\pm0.31^{***}$	$0.89\pm0.016^{***}\dagger\dagger\dagger$
3	Insulin treated diabetic	$0.25\pm0.26^{***}$	$1.25\pm0.032^{***aaa}$

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats.

***$p<0.001$ when compared to respective controls, †††$p<0.001$ when compared to 7 weeks old diabetic, $^{aaa}p<0.001$ when compared to 7 weeks old insulin treated diabetic

Figure-63

Real-Time PCR amplification of α_{2A}-adrenergic mRNA from the cerebral cortex of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Fig-63a (7 weeks old)

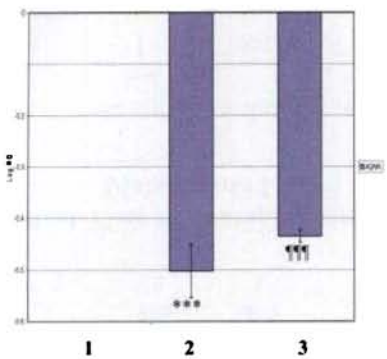


Fig-63b (90 weeks old)

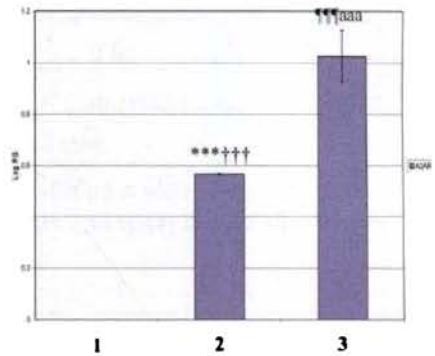


Table-47

Real-Time PCR amplification of α_{2A}-adrenergic mRNA from the cerebral cortex of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Sample No.	Animal status	Log RQ value	
		7 weeks old	90 weeks old
1	Control	0.0	0.0
2	Diabetic	-0.502±0.051***	0.565±0.002***†††
3	Insulin treated Diabetic	-0.435±0.012¶¶¶	1.025±0.101¶¶¶ aaa

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats.

***p<0.001 when compared to respective controls, ¶¶¶p<0.001 when compared to diabetic, †††p<0.001 when compared to 7 weeks old diabetic, aaap<0.001 when compared to 7 weeks old insulin treated diabetic

Figure-64

Real-Time PCR amplification of β_2-adrenergic mRNA from the Cerebral cortex of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Fig-64a (7 weeks old)

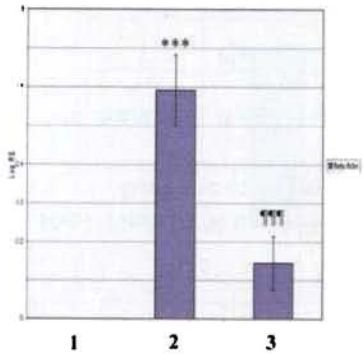


Fig-64b (90 weeks old)

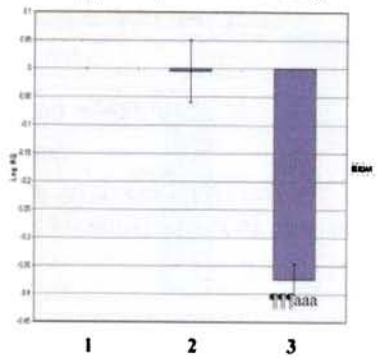


Table-48

Real-Time PCR amplification of β_2-adrenergic mRNA from the Cerebral cortex of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Sample No.	Animal status	Log RQ value	
		7 weeks old	90 weeks old
1	Control	0.0	0.0
2	Diabetic	$0.591\pm0.09^{***}$	-0.004 ± 0.055
3	Insulin treated diabetic	$0.144\pm0.06^{¶¶¶}$	$-0.373\pm0.027^{¶¶¶\ aaa}$

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats..

***p<0.001 when compared to respective controls, ¶¶¶p<0.001 when compared to diabetic, †††p<0.001 when compared to 7 weeks old diabetic, ^{aaa}p<0.001 when compared to 7 weeks old insulin treated diabetic

Figure-65

Real-Time PCR amplification of $GABA_{A\alpha 1}$ mRNA from the Cerebral cortex of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Fig-65a (7 weeks old)

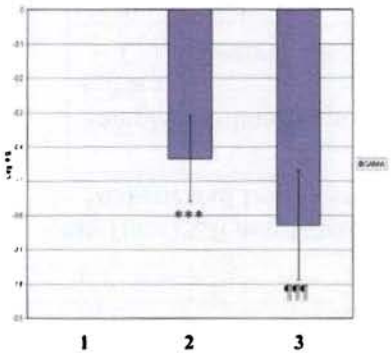


Fig-65b (90 weeks old)

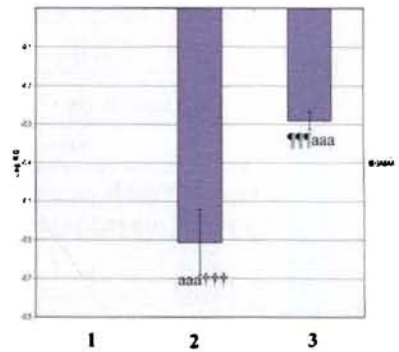


Table-49

Real-Time PCR amplification of $GABA_{A\alpha 1}$ mRNA from the Cerebral cortex of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Sample No.	Animal status	Log RQ value	
		7 weeks old	90 weeks old
1	Control	0.0	0.0
2	Diabetic	$-0.434\pm0.125^{***}$	$-0.606\pm0.084^{***}\dagger\dagger\dagger$
3	Insulin treated diabetic	$-0.628\pm0.158^{¶¶¶}$	$-0.290\pm0.022^{¶¶¶\,aaa}$

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats.

***p<0.001 when compared to respective controls, ¶¶¶p<0.001 when compared to diabetic, †††p<0.001 when compared to 7 weeks old diabetic, ^{aaa}p<0.001 when compared to 7 weeks old insulin treated diabetic

Figure-66

Real-Time PCR amplification of $GABA_B$ mRNA from the Cerebral cortex of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Fig-66a (7 weeks old)

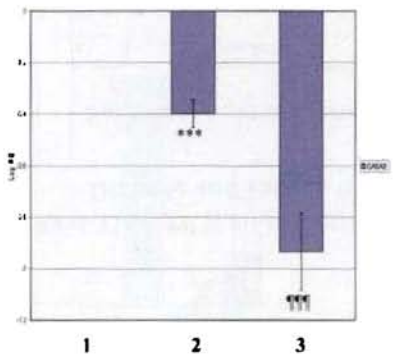


Fig-66b (90 weeks old)

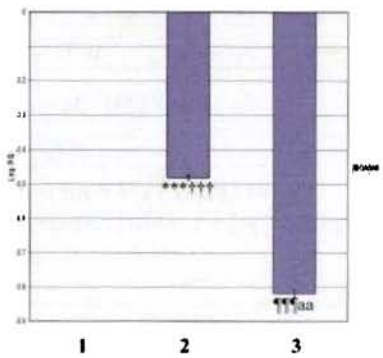


Table-50

Real-Time PCR amplification of $GABA_B$ mRNA from the Cerebral cortex of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Sample No.	Animal status	Log RQ value	
		7 weeks old	90 weeks old
1	Control	0.0	0.0
2	Diabetic	-0.398±0.05***	-0.481±0.006***†††
3	Insulin treated diabetic	-0.933±0.15¶¶¶	-0.819±0.010¶¶¶ aa

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats.

***p<0.001 when compared to respective controls, ¶¶¶p<0.001 when compared to diabetic, †††p<0.001 when compared to 7 weeks old diabetic, aap<0.01 when compared to 7 weeks old insulin treated diabetic

Figure-67

Real-Time PCR amplification of 5-HT_{2C} mRNA from the Cerebral cortex of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Fig-67a (7 weeks old)

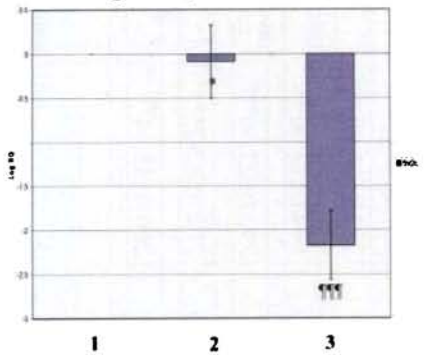


Fig-67b (90 weeks old)

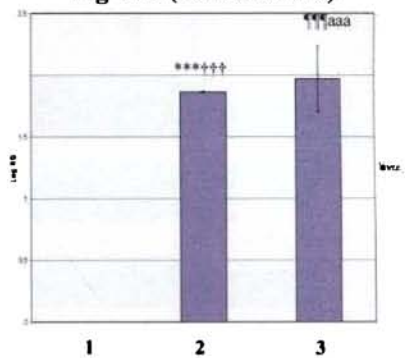


Table-51

Real-Time PCR amplification of 5-HT_{2C} mRNA from the Cerebral cortex of Control, Diabetic and Insulin treated diabetic 7 weeks (Young) and 90 weeks (Old) rats

Sample No.	Animal status	Log RQ value	
		7 weeks old	90 weeks old
1	Control	0.0	0.0
2	Diabetic	$-0.087\pm0.415^{*}$	$1.86\pm0.006^{***}\dagger\dagger\dagger$
3	Insulin treated diabetic	$-2.175\pm0.390^{¶¶¶}$	$1.96\pm0.267^{¶¶¶\,aaa}$

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats.
*p<0.05, ***p<0.001 when compared to respective controls, ¶¶¶p<0.001 when compared to diabetic, †††p<0.001 when compared to 7 weeks old diabetic, ^{aaa}p<0.001 when compared to 7 weeks old insulin treated diabetic.

Figure-68
IP3 content in the cerebral cortex of control and experimental 7 weeks (Young) and 90 weeks (Old) rats

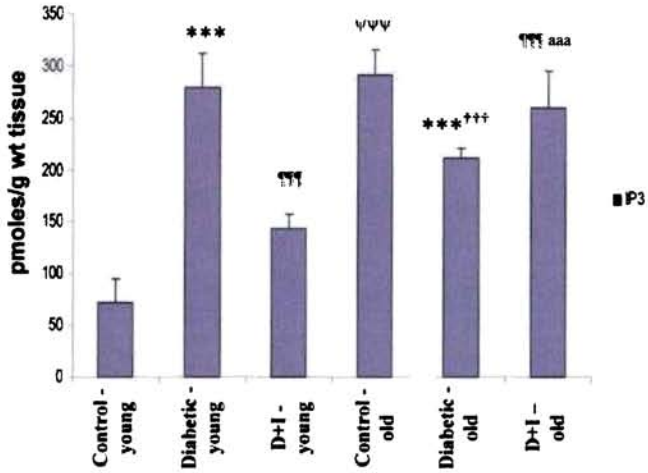


Table-52
IP3 content in the cerebral cortex of control and experimental 7 weeks (Young) and 90 weeks (Old) rats

Animal status	Concentration (pmoles/g wt tissue)
Control-young	72.0±23.45
Diabetic-young	280.0±32.00***
D+I-young	144.0±13.00¶¶¶
Control-old	292.0±23.50ψψψ
Diabetic- old	212.0±8.50***†††
D+I- old	260.0±35.64¶¶¶ aaa

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consisted of 6-8 rats.
***p<0.001 when compared to respective control groups, ¶¶¶p<0.001 when compared to respective diabetic groups, ψψψp<0.001 when compared to 7 weeks control, †††p<0.001 when compared to 7 weeks diabetic, aaap<0.001 when compared to 7 weeks insulin treated diabetic
D + I- Insulin treated Diabetic

Figure-69
IP3 content in the brainstem of control and experimental 7 weeks (Young) and 90 weeks (Old) rats

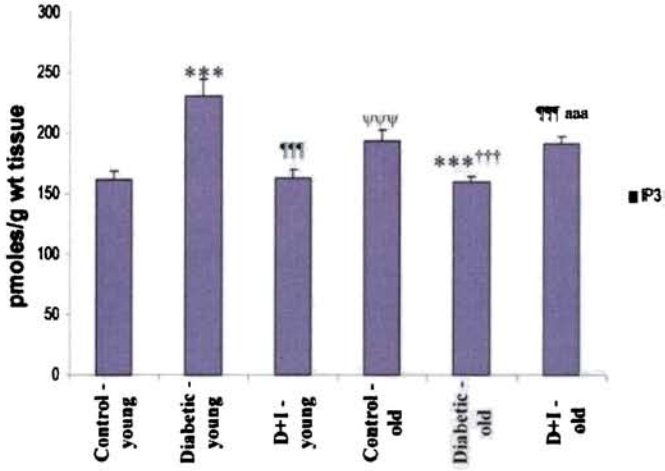


Table-53
IP3 content in the brainstem of control and experimental 7 weeks (Young) and 90 weeks (Old) rats

Animal status	Concentration (pmoles/g wt tissue)
Control-young	161.6±6.68
Diabetic-young	230.8±13.40***
D+I-young	162.4±7.25¶¶¶
Control-old	193.6±8.75ψψψ
Diabetic- old	158.8±4.67***†††
D+I- old	190.2±5.80¶¶¶ aaa

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consisted of 6-8 rats.
***p<0.001 when compared to respective control groups, ¶¶¶p<0.001 when compared to respective diabetic groups, ψψψp<0.001 when compared to 7 weeks control, †††p<0.001 when compared to 7 weeks diabetic, aaap<0.001 when compared to 7 weeks insulin treated diabetic
D + I- Insulin treated Diabetic

Figure-70

IP3 content in the corpus striatum of control and experimental 7 weeks (Young) and 90 weeks (Old) rats

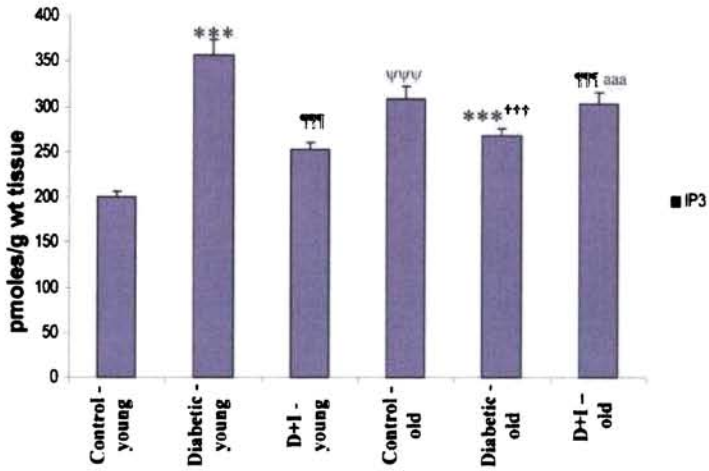


Table-54

IP3 content in the corpus striatum of control and experimental 7 weeks (Young) and 90 weeks (Old) rats

Animal status	Concentration (pmoles/g wt tissue)
Control-young	200.0±5.65
Diabetic-young	356.0±17.58***
D+I-young	252.0±7.80¶¶¶
Control-old	308.0±15.25ψψψ
Diabetic- old	268.0±8.50***†††
D+I- old	304.0±12.50¶¶¶ aaa

Values are Mean ± S.E.M of 6 separate experiments. Each group consisted of 6 rats.
***p<0.001 when compared to respective control groups, ¶¶¶p<0.001 when compared to respective diabetic groups, ψψψp<0.001 when compared to 7 weeks control, †††p<0.001 when compared to 7 weeks diabetic, aaap<0.001 when compared to 7 weeks insulin treated diabetic
D + I- Insulin treated Diabetic

Figure-71
IP3 content in the pancreas of control and experimental 7 weeks (Young) and 90 weeks (Old) rats

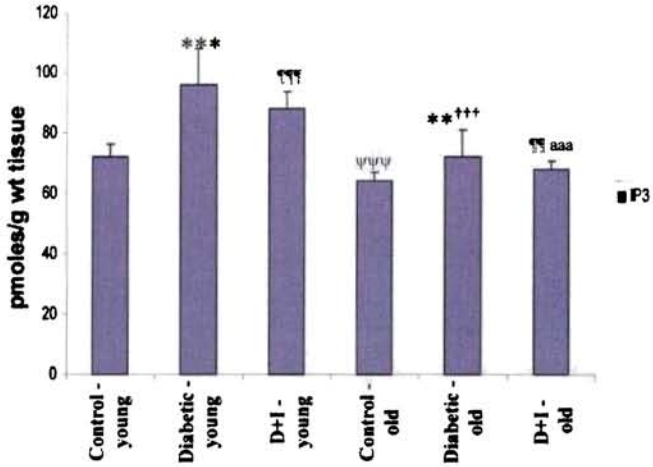


Table-55
IP3 content in the pancreas of control and experimental 7 weeks (Young) and 90 weeks (Old) rats

Animal status	Concentration (pmoles/g wt tissue)
Control-young	72.0±4.25
Diabetic-young	96.0±11.80***
D+I-young	88.0±5.26¶¶
Control-old	64.0±3.20^{ψψψ}
Diabetic- old	72.0±8.75**†††
D+I- old	68.0±2.50^{¶¶aaa}

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consisted of 6-8 rats.
**p<0.01, ***p<0.001 when compared to respective control groups, ¶p<0.01, ¶¶p<0.001 when compared to respective diabetic groups, ^{ψψψ}p<0.001 when compared to 7 weeks control, †††p<0.001 when compared to 7 weeks diabetic, ^{aaa}p<0.001 when compared to 7 weeks insulin treated diabetic
D + I- Insulin treated Diabetic

Figure-72
cGMP content in the cerebral cortex of control and experimental 7 weeks (Young) and 90 weeks (Old) rats

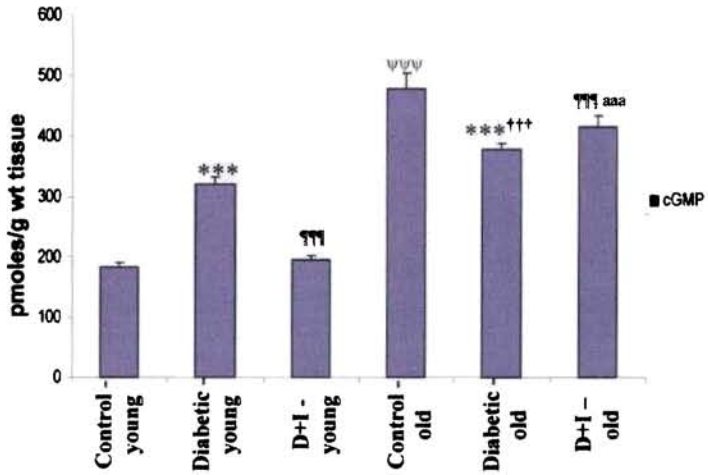


Table-56
cGMP content in the cerebral cortex of control and experimental 7 weeks (Young) and 90 weeks (Old) rats

Animal status	Concentration (pmoles/g wt tissue)
Control-young	184.0±7.50
Diabetic-young	320.0±12.45***
D+I-young	196.0±5.40¶¶¶
Control-old	480.0±25.60^{ΨΨΨ}
Diabetic- old	380.0±8.25***†††
D+I- old	416.0±18.34$^{¶¶¶\ aaa}$

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consisted of 6-8 rats.
***p<0.001 when compared to respective control groups, ¶¶¶p<0.001 when compared to respective diabetic groups, ^{ΨΨΨ}p<0.001 when compared to 7 weeks control, †††p<0.001 when compared to 7 weeks diabetic, ^{aaa}p<0.001 when compared to 7 weeks insulin treated diabetic
D + I- Insulin treated Diabetic

Figure-73
cGMP content in the brainstem of control and experimental 7 weeks (Young) and 90 weeks (Old) rats

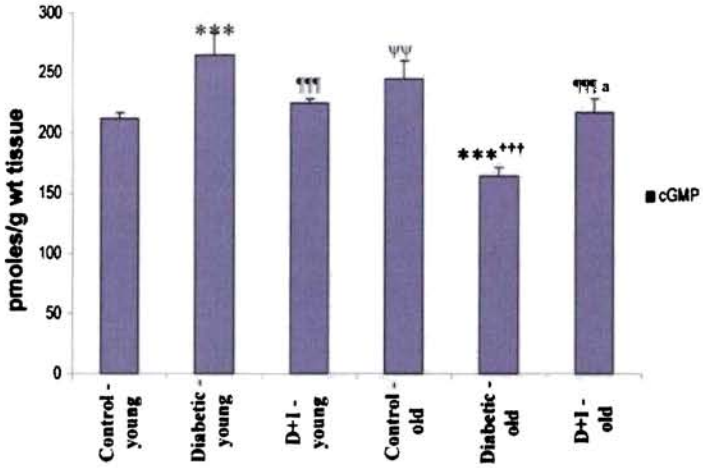


Table-57
cGMP content in the brainstem of control and experimental 7 weeks (Young) and 90 weeks (Old) rats

Animal status	Concentration (pmoles/g wt tissue)
Control-young	212.0±4.75
Diabetic-young	264.0±18.50***
D+I-young	224.0±3.85¶¶¶
Control-old	244.0±15.25ψψ
Diabetic- old	164.0±7.30***+++
D+I- old	216.0±12.45¶¶¶ a

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consisted of 6-8 rats.
***p<0.001 when compared to respective control groups, ¶¶¶p<0.001 when compared to respective diabetic groups, ψψψp<0.001 when compared to 7 weeks control, +++p<0.001 when compared to 7 weeks diabetic, aaap<0.001 when compared to 7 weeks insulin treated diabetic
D + I- Insulin treated Diabetic

Figure-74
cGMP content in the corpus striatum of control and experimental 7 weeks (Young) and 90 weeks (Old) rats

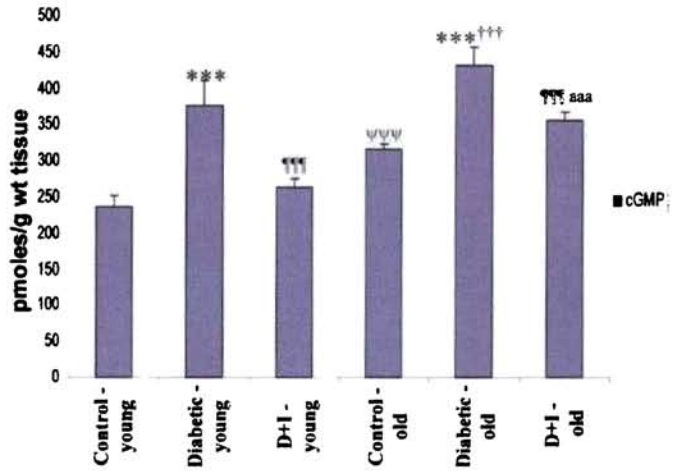


Table-58
cGMP content in the corpus striatum of control and experimental 7 weeks (Young) and 90 weeks (Old) rats

Animal status	Concentration (pmoles/g wt tissue)
Control-young	236.0±15.50
Diabetic-young	376.0±34.56***
D+I-young	264.0±10.25¶¶¶
Control-old	316.0±8.40ψψψ
Diabetic- old	432.0±25.80***†††
D+I- old	356.0±12.55¶¶¶ aaa

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consisted of 6-8 rats.
***p<0.001 when compared to respective control groups, ¶¶¶p<0.001 when compared to respective diabetic groups, ψψψp<0.001 when compared to 7 weeks control, †††p<0.001 when compared to 7 weeks diabetic, aaap<0.001 when compared to 7 weeks insulin treated diabetic
D + I- Insulin treated Diabetic

Figure-75
cGMP content in the pancreas of control and experimental 7 weeks (Young) and 90 weeks (Old) rats

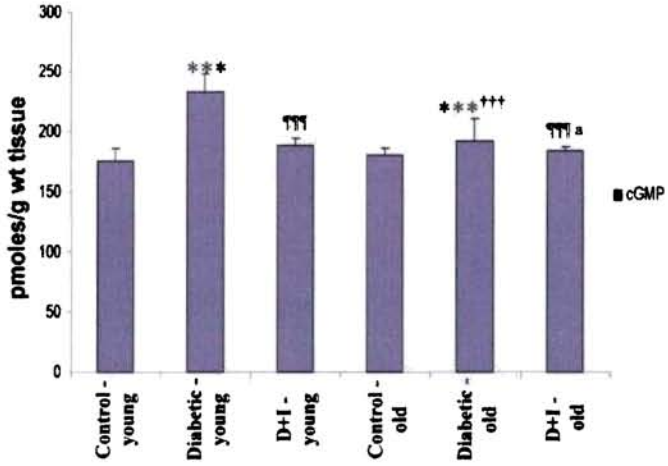


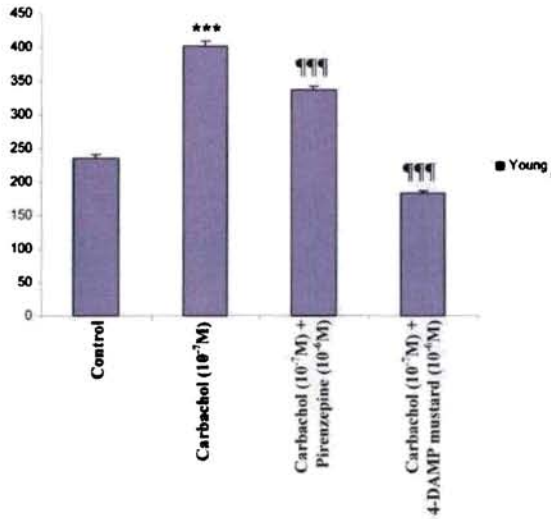
Table-59
cGMP content in the pancreas of control and experimental 7 weeks (Young) and 90 weeks (Old) rats

Animal status	Concentration (pmoles/g wt tissue)
Control-young	176.0±9.50
Diabetic-young	232.0±15.65***
D+I-young	188.0±5.70¶¶¶
Control-old	180.0±6.35
Diabetic- old	192.0±18.50***†††
D+I- old	184.0±3.25¶¶¶ a

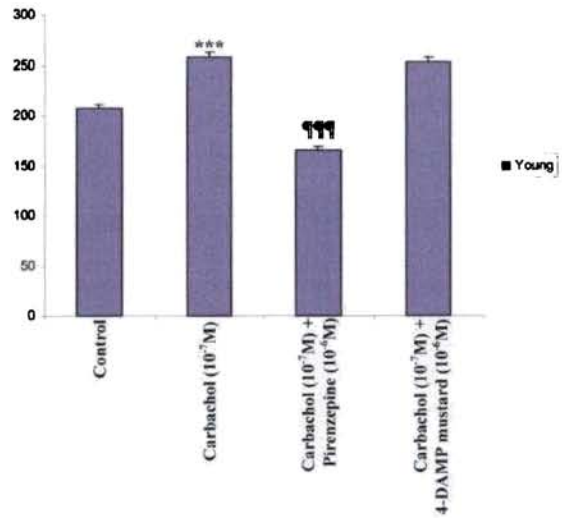
Values are Mean ± S.E.M of 4-6 separate experiments. Each group consisted of 6-8 rats.
**p<0.01, ***p<0.001 when compared to respective control groups, ¶p<0.01, ¶¶¶p<0.001 when compared to respective diabetic groups, ᵛᵛᵛp<0.001 when compared to 7 weeks control, †††p<0.001 when compared to 7 weeks diabetic, ᵃᵃᵃp<0.001 when compared to 7 weeks insulin treated diabetic
D + I- Insulin treated Diabetic

Figure-76
Effect of Carbachol & Muscarinic antagonists on insulin secretion in young pancreatic islets in one hour *in vitro*

4mM glucose



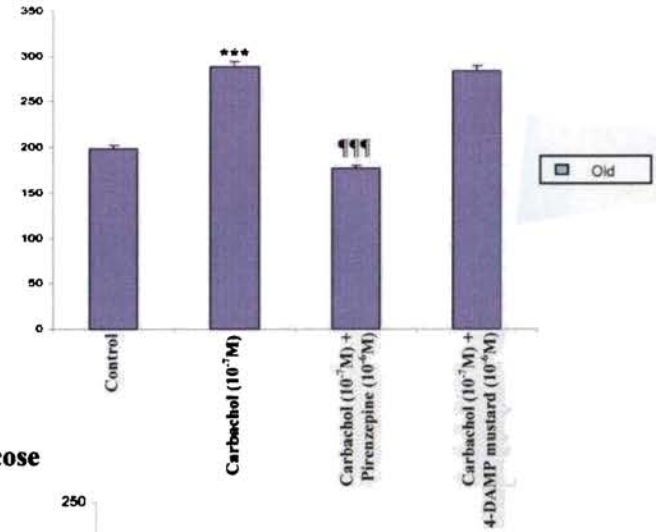
20mM glucose



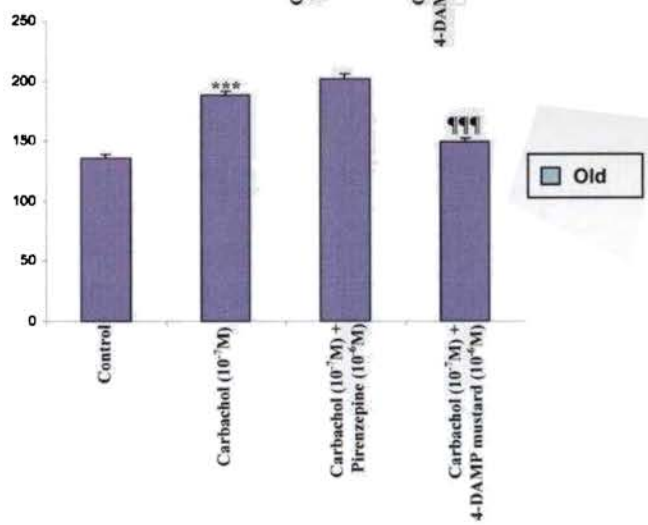
Values are Mean ± S.E.M of 4-6 separate experiments.
***p<0.001 when compared to respective control groups,
¶¶¶p<0.001 when compared to carbachol (10^{-7} M).

Figure-77
Effect of Carbachol & Muscarinic antagonists on insulin secretion in old pancreatic islets in one hour *in vitro*

4mM glucose



20mM glucose

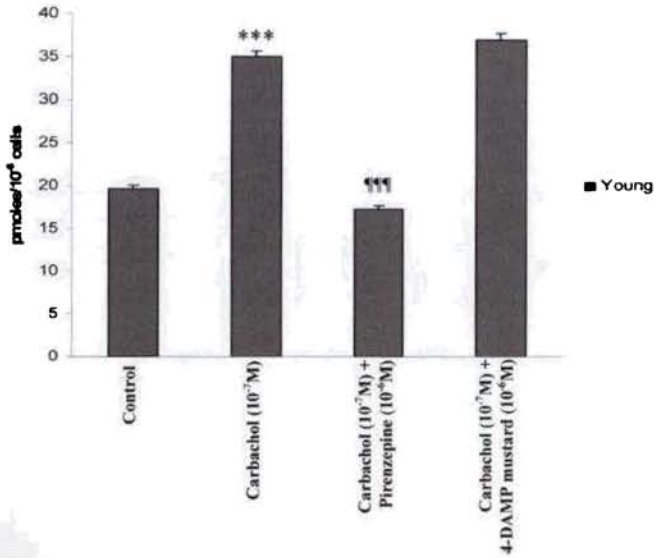


Values are Mean ± S.E.M of 4-6 separate experiments.
***p<0.001 when compared to respective control groups, ¶¶¶p<0.001 when compared to carbachol (10^{-7} M)

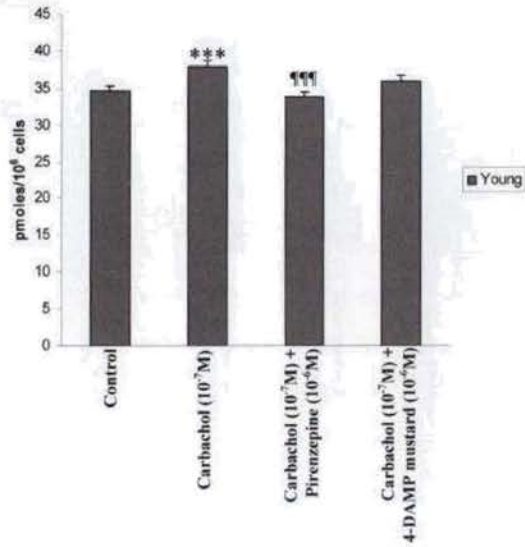
Figure-78

Effect of carbachol and muscarinic antagonists on IP3 levels in one hour young pancreatic islets *in vitro*

4mM glucose



20mM glucose



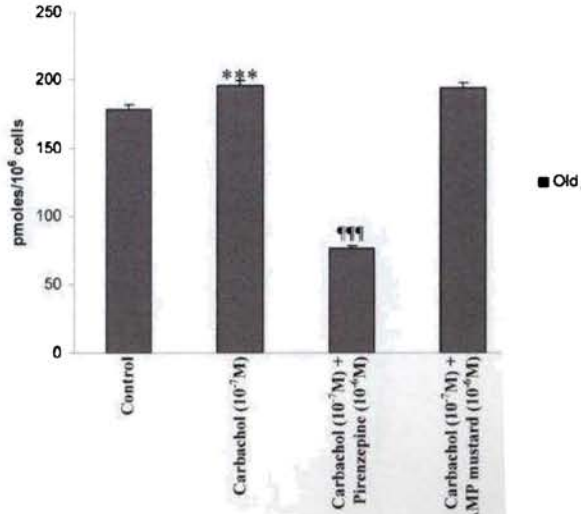
Values are Mean ± S.E.M of 4-6 separate experiments. .

***p<0.001 when compared to control, ¶¶¶p<0.001 when compared to carbachol (10^{-7}M)

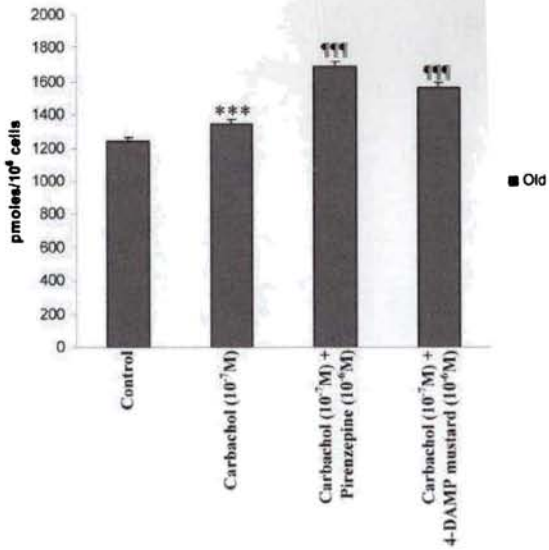
Figure- 79

Effect of carbachol and muscarinic antagonists on IP3 levels in one hour old pancreatic islets *in vitro*

4mM glucose



20mM glucose

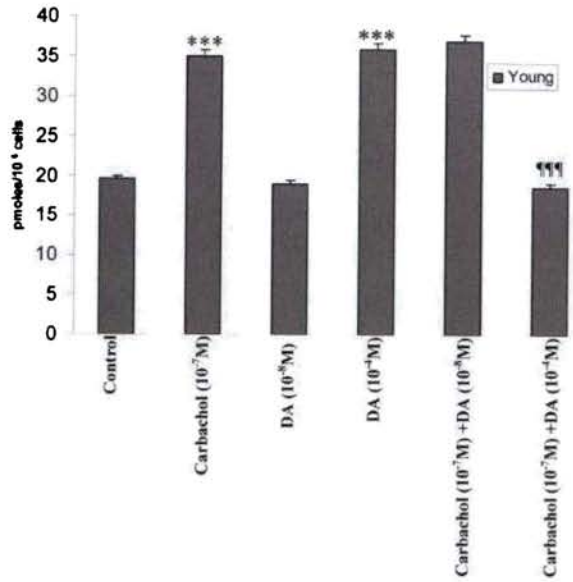


Values are Mean ± S.E.M of 4-6 separate experiments. .

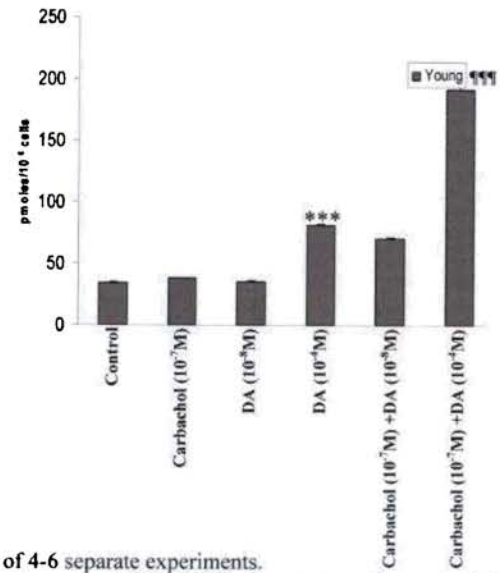
***p<0.001 when compared to control, ¶¶¶p<0.001 when compared to carbachol (10^{-7}M)

Figure-80
Effect of carbachol and dopamine on IP3 levels at one hour young pancreatic islets *in vitro*

4mM glucose



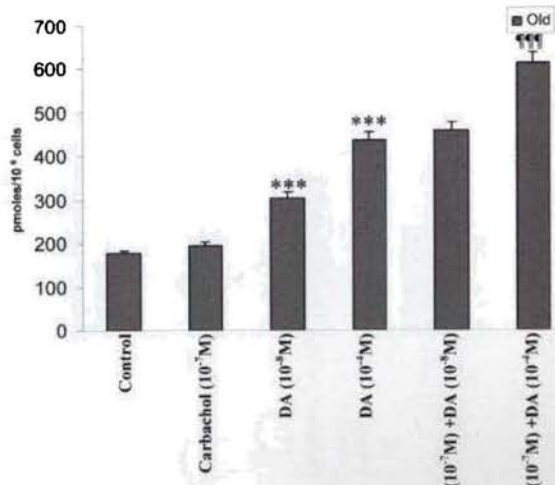
20mM glucose



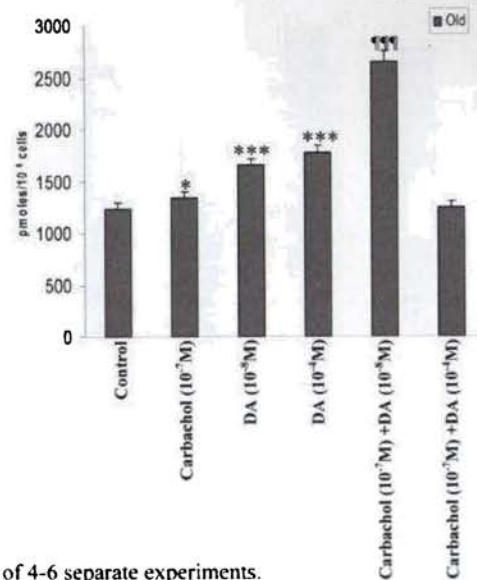
Values are Mean ± S.E.M of 4-6 separate experiments.
***p<0.001 when compared to control, ¶¶¶p<0.001 when compared to carbachol (10^{-7}M)

Figure-81
Effect of carbachol and dopamine on IP3 levels at one hour old pancreatic islets *in vitro*

4mM glucose



20mM glucose

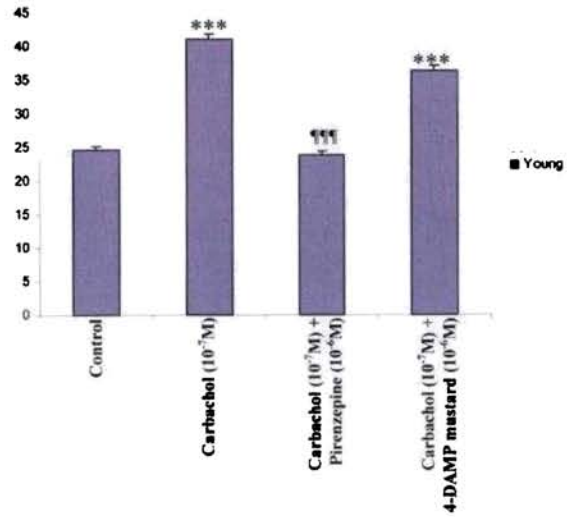


Values are Mean ± S.E.M of 4-6 separate experiments.
*$p<0.05$, ***$p<0.001$ when compared to control, ¶¶¶$p<0.001$ when compared to carbachol (10^{-7}M)

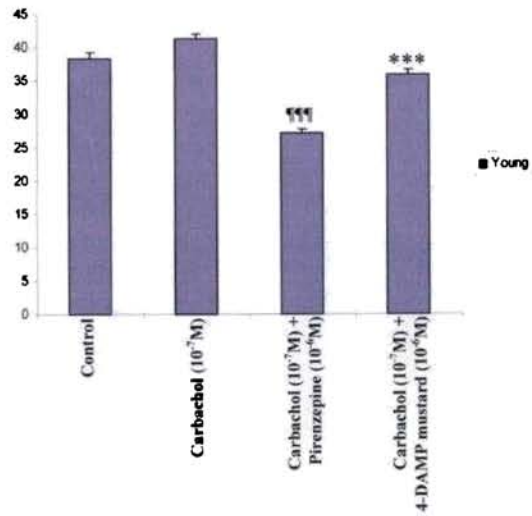
Figure-82

Effect of carbachol and muscarinic antagonists on cGMP contents in one hour young pancreatic islets *in vitro*

4mM glucose



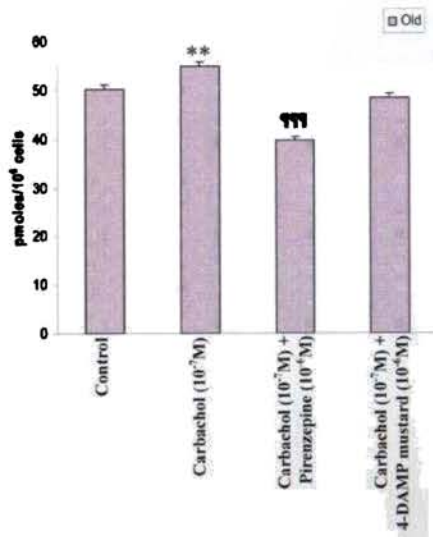
20mM glucose



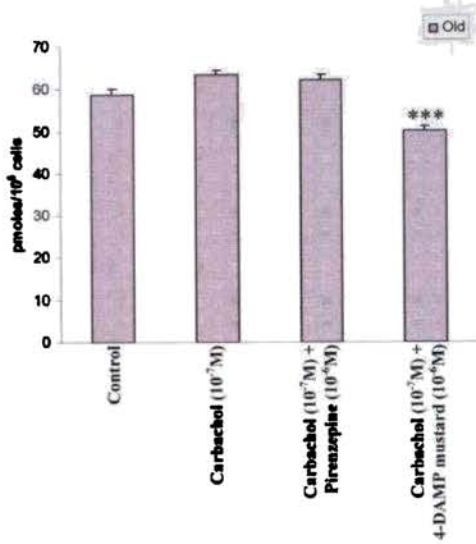
Values are Mean ± S.E.M of 4-6 separate experiments.
*p<0.05, ***p<0.001 when compared to control, ¶¶¶p<0.001 when compared to carbachol (10^{-7}M)

Figure-83
Effect of carbachol and muscarinic antagonists on cGMP levels in one hour old pancreatic islets *in vitro*

4mM glucose



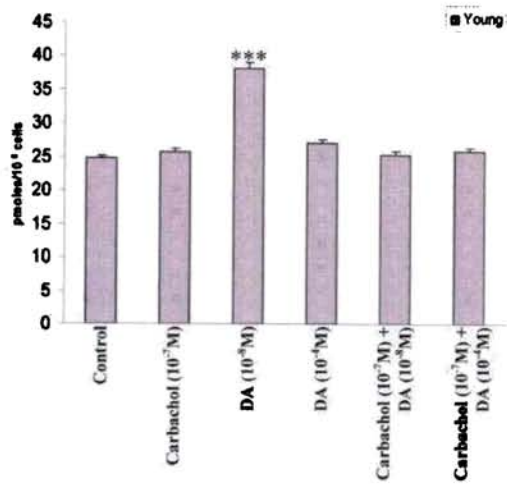
20mM glucose



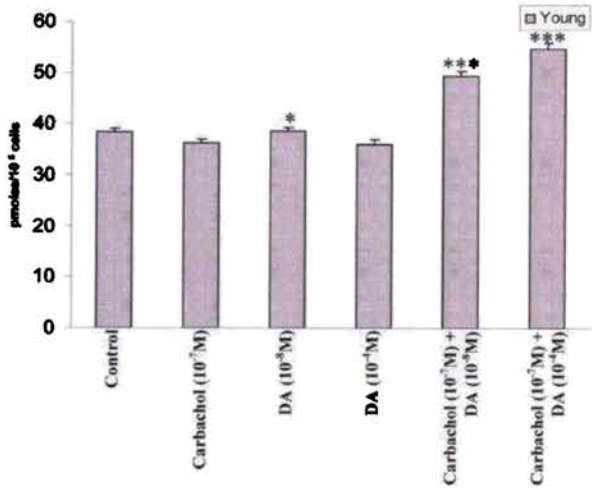
Values are Mean ± S.E.M of 4-6 separate experiments.
**p<0.01, ***p<0.001 when compared to control, ¶¶¶p<0.001 when compared to carbachol (10^{-7}M)

Figure-84
Effect of carbachol and dopamine on cGMP levels at one hour young pancreatic islets *in vitro*

4mM glucose



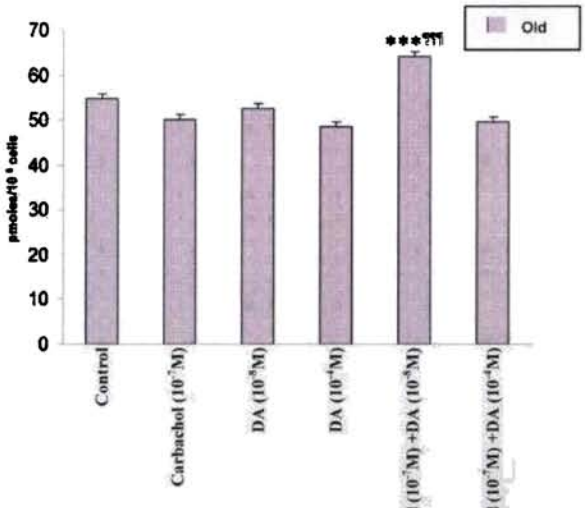
20mM glucose



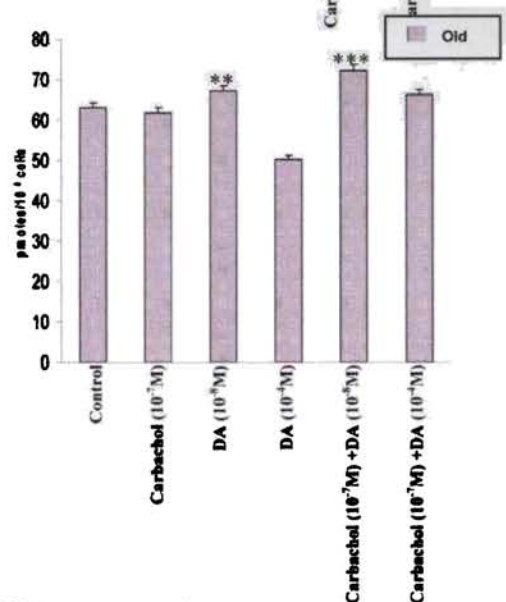
Values are Mean ± S.E.M of 4-6 separate experiments.
*p<0.05, ***p<0.001 when compared to carbachol (10^{-7} M)

Figure-85
Effect of carbachol and dopamine on cGMP levels at one hour old pancreatic islets *in vitro*

4mM glucose



20mM glucose



Values are Mean ± S.E.M of 4-6 separate experiments.
**p<0.01, ***p<0.001 when compared to control, ¶¶ p<0.001 when compared to carbachol (10^{-7} M)

Figure- 86
Triiodothyronine (T3) content (ng/ml) in the serum of experimental 7 weeks (Young) and 90 weeks (Old) rats

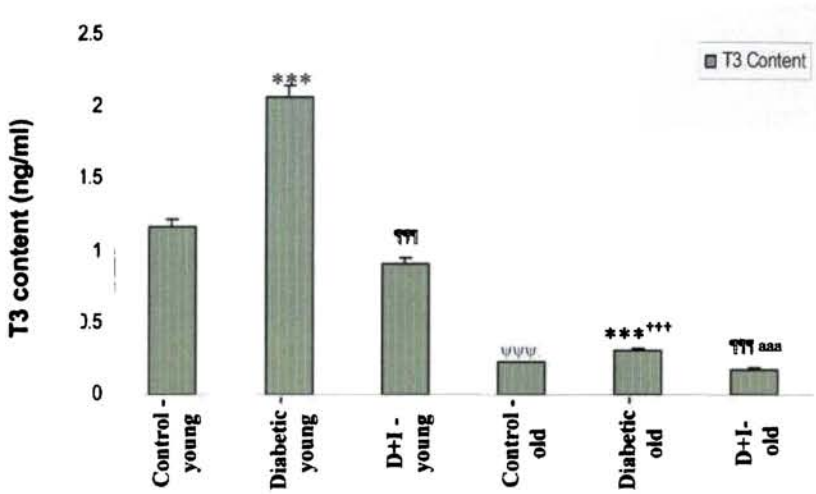


Table-60
Triiodothyronine (T3) content (ng/ml) in the serum of experimental 7 weeks (Young) and 90 weeks (Old) rats

Animal status	Concentration (ng/ml)
Control-7 weeks	1.171 ± 0.03
Diabetic-7 weeks	2.064 ± 0.05***
D+I-7 weeks	0.919 ± 0.15¶¶¶
Control-90 weeks	0.226 ± 0. 03^{ψψψ}
Diabetic-90 weeks	0.310 ± 0.09***†††
D+I -90 weeks	0.177 ± 0.05$^{¶¶¶\ aaa}$

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consisted of 6-8 rats.
***p<0.001 when compared to respective control groups, ¶¶¶p<0.001 when compared to respective diabetic groups, ^{ψψψ}p<0.01 when compared to 7 weeks control, †††p<0.001 when compared to 7 weeks diabetic, ^{aaa}p<0.001 when compared to 7 weeks insulin treated diabetic
D + I- Insulin treated Diabetic

Table-61

NE, EPI, DA and 5-HT content (nmoles/g wet weight of tissue) in the cerebral cortex of control and experimental young and old rats

Animal status	NE	EPI	DA	5-HT
Saline treated-young	3.65±0.05	3.82±0.17	2.23±0.14	2.60±0.25
STH treated-young	2.58±0.24***	3.12±0.15*	1.80±0.02***	1.18±0.07***
Insulin treated-young	0.73±0.02***	0.77±0.05***	0.37±0.03***	0.23±0.02***
Saline treated-old	2.38±0.58***	3.32±0.03*	0.99±0.05***	1.39±0.05***
STH treated-old	2.00±0.35	2.87±0.07¶¶¶ψψψ	0.79±0.04¶ψψψ	1.55±0.03¶
Insulin treated-old	1.01±0.03¶¶¶ aaa	1.36±0.02¶¶¶ aaa	0.39±0.05¶¶¶	0.68±0.01¶¶¶ aa

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consisted of 6-8 rats.
*p<0.05, ***p<0.001 when compared to saline treated young rats, ¶p<0.05, ¶¶¶p<0.001 when compared to saline treated old rats, ψp<0.05, ψψψp<0.001when compared to STH treated young rats, aap<0.01, aaap<0.001 when compared to INS treated young rats
Young- Treatment started at 4 weeks continued to 16 weeks
Old- Treatment started at 60 weeks continued to 90 weeks

Table-62

NE, EPI, DA and 5-HT content (nmoles/g wet weight of tissue) in the corpus striatum of control and experimental young and old rats

Animal status	NE	EPI	DA	5-HT
Saline treated-young	1.96±0.04	2.66±0.02	9.73±0.35	1.12±0.03
STH treated-young	1.90±0.03	3.05±0.15***	5.31±0.28***	1.49±0.02**
Insulin treated-young	1.92±0.05	3.15±0.04***	5.05±0.30***	1.31±0.05
Saline treated-old	0.87±0.07***	1.62±0.05***	2.58±0.10***	0.68±0.02***
STH treated-old	1.66±0.15^{¶¶¶ψ}	1.33±0.08^{¶ψψψ}	2.13±0.15^{ψψψ}	0.67±0.04^{ψψψ}
Insulin treated-old	1.36±0.23^{¶¶¶a}	2.17±0.17¶¶¶ aaa	4.86±0.32¶¶¶ aaa	0.92±0.07¶¶¶ aaa

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consisted of 6-8 rats.
**p<0.01, ***p<0.001 when compared to saline treated young rats, ¶p<0.05, ¶¶p<0.01, ¶¶¶p<0.001 when compared to saline treated old rats, ^{ψ}p<0.05, ^{ψψψ}p<0.001 when compared to STH treated young rats, ^{a}p<0.05, ^{aaa}p<0.001 when compared to INS treated young rats
Young- Treatment started at 4 weeks continued to 16 weeks
Old- Treatment started at 60 weeks continued to 90 weeks

Table-63
NE, EPI, DA and 5-HT content (nmoles/g wet weight of tissue) in the brainstem of control and experimental young and old rats

Animal status	NE	EPI	DA	5-HT
Saline treated-young	3.41±0.25	4.21±0.35	12.05±0.30	1.79±0.05
STH treated-young	2.01±0.10***	3.15±0.20***	4.05±0.25***	1.01±0.02*
Insulin treated-young	3.23±0.05	3.60±0.16***	4.93±0.18***	1.04±0.04*
Saline treated-old	3.00±0.09**	3.54±0.15***	3.32±0.04***	1.62±0.02
STH treated-old	1.61±0.02¶¶¶ψψψ	2.01±0.04¶¶¶ψψψ	2.49±0.05¶¶¶ψψψ	0.45±0.03¶¶¶ψψψ
Insulin treated-old	2.45±0.12¶¶ aaa	3.46±0.07	4.48±0.16¶¶	1.80±0.05 aa

Values are Mean + S.E.M of 4-6 separate experiments. Each group consisted of 6-8 rats.
*p<0.05, **p<0.01, ***p<0.001 when compared to saline treated young rats, ¶¶¶p<0.001 when compared to saline treated old rats, ψψψp<0.001 when compared to STH treated young rats, aap<0.01, aaap<0.001 when compared to INS treated young rats
Young- Treatment started at 4 weeks continued to 16 weeks
Old- Treatment started at 60 weeks continued to 90 weeks

Table-64
NE, EPI, DA and 5-HT content (nmoles/g wet weight of tissue) in the hypothalamus of control and experimental young and old rats

Animal status	NE	EPI	DA	5-HT
Saline treated-young	5.03±0.20	7.33±0.18	3.06±0.12	2.28±0.08
STH treated-young	4.37±0.12***	5.16±0.10***	2.89±0.05***	1.76±0.03***
Insulin treated-young	3.69±0.15***	4.73±0.14***	2.44±0.09***	1.78±0.02***
Saline treated-old	1.51±0.05***	1.86±0.02***	0.80±0.02***	0.78±0.05***
STH treated-old	3.52±0.07$^{\text{¶¶¶}\Psi\Psi\Psi}$	3.99±0.08$^{\text{¶¶¶}\Psi\Psi\Psi}$	2.52±0.03$^{\text{¶¶¶}\Psi}$	1.52±0.04$^{\text{¶¶¶}}$
Insulin treated-old	3.87±0.04$^{\text{¶¶¶ a}}$	5.18±0.05$^{\text{¶¶¶ aaa}}$	3.11±0.10$^{\text{¶¶¶ aaa}}$	1.61±0.03$^{\text{¶¶¶}}$

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consisted of 6-8 rats.
***p<0.001 when compared to saline treated young rats, $^{\text{¶¶¶}}$p<0.001 when compared to saline treated old rats, $^{\Psi}$p<0.05, $^{\Psi\Psi\Psi}$p<0.001 when compared to STH treated young rats, ^{a}p<0.05, ^{aaa}p<0.001 when compared to INS treated young rats
Young- Treatment started at 4 weeks continued to 16 weeks
Old- Treatment started at 60 weeks continued to 90 weeks

Figure-87
Acetylcholine esterase activity in the cerebral cortex of Control and Experimental Young and Old rats

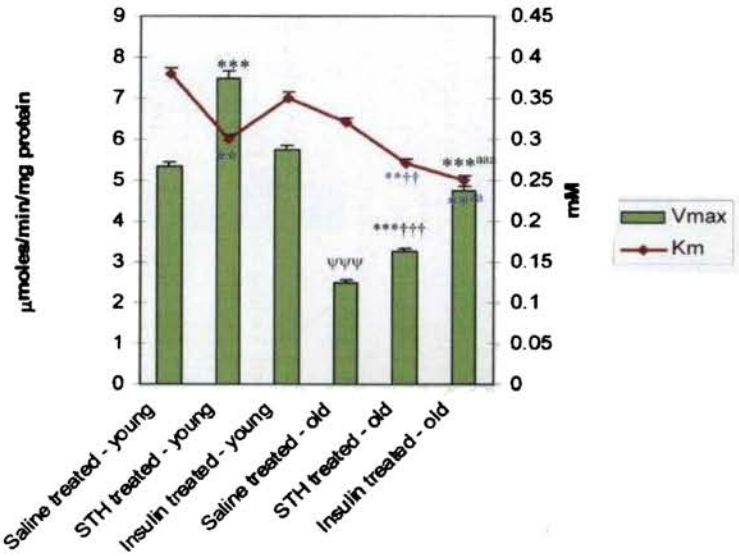


Table-65
Acetylcholine esterase activity in the cerebral cortex of Control and Experimental Young and Old rats

Animal status	V_{max} (µmoles/min/mg protein)		K_m (mM)	
	7 weeks	90 weeks	7 weeks	90 weeks
Saline treated	5.34± 0.13	2.50±0.08$^{\Psi\Psi\Psi}$	0.38±0.004	0.32±0.007
STH treated	7.50±0.02***	3.25±0.05***†††	0.30±0.002**	0.27±0.003**†††
INS treated	5.75±0.04	4.75±0.04^{***aaa}	0.035±0.007	0.25±0.014^{**aa}

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consists of 6-8 rats.
$^{**}p<0.01$, $^{***}p<0.001$ when compared to respective saline treated rat groups, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ when compared to STH treated young rats, $^{aa}p<0.01$, $^{aaa}p<0.001$ when compared to INS treated young rats, Young- Treatment started at 4 weeks continued to 16 weeks, Old- Treatment started at 60 weeks continued to 90 weeks, STH- Somatotropin treated, INS- Insulin treated.

Figure-88

Real-Time PCR amplification of Muscarinic M1 receptor mRNA from the Cerebral cortex of Saline treated, Somatotropin treated and Insulin treated Young and Old rats

Fig-88a (young)

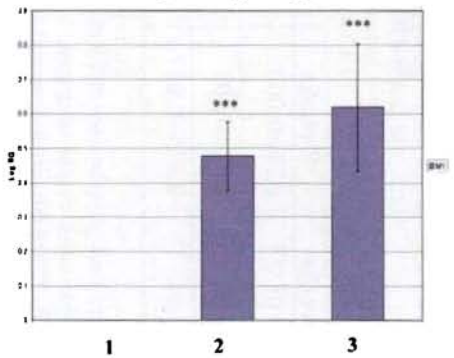


Fig-88b (old)

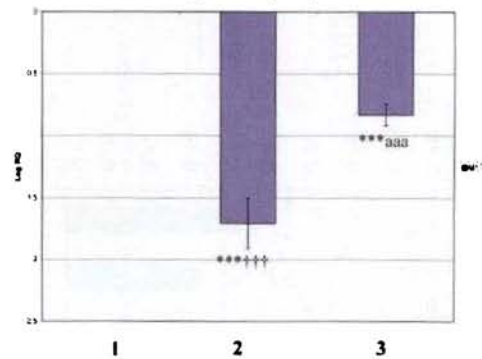


Table-66

Real-Time PCR amplification of Muscarinic M1 receptor mRNA from the Cerebral cortex of Saline treated, Somatotropin treated and Insulin treated Young and Old rats

Sample No.	Animal status	Log RQ value	
		young	old
1	Saline treated	0.0	0.0
2	STH treated	$0.48\pm0.01^{***}$	$-1.71\pm0.20^{***+++}$
3	INS treated	$0.62\pm0.02^{***}$	$-0.83\pm0.08^{***\,aaa}$

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats. ***p<0.001 when compared to respective saline treated rat groups, $^{+++}$p<0.001 when compared to STH treated young rats, ^{aaa} p<0.001 when compared to INS treated young rats, Young- Treatment started at 4 weeks continued to 16 weeks, Old- Treatment started at 60 weeks continued to 90 weeks, STH- Somatotropin treated, INS- Insulin treated.

Figure-89

Real-Time PCR amplification of Muscarinic M3 receptor mRNA from the Cerebral cortex of Saline treated, Somatotropin treated and Insulin treated Young and Old rats

Fig-89a (young)

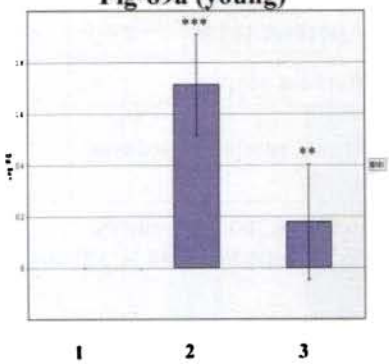


Fig-89b (old)

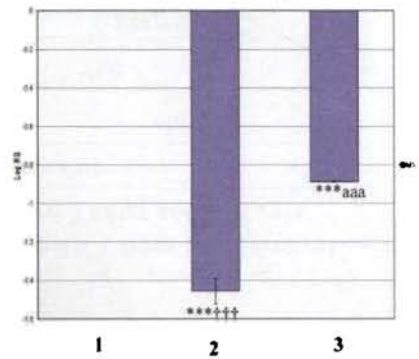


Table-67

Real-Time PCR amplification of Muscarinic M3 receptor mRNA from the Cerebral cortex of Saline treated, Somatotropin treated and Insulin treated Young and Old rats

Sample No.	Animal status	Log RQ value	
		young	old
1	Saline treated	0.0	0.0
2	STH treated	$0.71\pm0.19^{***}$	$-1.45\pm0.0.65^{***\dagger\dagger\dagger}$
3	INS treated	$0.17\pm0.22^{**}$	$-0.88\pm0.01^{***\,aaa}$

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats. **p<0.01, ***p<0.001 when compared to respective saline treated rat groups. †††p<0.001 when compared to STH treated young rats, ^{aaa}p<0.001 when compared to INS treated young rats, Young- Treatment started at 4 weeks continued to 16 weeks, Old- Treatment started at 60 weeks continued to 90 weeks, STH- Somatotropin treated, INS- Insulin treated.

Figure-90

Real-Time PCR amplification of Glutamate receptor (NMDAR1) mRNA from the Cerebral cortex of Saline treated, Somatotropin treated and Insulin treated Young and Old rats

Fig-90a (young)

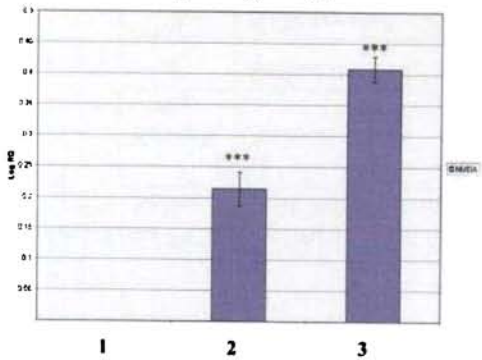


Fig-90b (old)

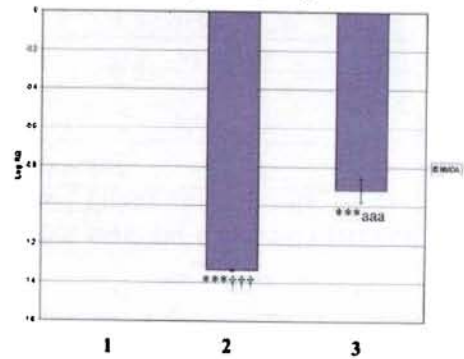


Table-68

Real-Time PCR amplification of Glutamate receptor (NMDAR1) mRNA from the Cerebral cortex of Saline treated, Somatotropin treated and Insulin treated Young and Old rats

Sample No.	Animal status	Log RQ value	
		young	old
1	Saline treated	0.0	0.0
2	STH treated	$0.21\pm0.02^{***}$	$-1.33\pm0.01^{***\dagger\dagger\dagger}$
3	INS treated	$0.40\pm0.02^{***}$	$-0.91\pm0.06^{***aaa}$

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats. ***p<0.001 when compared to respective saline treated rat groups, †††p<0.001 when compared to STH treated young rats, ^{aaa} p<0.001 when compared to INS treated young rats, Young- Treatment started at 4 weeks continued to 16 weeks, Old- Treatment started at 60 weeks continued to 90 weeks, STH- Somatotropin treated, INS- Insulin treated.

Figure-91

Real-Time PCR amplification of Glutamate receptor (mGlu-5) mRNA from the Cerebral cortex of Saline treated, Somatotropin treated and Insulin treated Young and Old rats

Fig-91a (young)

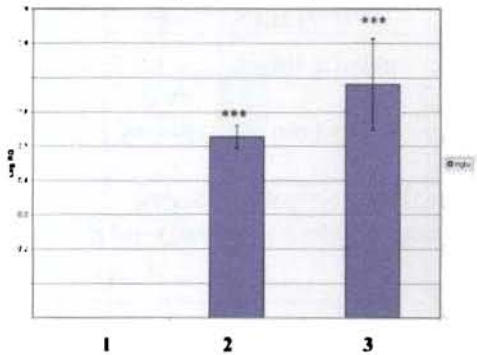


Fig-91b (old)

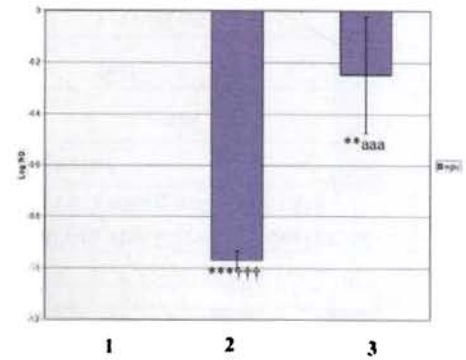


Table-69

Real-Time PCR amplification of of Glutamate receptor (mGlu-5) mRNA from the Cerebral cortex of Saline treated, Somatotropin treated and Insulin treated Young and Old rats

Sample No.	Animal status	Log RQ value	
		young	old
1	Saline treated	0.0	0.0
2	STH treated	$0.52\pm0.03^{***}$	$-0.96\pm0.03^{***\dagger\dagger\dagger}$
3	INS treated	$0.68\pm0.13^{***}$	$-0.24\pm0.22^{**aaa}$

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats. $^{**}p<0.01$ when compared to respective saline treated rat groups, $^{\dagger\dagger\dagger}p<0.001$ when compared to STH treated young rats, $^{aaa}p<0.001$ when compared to INS treated young rats, Young- Treatment started at 4 weeks continued to 16 weeks, Old- Treatment started at 60 weeks continued to 90 weeks, STH- Somatotropin treated, INS- Insulin treated.

Figure-92
Real-Time PCR amplification of DA D2 receptor mRNA from the Cerebral cortex of Saline treated, Somatotropin treated and Insulin treated Young and Old rats

Fig-92a (young)

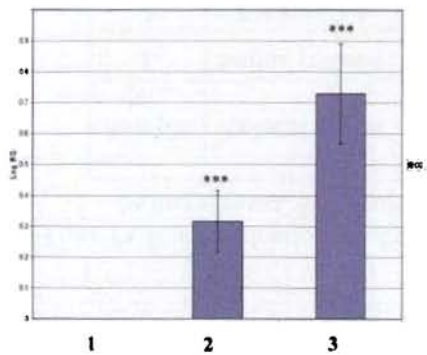


Fig-92b (old)

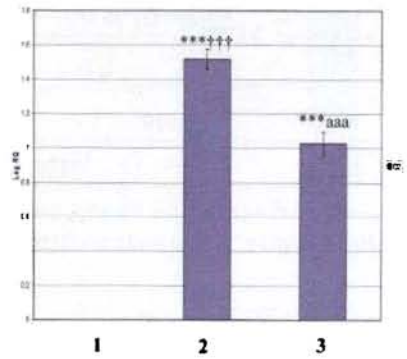


Table-70
Real-Time PCR amplification of DA D2 receptor mRNA from the Cerebral cortex of Saline treated, Somatotropin treated and Insulin treated Young and Old rats

Sample No.	Animal status	Log RQ value	
		young	old
1	Saline treated	0.0	0.0
2	STH treated	$0.316\pm0.10^{***}$	$1.51\pm0.05^{***\dagger\dagger\dagger}$
3	INS treated	$0.727\pm0.16^{***}$	$1.02\pm0.06^{***aaa}$

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats. ***p<0.001 when compared to respective saline treated rat groups, †††p<0.001 when compared to STH treated young rats, ^{aaa} p<0.001 when compared to INS treated young rats, Young- Treatment started at 4 weeks continued to 16 weeks, Old- Treatment started at 60 weeks continued to 90 weeks. STH- Somatotropin treated, INS- Insulin treated.

Figure-93

Real-Time PCR amplification of α_{2A}-adrenergic mRNA from the Cerebral cortex of Saline treated, Somatotropin treated and Insulin treated Young and Old rats

Fig-93a (young)

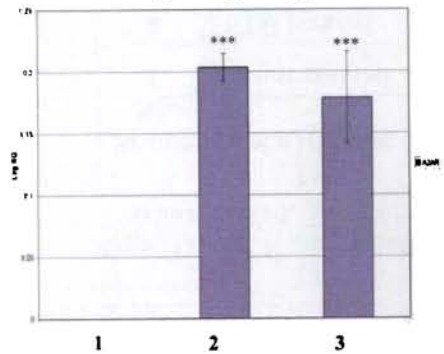


Fig-93b (old)

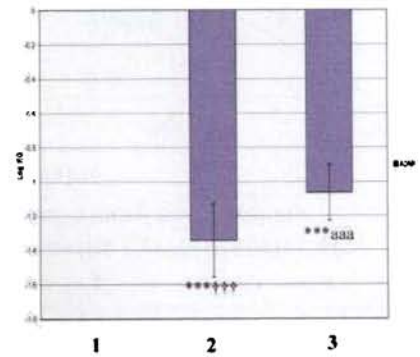


Table-71

Real-Time PCR amplification of α_{2A}-adrenergic mRNA from the Cerebral cortex of Saline treated, Somatotropin treated and Insulin treated Young and Old rats

Sample No.	Animal status	Log RQ value	
		young	old
1	Saline treated	0.0	0.0
2	STH treated	$0.20\pm0.01^{***}$	$-1.34\pm0.21^{***\dagger\dagger\dagger}$
3	INS treated	$0.17\pm0.03^{***}$	$-1.06\pm0.16^{***aaa}$

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats. ***p<0.001 when compared to respective saline treated rat groups, †††p<0.001 when compared to STH treated young rats, ^{aaa} p<0.001 when compared to INS treated young rats, Young- Treatment started at 4 weeks continued to 16 weeks, Old- Treatment started at 60 weeks continued to 90 weeks, STH- Somatotropin treated, INS- Insulin treated.

Figure-94

Real-Time PCR amplification of β_2-adrenergic mRNA from the Cerebral cortex of Saline treated, Somatotropin treated and Insulin treated Young and Old rats

Fig-94a (young)

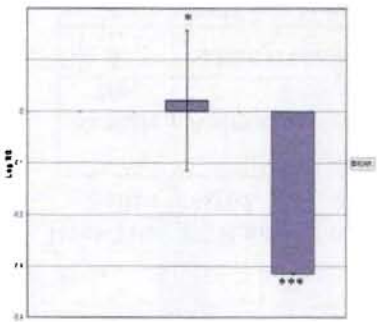


Fig-94b (old)

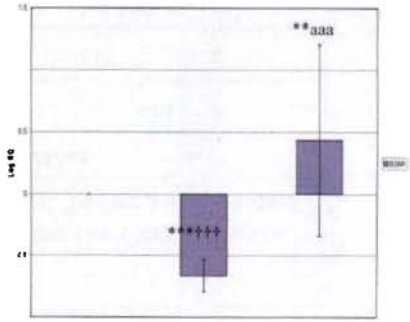


Table-72

Real-Time PCR amplification of β_2-adrenergic mRNA from the Cerebral cortex of Saline treated, Somatotropin treated and Insulin treated Young and Old rats

Sample No.	Animal status	Log RQ value	
		young	old
1	Saline treated	0.0	0.0
2	STH treated	$0.021\pm0.135^{*}$	$-0.66\pm0.12^{***\dagger\dagger\dagger}$
3	INS treated	$-0.315\pm0.001^{***\P\P\P}$	$0.43\pm0.77^{**aaa}$

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats. *p<0.05,**p<0.01,***p<0.001 when compared to respective saline treated rat groups, †††p<0.001 when compared to STH treated young rats, ^{aaa}p<0.001 when compared to INS treated young rats, Young- Treatment started at 4 weeks continued to 16 weeks, Old- Treatment started at 60 weeks continued to 90 weeks, STH- Somatotropin treated, INS- Insulin treated.

Figure-95

Real-Time PCR amplification of $GABA_{Aα1}$ mRNA from the Cerebral cortex of Saline treated, Somatotropin treated and Insulin treated Young and Old rats

Fig-95a (young)

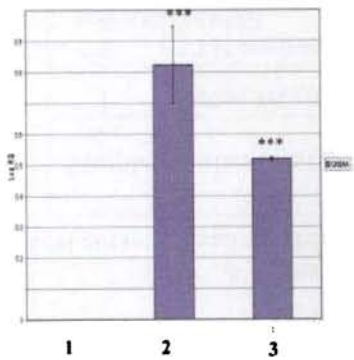


Fig-95b (old)

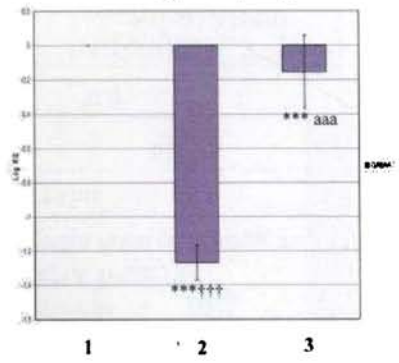


Table-73

Real-Time PCR amplification of $GABA_{Aα1}$ mRNA from the Cerebral cortex of Saline treated, Somatotropin treated and Insulin treated Young and Old rats

Sample No.	Animal status	Log RQ value	
		young	old
1	Saline treated	0.0	0.0
2	STH treated	$0.82\pm0.12^{***}$	$-1.269\pm0.101^{***\dagger\dagger\dagger}$
3	INS treated	$0.51\pm0.01^{***}$	$-0.155\pm0.211^{***\ aaa}$

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats.***$p<0.001$ when compared to respective saline treated rat groups. $^{\dagger\dagger\dagger}p<0.001$ when compared to STH treated young rats, $^{aaa}p<0.001$ when compared to INS treated young rats, Young- Treatment started at 4 weeks continued to 16 weeks, Old- Treatment started at 60 weeks continued to 90 weeks, STH- Somatotropin treated, INS- Insulin treated.

Figure-96

Real-Time PCR amplification of $GABA_B$ mRNA from the Cerebral cortex of Saline treated, Somatotropin treated and Insulin treated Young and Old rats

Fig-96a (young)

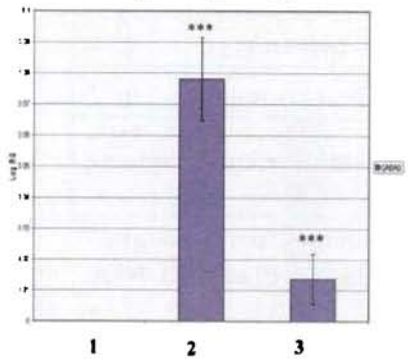


Fig-96b (old)

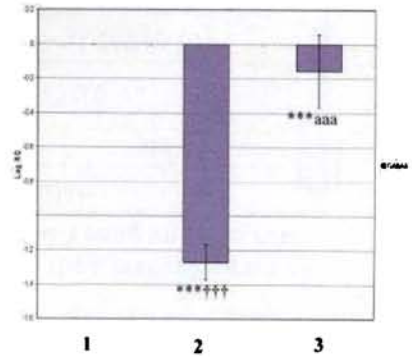


Table-74

al-Time PCR amplification of $GABA_B$ mRNA from the Cerebral cortex of Saline treated, Somatotropin treated and Insulin treated Young and Old rats

Sample No.	Animal status	Log RQ value	
		young	old
1	Saline treated	0.0	0.0
2	STH treated	$0.078\pm0.013^{***}$	$-1.191\pm0.006^{***+++}$
3	INS treated	$0.013\pm0.008^{***}$	$-0.203\pm0.040^{***aaa}$

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats. ***p<0.001 when compared to respective saline treated rat groups, $^{+++}$p<0.001 when compared to STH treated young rats, ^{aaa} p<0.001 when compared to INS treated young rats, Young- Treatment started at 4 weeks continued to 16 weeks, Old- Treatment started at 60 weeks continued to 90 weeks, STH- Somatotropin treated, INS- Insulin treated.

Figure-97

Real-Time PCR amplification of 5-HT_{2C} mRNA from the Cerebral cortex of Saline treated, Somatotropin treated and Insulin treated Young and Old rats

Fig-97a (young)

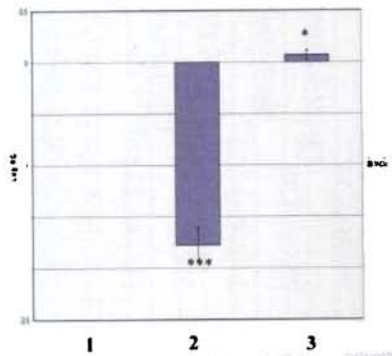


Fig-97b (old)

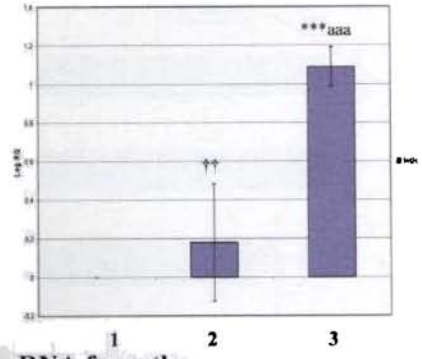


Table-75

Real-Time PCR amplification of 5-HT_{2C} mRNA from the Cerebral cortex of Saline treated, Somatotropin treated and Insulin treated Young and Old rats

Sample No.	Animal status	Log RQ value	
		young	old
1	Saline treated	0.0	0.0
2	STH treated	$-1.77\pm0.16^{***}$	$0.18\pm0.30^{\dagger\dagger}$
3	INS treated	$0.06\pm0.04^{*}$	$1.08\pm0.10^{***aaa}$

Values are Mean ± S.D of 4-6 separate experiments. Each group consists of 6-8 rats. *p<0.05,***p<0.001 when compared to respective saline treated rat groups, ††p<0.01 when compared to STH treated young rats, ^{aaa} p<0.001 when compared to INS treated young rats, Young- Treatment started at 4 weeks continued to 16 weeks, Old- Treatment started at 60 weeks continued to 90 weeks, STH- Somatotropin treated, INS- Insulin treated.

Figure- 98
IP3 content in the cerebral cortex of control and experimental Young and Old rats

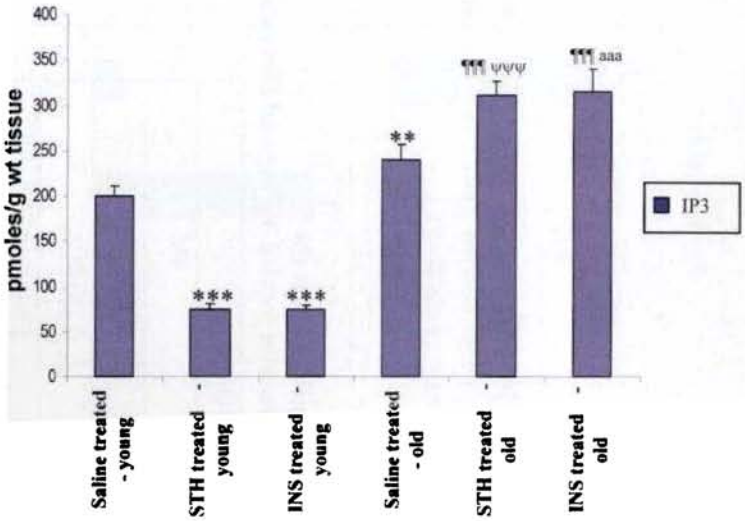


Table-76
IP3 content in the cerebral cortex of control and experimental Young and Old rats

Animal status	Concentration (pmoles/g wt tissue)
Saline treated-young	200.00±11.50
STH treated-young	74.00±6.36***
Insulin treated-young	75.00±4.25***
Saline treated-old	240.00±17.00**
STH treated-old	312.00±15.23¶¶¶ ψψψ
Insulin treated-old	316.00±25.50¶¶¶ aaa

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consisted of 6-8 rats.
**p<0.01, ***p<0.001 when compared to saline treated young rats, ¶¶¶p<0.001 when compared to saline treated old rats, ψψψp<0.01 when compared to STH treated young rats, aaa p<0.001 when compared to INS treated young rats
Young- Treatment started at 4 weeks continued to 16 weeks
Old- Treatment started at 60 weeks continued to 90 weeks

Figure-99

IP3 content in the brainstem of control and experimental Young and Old rats

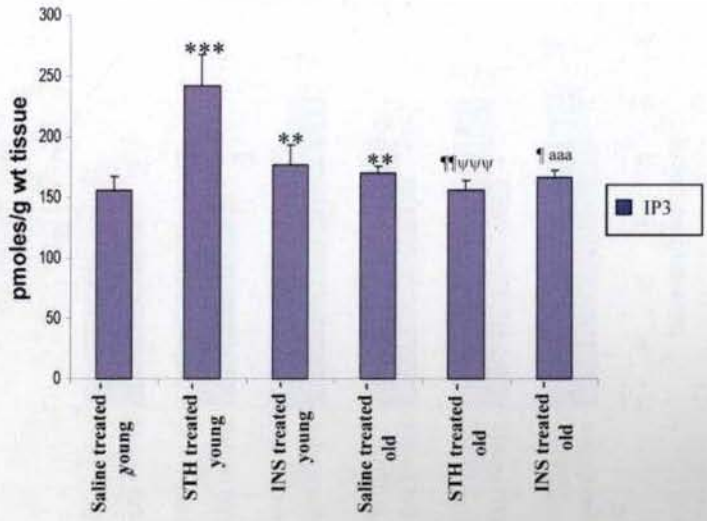


Table-77

IP3 content in the brainstem of control and experimental young and old rats

Animal status	Concentration (pmoles/g wt tissue)
Saline treated-young	156.00±11.25
STH treated-young	242.00±25.50***
Insulin treated-young	177.20±15.50**
Saline treated-old	169.20±6.56**
STH treated-old	156.00±7.85¶¶ψψψ
Insulin treated-old	166.40±5.69¶aaa

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consisted of 6-8 rats.
**p<0.01, ***p<0.001 when compared to saline treated young rats, ¶p<0.05, ¶¶p<0.01 when compared to saline treated old rats, ψψψp<0.001 when compared to STH treated young rats, aaa p<0.001 when compared to INS treated young rats.
Young- Treatment started at 4 weeks continued to 16 weeks
Old- Treatment started at 60 weeks continued to 90 weeks

Figure-100
IP3 content in the corpus striatum of control and experimental Young and Old rats

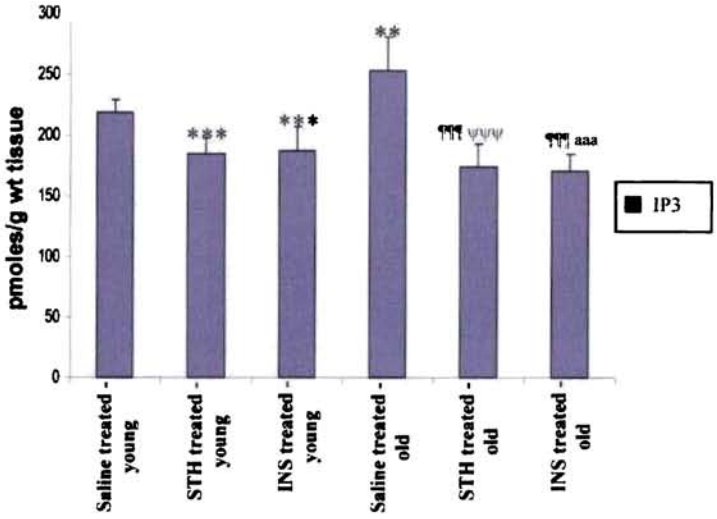


Table- 78
IP3 content in the corpus striatum of control and experimental Young and Old rats

Animal status	Concentration (pmoles/g wt tissue)
Saline treated-young	218.80±10.15
STH treated-young	185.20±12.05***
Insulin treated-young	187.20±19.50***
Saline treated-old	253.20±27.65**
STH treated-old	174.80±18.25¶¶¶ψψψ
Insulin treated-old	170.80±14.40¶¶¶ aaa

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consisted of 6-8 rats.
**p<0.01, ***p<0.001 when compared to saline treated young rats, ¶¶¶p<0.001 when compared to saline treated old rats, ψψψp<0.01 when compared to STH treated young rats, aaa p<0.001 when compared to INS treated young rats
Young- Treatment started at 4 weeks continued to 16 weeks
Old- Treatment started at 60 weeks continued to 90 weeks

Figure-101
cGMP content in the cerebral cortex of control and experimental Young and Old rats

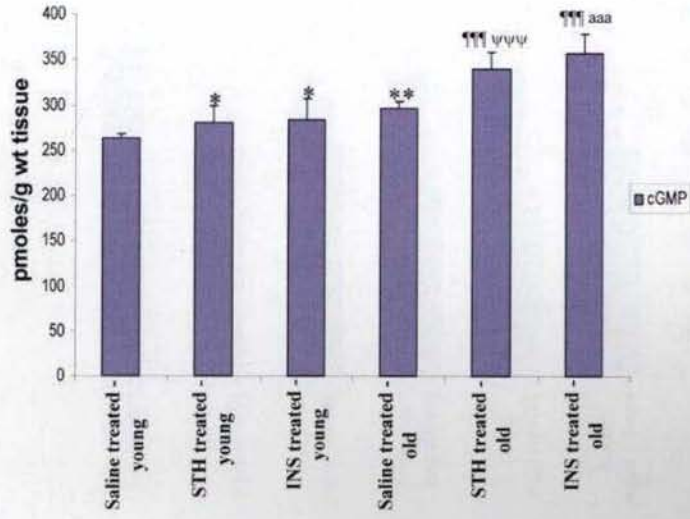


Table-79
cGMP content in the cerebral cortex of control and experimental young and old rats

Animal status	Concentration (pmoles/g wt tissue)
Saline treated-young	264.00±4.67
STH treated-young	280.00±18.50*
Insulin treated-young	284.00±23.56*
Saline treated-old	296.00±7.54**
STH treated-old	340.00±17.45¶¶¶ ψψψ
Insulin treated-old	356.00±22.50¶¶¶ aaa

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consisted of 6-8 rats.
*p<0.05, **p<0.01 when compared to saline treated young rats, ¶¶¶p<0.001 when compared to saline treated old rats, ψψψp<0.01 when compared to STH treated young rats, aaa p<0.001 when compared to INS treated young rats
Young- Treatment started at 4 weeks continued to 16 weeks
Old- Treatment started at 60 weeks continued to 90 weeks

Figure-102
cGMP content in the brainstem of control and experimental Young and Old rats

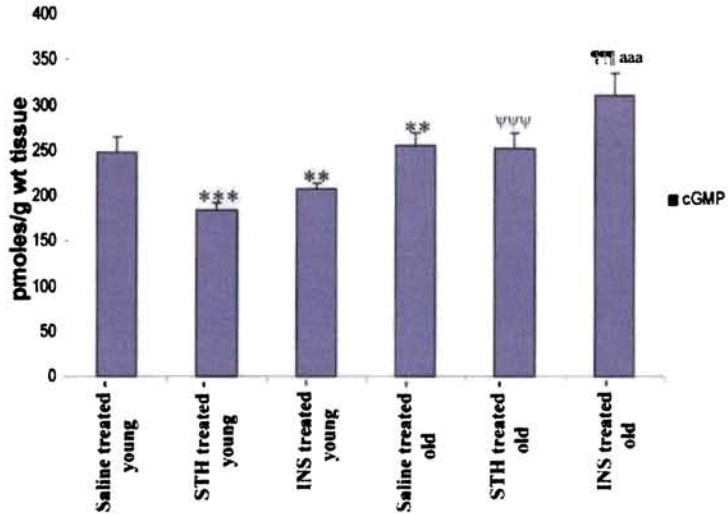


Table-80
cGMP content in the brainstem of control and experimental Young and Old rats

Animal status	Concentration (pmoles/g wt tissue)
Saline treated-young	248.00±17.50
STH treated-young	184.00±8.26***
Insulin treated-young	208.00±5.48**
Saline treated-old	256.00±13.50**
STH treated-old	252.00±18.25 ΨΨΨ
Insulin treated-old	312.00±24.70¶¶¶ aaa

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consisted of 6-8 rats.
**p<0.01, ***p<0.001 when compared to saline treated young rats, ¶¶¶p<0.001 when compared to saline treated old rats, ΨΨΨp<0.001 when compared to STH treated young rats, aaa p<0.001 when compared to INS treated young rats
Young- Treatment started at 4 weeks continued to 16 weeks
Old- Treatment started at 60 weeks continued to 90 weeks

Figure- 103
cGMP content in the corpus striatum of control and experimental Young and Old rats

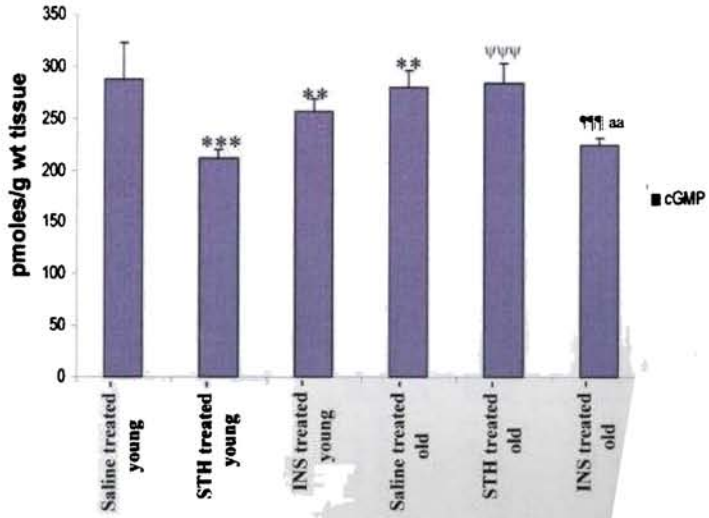


Table-81
cGMP content in the corpus striatum of control and experimental Young and old rats

Animal status	Concentration (pmoles/g wt tissue)
Saline treated-young	288.00±35.50
STH treated-young	212.00±7.45***
Insulin treated-young	256.00±12.25**
Saline treated-old	280.00±15.20**
STH treated-old	284.00±18.40ΨΨΨ
Insulin treated-old	224.0±6.50¶¶¶ aa

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consisted of 6-8 rats.
**p<0.01, ***p<0.001 when compared to saline treated young rats, ¶¶¶p<0.001 when compared to saline treated old rats, ΨΨΨp<0.001 when compared to STH treated young rats, aap<0.001 when compared to INS treated young rats
Young- Treatment started at 4 weeks continued to 16 weeks
Old- Treatment started at 60 weeks continued to 90 weeks

Figure-104
Triiodothyronine (T3) content (ng/ml) in the serum of experimental Young and Old rats

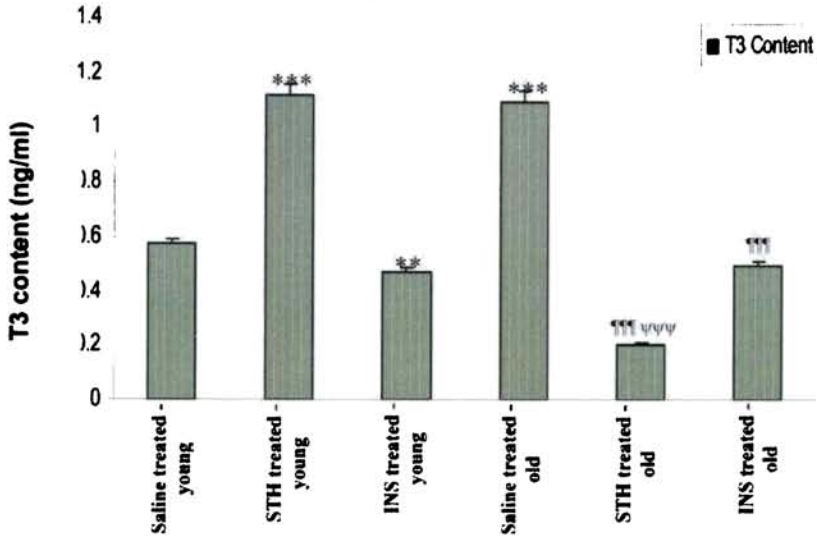


Table-82
Triiodothyronine (T3) content (ng/ml) in the serum of control and experimental young and old rats

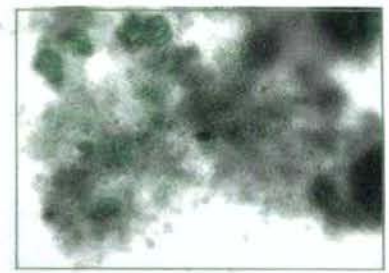
Animal status	Concentration (ng/ml)
Saline treated-young	0.571±0.03
STH treated-young	1.115±0.12***
Insulin treated-young	0.469±0.05**
Saline treated-old	1.091±0.10***
STH treated-old	0.199±0.02¶¶¶ ψψψ
Insulin treated-old	0.493±0.06¶¶¶

Values are Mean ± S.E.M of 4-6 separate experiments. Each group consisted of 6-8 rats.
**p<0.01, ***p<0.001 when compared to saline treated young rats, ¶¶¶p<0 .001 when compared to saline treated old rats, ψψψp<0.001 when compared to STH treated young rats.
Young- Treatment started at 4 weeks continued to 16 weeks
Old- Treatment started at 60 weeks continued to 90 weeks

Figure-105
Effect of Carbachol (10^{-7}M) and muscarinic M1 receptor antagonist (10^{-6} M) on calcium release from young pancreatic islets *in vitro*

Control Young –
Fluorescence at zero time (T_0)

Control Young - Carbachol
Fluorescence at 3 min



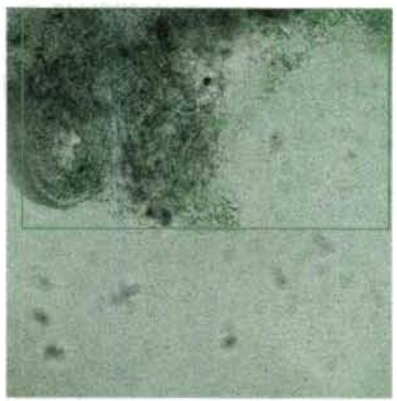
Control Young –
(Carbachol + Pirenzepine)
Fluorescence at 6 min

Table-83

Time in min	Pixel Intensity
0	4455219
3	9301764
6	5855785

Figure-106
Effect of Carbachol (10^{-7}M) and muscarinic M1 receptor antagonist (10^{-6} M) on calcium release from old pancreatic islets *in vitro*

Control Old –
Fluorescence at zero time (T_0)



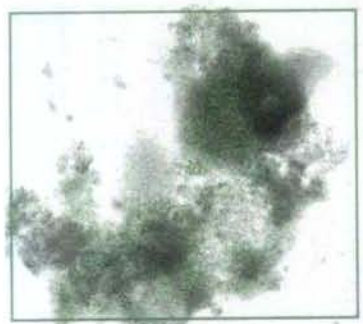
Control Old - Carbachol
Fluorescence at 3 min

Control Old –
(Carbachol + Pirenzepine)
Fluorescence at 6 min

Table-84

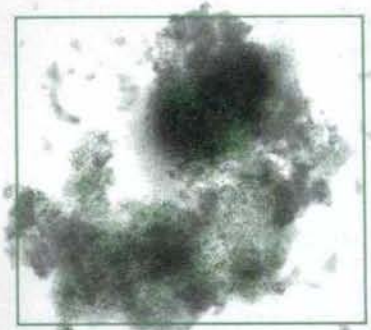
Time in min	Pixel Intensity
0	4720102
3	4967833
6	4348811

Figure-107
Effect of Carbachol (10^{-7}M) and muscarinic M3 receptor antagonist (10^{-6} M) on calcium release from young pancreatic islets *in vitro*



Control Young –
Fluorescence at zero time (T_0)

Control Young - Carbachol
Fluorescence at 3 min

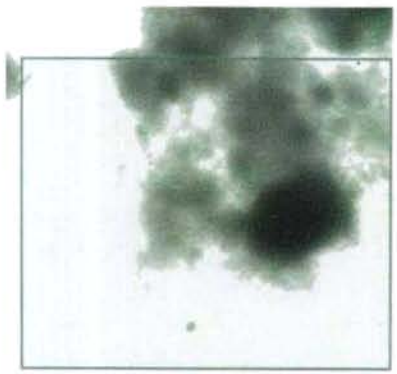


Control Young-
(Carbachol + 4-DAMP mustard)
Fluorescence at 6 min

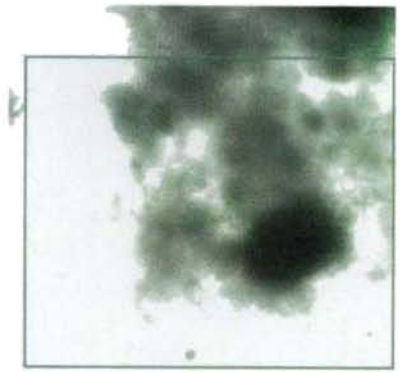
Table-85

Time in min	Pixel Intensity
0	4896515
3	5090717
6	4554568

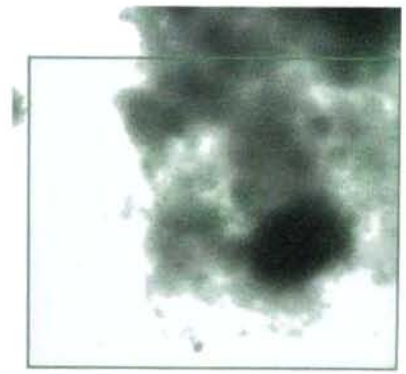
Figure-108
Effect of Carbachol (10^{-7}M) and muscarinic M3 receptor antagonist (10^{-6} M) on calcium release from old pancreatic islets *in vitro*



Control Old –
Fluorescence at zero time (T_0)



Control Old - Carbachol
Fluorescence at 3 min



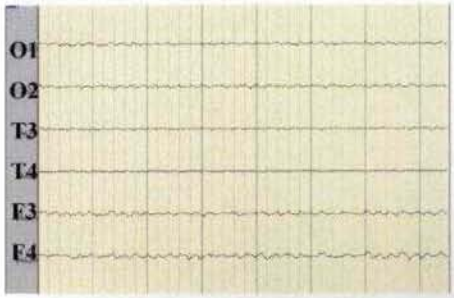
Control Old-
(Carbachol + 4-DAMP mustard)
Fluorescence at 6 min

Table-86

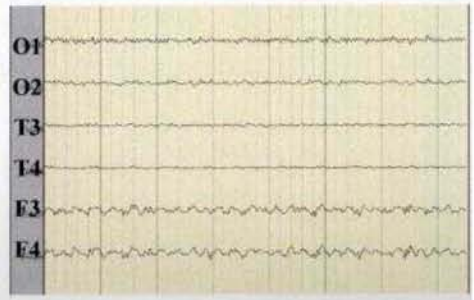
Time in min	Pixel Intensity
0	5432455
3	5666970
6	5442450

Figure- 109
EEG brain wave pattern of Control, Diabetic and Insulin treated diabetic rats

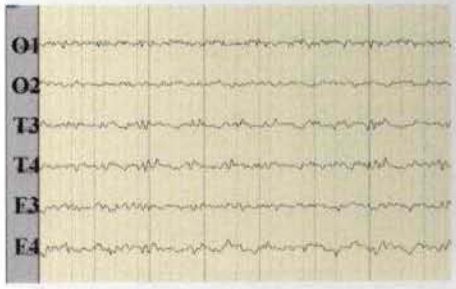
Control –7w



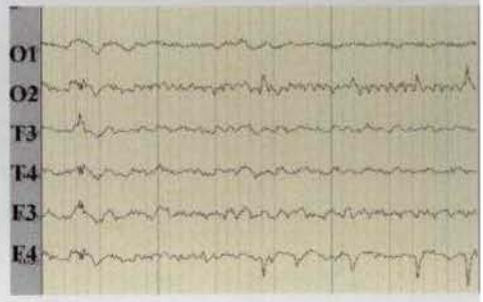
Control –90w



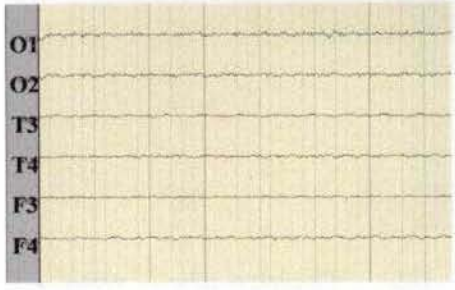
Diabetic –7w



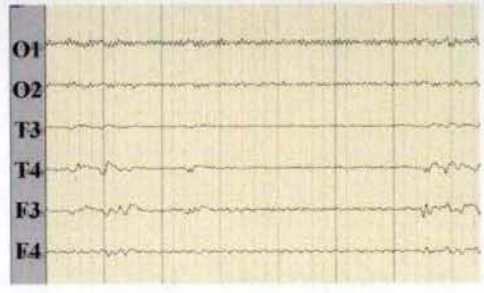
Diabetic –90w



D + I –7w



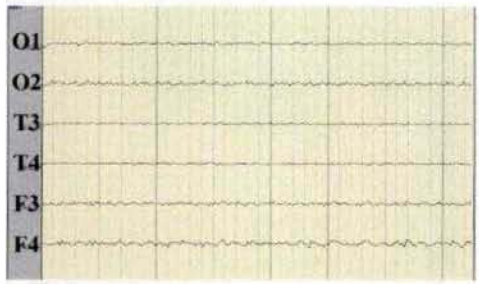
D + I –90w



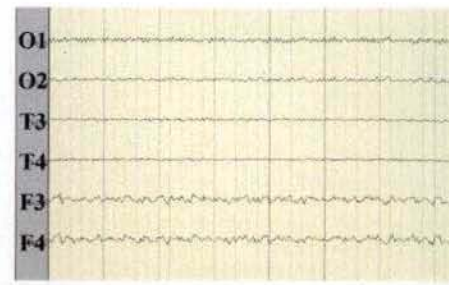
O1	**Occipital lobe**	**left**	**O2**	**Occipital lobe**	**right**
T3	**Temporal lobe**	**left**	**T4**	**Temporal lobe**	**right**
F3	**Frontal lobe**	**left**	**F4**	**Frontal lobe**	**right**

Figure- 110
EEG brain wave pattern of Control and Experimental Young and Old rats

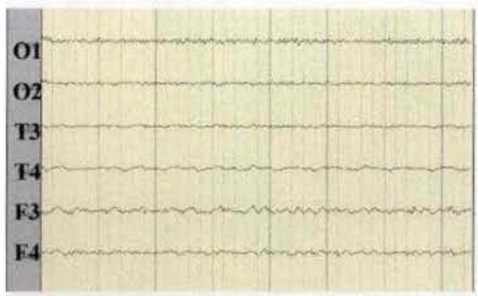
Saline treated – young



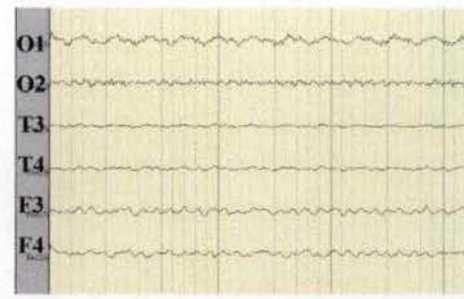
Saline treated – old



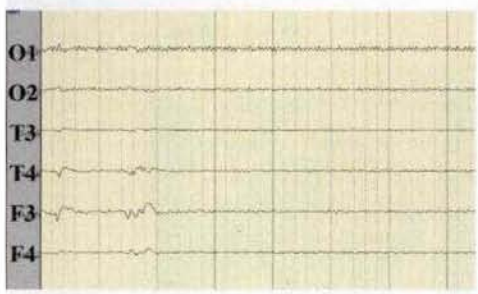
STH treated – young



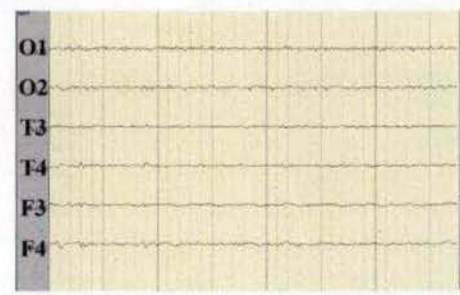
STH treated – old



INS treated – young



INS treated – old



O1	**Occipital lobe**	**left**	**O2**	**Occipital lobe**	**right**
T3	**Temporal lobe**	**left**	**T4**	**Temporal lobe**	**right**
F3	**Frontal lobe**	**left**	**F4**	**Frontal lobe**	**right**