DOPAMINE D_1 AND D_2 RECEPTOR FUNCTIONAL REGULATION: GLUTAMATE RECEPTOR GENE EXPRESSION IN THE BRAIN OF HYPOGLYCAEMIC AND HYPERGLYCAEMIC RATS

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CERTIFICATE

This is to certify that the thesis entitled "**DOPAMINE D_1 AND D_2 RECEPTOR FUNCTIONAL REGULATION: GLUTAMATE RECEPTOR GENE EXPRESSION IN THE BRAIN OF HYPOGLYCAEMIC AND HYPERGLYCAEMIC RATS**" is a bonafide record of the research work carried out by **Ms. Remya Robinson**, 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 any other degree.

(C. S. Paulose)

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Date: 26 April, 2007

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DECLARATION

I hereby declare that the thesis entitled "**DOPAMINE D_1 AND D_2 RECEPTOR FUNCTIONAL REGULATION: GLUTAMATE RECEPTOR GENE EXPRESSION IN THE BRAIN OF HYPOGLYCAEMIC AND HYPERGLYCAEMIC RATS**" 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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Remya Robinson

Dedicated To My Beloved Parents ...

ABBREVIATIONS USED IN THE TEXT

3-MT	3-methoxytyramine
5-HIAA	5-hydroxy indole - 3 acetic acid
5-HT	5-Hydroxy tryptamine
7-OH DPAT	7-Hydroxy-2(di-n-propylamino)-tetralin
Ach	Acetylcholine
ADP	Adenosine diphosphate
AD	Aldehyde dehydrogenase
AMPA	amino-3-hydroxy-5-methysoxazole-4-propionic acid
ANS	Autonomic nervous system
ARAS	Ascending reticular activating system
AAD	Aromatic L-amino decarboxylase
BBB	Blood brain barrier
B_{max}	Maximal binding
BRC	Bromocriptine
BS	Brainstem
CA	Cornu Ammonis
cAMP	Cyclic adenosine mono phosphate
COMT	catechol-*O*-methyltransferase
CBF	Cerebral blood flow
CC	Cerebral cortex
CRH	Corticotropin-releasing hormone
CNS	Central nervous system
CREB	cAMP regulatory element binding protein
CS	corpus striatum
Ct	Crossing threshold

CT	Computed tomography
DA	Dopamine
DAG	Diacylglycerol
DARPP	DA receptor phosphor protein
DAT	Dopamine Transporter
dATP	Deoxy adenosine triphosphate
DDC	Dihydroxy phenyl acetic acid decarboxylase
DEPC	Diethyl pyrocarbonate
dGTP	Deoxy guanosine triphosphate
DNTP	Deoxynucleotide triphosphate
DOI	1-(2,5-di-methoxy-4-iodophenyl)-2-aminopropane
DOPAC	Dihydroxy phenyl acetic acid
DBH	Dopamine β hydroxylase
dTTP	Deoxynucleotide thymidine triphosphate
EEG	Electro encephalogram
ECD	Electrochemical detector
EGF	Epidermal growth factor
EPI	Epinephrine
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
FGF	Fibroblast growth factor
GABA	Gamma aminobutyric acid
GAP	GTPase activating protein
GD	Glucose deprivation
Glu	Glutamate
GLUT	Glucose transporter

(+) MK-801	(+)5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine maleate
mRNA	messenger ribonucleic acid
MuMLV	Murine moloney leukemia virus reverse transcriptase
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NE	Norepinephrine
NIDDM	Non-Insulin dependant diabetes mellitus
NMDA	N-methyl-D-aspartic acid
NO	Nitric oxide
p	Level of significance
PFC	Prefrontal cortex
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PIP_2	Phosphatidylinositol-4,5-biphosphate
PKC	Protein kinase C
PLC	Phospholipase C
RH	Recurrent hypoglycaemia
RIA	Radioimmunoassay
RT-PCR	Reverse-transcription-polymerase chain reaction
SCH 23390	[methyl]-(R)-(+)-8-chloro-2,3,4,5-tetra-hydro-3-methyl-5-phenyl-1H-3-benzazepin-7-ol
S.E.M.	Standard error of mean

GDH	Glutamate dehydrogenase
G_i	G protein inhibitory
G_s	G protein stimulatory
GSIS	Glucose stimulated insulin secretion
GTP	Guanosine triphosphate
HBSS	Hank's balanced salt solution
Hippo	Hippocampus
HPA	Hypothalamic pituitary axis
HPLC	High performance liquid chromatography
HYPO	Hypothalamus
IC	Inferior colliculus
IDDM	Insulin dependant diabetes mellitus
IIH	Insulin induced hypoglycaemia
i.p	Intraperitoneally
IP_3	Inositol triphosphate
KA	Kainate
K_d	Dissociation constant
Ki	Inhibitory coefficient
K_m	Michaelis constant
KRB	Krebs Ringer Bicarbonate
L-DOPA	L-3,4 Dihydroxy phenyl alanine
LDH	Lactate dehydrogenase
LTP	Long term potentiation
MAO	Monoamine oxidase
MDH	Malate dehydrogenase

SN	Substantia nigra
STZ	Streptozotocin
TCA	Tricarboxylic acid
TH	tyrosine hydroxylase
TM	Transmembrane domain
V_{max}	Maximal velocity
VMH	Ventromedial hypothalamus
VTA/ SNc	Ventral tegmental area/ Substantia nigra compacta
YM-09151-2	cis-N-(1-benzyl-2-methylpyrrolidin-3-yl)-5-chloro-2-methoxy-4-methylaminobenzamide

CONTENTS

MATERIALS AND METHODS

Introduction

Glucose is the primary source of fuel for the cells of the brain. Brain is dependent on a continuous supply of glucose diffusing from the blood into the interstitial tissue within the central nervous system and into neurons themselves. The physiological concentration of glucose in blood is maintained through highly regulated systemic mechanisms. Changes in glucose levels elicit a complex neuroendocrine response that prevents or rapidly corrects hyper or hypoglycaemia. Diabetes Mellitus is a chronic metabolic disorder resulting in hyperglycaemia (high plasma glucose level). Hypoglycaemia (low level of plasma glucose) is a common adverse effect of insulin treatment in individuals with diabetes. It is one of the most common and serious stress conditions challenging the body homeostasis. Severe hypoglycaemia with cognitive dysfunction is three times more common in intensively, rather than conventionally, treated insulin-dependent diabetes mellitus (IDDM) (Maran *et al.*, 1995). When glucose levels fall below threshold glycaemic levels, neuroendocrine, autonomic nervous system (ANS) and metabolic glucose counter regulatory mechanisms are activated. These hypoglycaemic counter regulatory mechanisms can be blunted irreversibly by disease duration or by acute episodes of prior stress (Ertl & Davis, 2004). Although hypoglycaemia is associated with a number of physiological changes, the most profound effects are seen in the brain, where glucose is the major substrate for energy metabolism. Lack of glucose produces brain damage or even death if the deficit is prolonged.

The central nervous system (CNS) neurotransmitters play an important role in the regulation of glucose homeostasis. 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 (Julius *et al.,* 1989). Neurotransmitters have been reported to show significant alterations during hyperglycaemia resulting in altered functions causing neuronal degeneration (Bhardwaj *et al.,* 1999). Chronic hyperglycaemia during diabetes mellitus is a major initiator of diabetic micro-vascular complications like retinopathy, neuropathy and nephropathy (Sheetz & King, 2002). Glucose processing uses a variety of diverse metabolic pathways. Chronic hyperglycaemia can induce multiple cellular changes leading to metabolic disorders.

Hypoglycaemia during insulin therapy causes damage to the brain specifically because that is the organ which cannot withstand glucose deficiency. The functional capability of the brain will deteriorate due to the frequent hypoglycaemic shock. If glucose supply to the brain is not maintained, there is a decrease in cerebral electrical activity, membrane breakdown with release of free fatty acids and altered amino acid metabolism, including increased production of glutamate. Pathological studies in humans and animals show that hypoglycaemia-induced neuronal death occurs preferentially in the hippocampus, superficial layers of the cortex and striatum (Auer *et al.*, 1988; Auer & Siesjo, 1993; Auer, 2004). Because of the extensive neuronal loss, one of the neurological sequalae associated with hypoglycaemia is cognitive decline. According to clinical studies, significant learning and memory deficits correlate with the frequency of hypoglycaemia not only in patients with type 1 diabetes, but also in the relatively younger group among the population with type 2 diabetes (Dey *et al.*, 1997). Acute neuroglycopenia causes rapid deterioration of cognitive function in humans with and without diabetes. Numerous clinical studies

suggest that intensive insulin treatment of type 1 diabetes is associated with an increased frequency of hypoglycaemic coma (Hannonen *et al.*, 2003; Rovet, 1999) and cognitive impairment (Ryan *et al.*, 1985; Langan *et al.*, 1991; Wredling *et al.*, 1990).

Glutamate, which is one of the excitatory amino acid neurotransmitters found only in the central nervous system, is believed to play a major role in the pathophysiology of hypoglycaemic brain injury. Numerous reports have documented that excessive glutamate, through NMDA/AMPA receptors, activate the excitotoxic process, which play an important role in the hypoglycaemic brain damage (Choi *et al.*, 1998; Lipton & Nicotera, 1998; Duchen, 2000). 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. Hypoglycaemia specifically increases the sensitivity of NMDA receptors to activation by glutamate, which results in a lower threshold for glutamate induced excitotoxicity (Jane *et al.*, 1999).

Dopamine (DA), a major neurotransmitter in central nervous system is involved in the control of both motor and emotional behaviour (Vallone *et al.*, 2000) and peripherally modulates insulin secretion in the pancreatic islets (Nogueira *et al.*, 1994). DA is synthesised from tyrosine, stored in vesicles in axon terminals and released when the neuron is depolarised. DA interacts with specific membrane receptors to produce its effects. These effects are terminated by reuptake of dopamine into the presynaptic neuron by a dopamine transporter or by metabolic inactivation by monoamine oxidase B (MAO-B) or catechol-*O*-methyltransferase (COMT). DA plays an important role both centrally and peripherally. The recent identification of five dopamine receptor subtypes provides a basis for understanding dopamine's

central and peripheral actions. DA receptors are classified into two major groups: DA D_1 like and DA D_2 like. DA D_1 like receptors consists of DA D_1 and DA D_5 receptors. DA D_2 like receptors consists of DA D_2, DA D_3 and DA D_4 receptors. Stimulation of the DA D_1 receptor gives rise to increased production of cAMP. DA D_2 receptors inhibit cAMP production, but activate the inositol phosphate second messenger system (Seeman, 1980). An imbalance between dopaminergic neurotransmission and DA receptors is known to be associated with the symptomatology of numerous neuropsychiatric disorders, like schizophrenia, psychosis, mania and depression as well as neuropathological disorders, like Parkinson's disease and Huntington's disease (Carlsson, 1988, 1993; Bermanzohn & Siris, 1992; Brown & Gershon, 1993; Jakel & Maragos, 2000; Kostrzewa & Segura-Aguilar, 2003). Hyperglycaemia during diabetes is reported to damage dopaminergic functions. 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). The dopaminergic cells in particular are highly sensitive to excitotoxicity and oxidative stress when the energy metabolism is impaired (Callahan *et al.*, 1998).

Despite our advances in the treatment of diabetes, hypoglycaemic episodes are often the limiting factor in achieving optimal blood sugar control. Recent therapeutic strategies aimed at closely controlling elevated glucose levels in diabetic individuals put them at risk for experiencing episodes of hypoglycaemia. Acute and recurrent hypoglycaemia cause transient or persistent alteration of cognitive functions, and can result in seizures or coma. The effects of acute or recurrent episodes of hypoglycaemia on the cells of the CNS are potentially harmful, and impose long-lasting damaging effects on the brain. The pathogenesis of hypoglycaemia induced nerve cell injury is largely unknown, but mechanisms that could result in damage to

cells of the CNS include excitotoxicity related to a dysregulation of the glutamate-glutamine cycle. To understand the effects of hypoglycaemia on the cells of the CNS, it is essential to characterize the response of CNS cells to reduced glycemic levels, to determine the extent of CNS cell injury induced by hypoglycaemia and to identify the mechanisms involved in hypoglycaemia induced cell or tissue damage in brain. The reports so far stated did not attempt to emphasis the functional correlation of dopaminergic and glutamergic receptors in hypoglycaemic and hyperglycaemic brain.

In the present study a detailed investigation on the alterations of dopamine and its receptors in the brain regions of streptozotocin induced diabetic and insulin induced hypoglycaemic rats were carried out. Glutamate receptor, NMDAR1 gene expression in the hypoglycaemic and hyperglycaemic brain was also studied. EEG recording in hypoglycaemic and hyperglycaemic will be carried out to measure brain activity. *In vitro* studies on glucose uptake and insulin secretion, with and without specific antagonists were carried out to confirm the specific receptor subtypes - DA D_1, DA D_2 and NMDA involved in the functional regulation during hyperglycaemic and hypoglycaemic brain damage. The molecular studies on the brain damage through dopaminergic and glutamergic receptors will elucidate the therapeutic role in the corrective measures of the damage to the brain during hypoglycaemia and hyperglycaemia. This has importance in the management of diabetes and anti-diabetic treatment for better intellectual functioning of the individual.

OBJECTIVES OF THE PRESENT STUDY

1. To quantify dopamine (DA) and homovanillic acid (HVA) content in the brain regions - hippocampus (Hippo), brainstem (BS), cerebral cortex (CC) and corpus striatum (CS) - of control and experimental rats using High Performance Liquid Chromatography (HPLC).

2. To study DA, DA-D_1, DA-D_2 receptors changes in the brain regions - Hippo, BS, CC and CS - of control and experimental rats.

3. To study DA- D_1 and DA-D_2 receptor gene expression in the brain regions - Hippo, BS, CC and CS of control and experimental rats using Real-Time PCR.

4. To study glutamate dehydrogenase (GDH) and malate dehydrogenase (MDH) activity in the brain regions- BS and CC - of control and experimental rats.

5. To study glutamate receptor, NMDAR1 gene expression in the brain regions - Hippo, BS, CC and CS of control and experimental rats.

6. To study the neurophysiologial analysis of electrical activity in the frontal lobe of control and experimental rat brain using EEG recorder.

7. To study the role of DA, DA-D_1 and DA-D_2 receptors on glucose uptake by pancreatic islets *in vitro*.

8. To study the role of glutamate and NMDA receptors on glucose uptake by pancreatic islets *in vitro*.

9. To study the role of DA, DA-D_1 and DA-D_2 receptors on glucose induced insulin secretion by pancreatic islets *in vitro*.

10. To study the role of glutamate and NMDA receptors on glucose induced insulin secretion by pancreatic islets *in vitro*.

Literature Review

Glucose is the only fuel that neuronal tissue can use for energy under normal circumstances (Sokoloff, 1981). The brain can neither synthesize nor store more than a few minutes worth of glucose; thus a continuous systemic supply is essential for normal cerebral metabolism (Pardridge, 1983). Chronic changes in the antecedent level of glycaemia (either sustained hyperglycaemia or hypoglycaemia) induce alterations in brain glucose metabolism in rodents (McCall *et al.*, 1982; Boyle *et al.*, 1994). Diabetes mellitus is a major global health problem that affects more than 185 million people around the world (Amos *et al.*, 1997; Zimmet, 1999; Zimmet *et al.*, 2001). This disease is an increasingly prevalent metabolic disorder in humans and is characterised by hyperglycaemia (Kumar *et al.*, 2002; Dunne *et al.*, 2004). 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 dopamine plays a prominent role.

There is an increased incidence of hypoglycaemia when attempts are made to institute tight glycaemic control using currently available regimens of subcutaneous insulin administration in diabetic patients (Cryer *et al.*, 1994). Tight blood glucose control can reduce the risk of diabetes complications but also increases the risk of

hypoglycaemic episodes. Symptomatic hypoglycaemia occurs frequently in insulin-treated patients, and 36% of patients were found in one study to have experienced hypoglycaemic coma in their lifetime (Pramming *et al.*, 1991). Upto 10% of patients practicing conventional insulin therapy and 25% of those practicing intensive therapy suffer at least one episode of severe, temporarily disabling hypoglycaemia, often with seizure or coma, in a given year (Cryer *et al.*, 1994, DCCT, 1987, 1991), and hypoglycaemia causes recurrent and even persistent psychological morbidity in many diabetic patients (Cryer *et al.*, 1994). Speculation that an adaptation in the CNS might exist in patients with diabetes, depending upon antecedent glycaemia, appeared nearly a decade ago (Cryer, 1985, 2003). Amiel *et al.* (1988) observed that lower glucose concentrations were required to initiate epinephrine secretion following a period of intensified diabetes management with its attendant increase in hypoglycaemia. Similar hormonal defects with unawareness of symptoms can be induced in patients with diabetes (Dagogo *et al.*, 1993; Hepburn *et al.*, 1991) and nondiabetics (Veneman *et al.*, 1993; Heller & Cryer, 1991; Davis & Shamoon, 1991), some after a solitary episode of hypoglycaemia.

Insulin and sulfonylurea therapy for diabetes mellitus carries the risk of hypoglycaemic brain injury, and this risk is a major impediment to optimal glucose regulation in diabetic patients (Davis *et al.*, 1998). Depending upon its severity, hypoglycaemia can cause irritability, impaired concentration, focal neurological deficits, seizures, coma, and, with profound hypoglycaemia, neuronal death (McCrimmon *et al.*, 1997; Auer & Siesjo, 1988; Ben-Ami *et al.*, 1999). Symptoms of hypoglycaemia result from the actions of hormones and neurotransmitters in the process of restoring blood glucose levels. Declining glucose levels in the brain stimulate the autonomic nervous system, causing epinephrine and norepinephrine to

be released from the adrenal medulla. Norepinephrine and acetylcholine from the sympathetic nervous system is also involved in glucose control. Symptoms occur as these hormones and neurotransmitters simultaneously stimulate α-cells in the pancreas to release glucagon, which consequently induces new glucose production in the liver (Cryer *et al.*, 1999, 2002 a, b, 2003). In this homeostatic mechanism, rising blood glucose levels shut down the neoglucogenesis activities of autonomic nervous system (Cryer *et al.*, 1997; Towler *et al.*, 1993; McAulay *et al.*, 2001, Charles *et al.*, 2005). Recent studies indicate that neuronal NADPH oxidase is the primary source of neuronal oxidative stress after hypoglycaemia and the rate of superoxide production is influenced by the blood glucose concentration achieved in the immediate posthypoglycaemic period. Restoring blood glucose to 1–2 mM during the first hour after hypoglycaemia resulted in less superoxide production and less neuronal death than restoration to higher glucose levels (>5 mM). It is suggested that a gradual correction of blood glucose in patients with hypoglycaemic coma may be preferable to more rapid correction and hyperglycaemia (Sang *et al.*, 2007). Symptoms of hypoglycaemia result primarily from a lowered glucose level in the brain and its effects on the central and autonomic nervous systems (Charles *et al.*, 2005). Although hypoglycaemia is associated with a number of physiological changes, the most profound effects are seen in the brain, where glucose is the major substrate for energy metabolism and both local energy store and the supply of alternate sources are limited. The initiating events in hypoglycaemic encephalopathy still are not understood completely. But brain injury appears to result from a number of processes that are initiated when blood glucose concentration decrease. Severe hypoglycaemia, whether in patients with type 1 or type 2 diabetes, can have debilitating consequences, including seizures or coma or even death (Jane, 1999).

Hypoglycaemia and brain

Hypoglycaemia constitutes a unique metabolic brain insult (Auer, 2004). Glucose arrives in the central nervous system (CNS) through the specific brain capillary endothelial transporter, GLUT 1 (Pardridge *et al.*, 1990), at a rate that is generally far in excess of the phosphorylation rate by hexokinase (Pardridge, 1983). Therefore, at euglycaemia, glucose transport is not rate-limiting for brain metabolism; but during an acute reduction in the glucose concentration, a level is reached where transport assumes a rate-limiting role. Beyond this critical point, hexokinase is not fully saturated and brain energy metabolism deteriorates. Among the ultimate consequences of neuroglycopenia are initial elevations in epinephrine and glucagon, which serve to increase systemic glucose production and restore glucose provision to the brain. Widespread regions of the brain have been shown to direct this hormonal response during acute CNS fuel deprivation (Frizzell *et al.*, 1993). Maintaining cerebral normoglycaemia while inducing systemic hypoglycaemia greatly attenuates this counterregulatory hormone response (Frizzell *et al.*, 1993, Biggers *et al.*, 1989). The incidence of severe hypoglycaemia in patients with diabetes treated by intensive insulin therapy is two to six times higher as in conventionally treated patients with diabetes. In particular, recurrent hypoglycaemic episodes during the night represent a relevant risk for the patient, because they are often not realized and lead to a deterioration in the awareness for subsequent hypoglycaemic episodes. Recent data show that recurrent hypoglycaemia not only affects neuroendocrine counter regulation but also autonomic and neuroglucopenic symptoms (Minna *et al.*, 2005; Karen *et al.*, 2006).

Clinical signs and symptoms of metabolic encephalopathies consist of a generalized depression of cerebral function, including consciousness. The effect on consciousness is a consequence of decreased integrative capacity of the neocortex (Jane, 1999). Arousal of the neocortex and other forebrain structures involved in cognition is mediated by specific brainstem nuclei and their projecting fiber tracts, which together constitute the ascending reticular activating system (ARAS). Activating pathways ascend from the ARAS *via* thalamic synaptic relays to the neocortex. Metabolic encephalopathies result from alterations of brain chemistry at both neocortical and brainstem ARAS centers (Pulsinelli & Cooper, 1994). If the glucose supply to the brain is not maintained, there is a decrease in cerebral electrical activity, membrane breakdown and release of free fatty acids and altered amino acid metabolism, including increased production of glutamate which is one of the excitatory amino acid neurotransmitter found only in the central nervous system. It is believed to play a major role in the pathophysiology of hypoglycaemic brain injury. There is increasing evidence that specific changes in mitochondrial function also play a major role in the early events leading to hypoglycaemic encephalopathy. Hypoglycaemic brain injury is a common and serious complication of insulin therapy. Not surprisingly, hypoglycaemic brain injury occurs most frequently in patients attempting tight glucose control (Davis *et al.*, 1998, Auer *et al.*, 1992). The only treatment for hypoglycaemia is blood glucose repletion and there is no currently available intervention for preventing the neuronal death that develops after hypoglycaemia is corrected.

Hypoglycaemic coma induces a purely neuronal lesion of neo cortex and the hippocampus in rat brain (Wieloch *et al.*, 1984). CT studies show that hypoglycaemia predominantly affects cerebral gray matter in the brain. Analysis of regional cerebral

blood flow (CBF) differences identified neuronal activation during hypoglycaemia in bilateral medial prefrontal cortex (Auer & Siesjo, 1993). Hypoglycaemic neuronal death is most pronounced in specific neuron populations: neurons in the hippocampal CA1, subiculum, and dentate granule cell layer; cortical layers 2 and 3 of cerebral cortex; and the dorsolateral striatum (Auer *et al.*, 1989; Auer & Siesjo, 1993). These neurons receive a rich glutamergic innervation, and evidence suggests that hypoglycaemic injury in these neurons is precipitated almost entirely by sustained glutamate receptor activation (excitotoxicity) (Auer *et al.*, 1985b). The hippocampal neurons in particular are important for learning and memory, and patients who survive hypoglycaemic coma may be left with significant cognitive impairment (Patrick & Campbell, 1990; Kalimo & Olsson, 1980).

Dopamine, a neurotransmitter in the central nervous system

Dopamine (DA) is the predominant catecholamine neurotransmitter in the mammalian brain, where it controls a variety of functions including locomotor activity, cognition, emotion, positive reinforcement, food intake, and endocrine regulation. This catecholamine 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).

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). Dopaminergic system is organized into four major subsystems (i) the *nigrostriatal* system involving neurons projecting from the substantia nigra, pars compacta to the caudate-putamen of the basal ganglia. This is

the major DA system in the brain as it accounts for about 70% of the total DA in the brain, and its degeneration makes a major contribution to the pathophysiology of Parkinson's disease; (ii) *the mesolimbic system* that originates in the midbrain tegmentum and projects to the nucleus accumbens septi and lateral septal nuclei of the basal forebrain as well as the amygdala, hippocampus and the entorhinal cortex, all of which are considered components of the limbic system and so are of particular interest for the patho-physiology of idiopathic psychiatric disorders; (iii) the *mesocortical* system, which also arises from neuronal cell bodies in the tegmentum which project their axons to the cerebral cortex, particularly the medial prefrontal regions; (iv) the *tuberinfundibular* pathway, which is a neuroendocrinological pathway arising from the arcuate and other nuclei of the hypothalamus and ending in the median eminence of the inferior hypothalamus. DA released in this system exerts regulatory effects in the anterior pituitary and inhibits the release of prolactin. DA is involved in the control of both motor and emotional behaviour. Despite the large number of crucial functions it performs, this chemical messenger is found in a relatively small number of brain cells. In fact, while there are a total of 10 billion cells in the cerebral cortex alone, there are only one million dopaminergic cells in the entire brain (Missale *et al.*, 1998).

Biosynthesis of dopamine

Dopamine is synthesized from the amino acid L-tyrosine. L-tyrosine is hydroxylated by the enzyme tyrosine hydroxylase (TH) to give L-3, 4-dihydroxyphenylalanine (L-DOPA) which is the rate limiting step. L-DOPA is subsequently decarboxylated to dopamine by the enzyme aromatic L-amino acid decarboxylase. Therefore, it is not possible to enhance the levels of DA by providing L-tyrosine. The activity of tyrosine hydroxylase is regulated by several endogenous

mechanisms. For example, the enzyme is activated by increased neuronal impulse flow, but is inactivated either by DA itself as an end-product inhibitor, or by activation of presynaptic DA receptors. On the other hand, the enzyme aromatic L-amino acid decarboxylase converts L-DOPA to DA instantaneously. Therefore, providing L-DOPA creates a possibility to enhance the formation of DA.

Dopamine reuptake and metabolism

Dopamine exerts its functions mediated through various receptors and these actions are terminated to prevent continuous stimulation of the receptors. This inactivation is brought about by reuptake mechanisms and metabolism of DA. Reuptake of DA is accomplished by a high affinity carrier present in the membrane, the dopamine transporter (DAT). The dopamine transporter recycles extracellular DA by actively pumping it back into the nerve terminal. The dopamine content which is about 70 to 80 % in the striatal synaptic cleft is inactivated by this process. Drugs, such as cocaine, are able to block the action of the dopamine transporter, thereby sustaining the presence of dopamine in the synaptic cleft and its action on dopamine receptors. Part of the dopamine is inactivated by conversion to inactive compounds by metabolic enzymes, which are present both intra- and extraneuronally. Monoamine oxidase (MAO), aldehyde dehydrogenase (AD) and catechol-*O*-methyltransferase (COMT) are responsible for the metabolism of DA. Dopamine after reuptake is intraneuronally deaminated by MAO to give dihydroxyphenyl acetaldehyde, which subsequently is converted to 3, 4-dihydroxyphenylacetic acid (DOPAC) by AD. DOPAC is then methylated by COMT to give homovanillic acid (HVA). Extraneuronally, DA is metabolized by an alternative route in which it is first *O*-methylated to 3-methoxytyramine (3-MT) through the action of COMT and subsequently oxidized by MAO and AD to HVA.

Dopamine receptors

Dopamine mediates its actions via membrane receptor proteins. DA receptors are found on postsynaptic neurons in brain regions that are DA-enriched. In addition, they reside presynaptically on DA neuronal cell bodies and dendrites in the midbrain as well as on their terminals in the forebrain. Dopamine receptors belong to a family of large peptides that are coupled to G-proteins which are modified by attached carbohydrate, lipid-ester or phosphate groups. The topologies of the five dopamine receptors are predicted to be the same as all the other G-protein-coupled receptors. They are characterized by having seven hydrophobic transmembrane-spanning regions. The third intracytoplasmic loop is functionally critical and interacts with G-proteins and other effector molecules to mediate the physiological and neurochemical effects (Tarazi *et al.*, 1997 a, b, 1998 a, b; Tepper, *et. al.*, 1997; Royh, *et. al.*, 1991; Carlsson, 1993). In their putative transmembrane domains, the DA D_1 and D_5 receptors are 79% identical to each other, while they are only 40–45% identical to the DA D_2, D_3, and D_4 receptors. Conversely, the DA D_2, D_3, and D_4 receptors are between 75% and 51% identical to each other. They contain seven putative membrane-spanning helices which would form a narrow dihedral hydrophobic cleft surrounded by three extracellular and three intracellular loops. The receptor polypeptides are probably further anchored to the membranes through palmitoylation of a conserved Cys residue found in their carboxy tails, 347 in DA D_1, the C-terminus in DA D_2 like receptors. The dopamine receptors are glycosylated in their N-terminal domains. Dopamine D_1 like subtypes have potential glycosylation sites in their first extra cytoplasmic loop.

Dopamine receptors are divided into two families on the presence or absence of ability of DA to stimulate adenylyl cyclase and produce the second-messenger

molecule cyclic-AMP (cAMP) (Kebabian & Calne, 1979; Schwartz *et al* 1992; Civelli *et al*, 1993; O'Dowd, 1993; Jackson & Westlind, 1994; Ogawa, 1995; Strange, 1996). This classification is based on similarities in structure, pharmacology, function and distribution. Dopamine D_1 like receptors are characterized initially as mediating the stimulation of cAMP production. Dopamine D_2 like receptors inhibit the production of cAMP. This pharmacological characterization is based on the ability of some DA agents to block adenylyl cyclase activity to inhibit the release of prolactin *in vivo* and *in vitro* in a cAMP-independent fashion (Seeman, 1980). Applications of recent technical advances in molecular genetics have greatly facilitated the isolation and characterization of novel DA receptors, DA D_3, D_4 and D_5, with different anatomical localization from traditional DA D_1 or DA D_2 receptors. Based upon their pharmacological profiles, including their effects on different signal transduction cascades, these receptors are currently divided into two families: the DA D_1like family which includes dopamine D_1 and D_5 receptors. The DA D_2 like family includes dopamine D_2, D_3 and D_4 receptors (Grandy *et al.*, 1993; Sibley *et al.*, 1993; Schwartz *et al.*, 1992). The genomic organizations of the DA receptors demonstrate that they are derived from the divergence of two gene families that mainly differ in the absence or the presence of introns in their coding sequences. Dopamine D_1 like receptors genes do not contain introns in their coding regions, a characteristic shared with most G protein-coupled receptors. The genes encoding the dopamine D_2 like receptors are interrupted by introns (Gingrich & Marc, 1993). Furthermore, most of the introns in the DA D_2-like receptor genes are located in similar positions.

Dopamine D_1-like family

The DA D_1 receptor is the most abundant DA receptor in the central nervous system. The DA D_1 like receptors are characterized by a short third loop as in many

receptors coupled to Gs protein (Civelli *et al.*, 1993; Gingrich & Canon, 1993; O'Dowd, 1993). The DA D_1 like receptors have short third intracellular loops and long carboxy terminal tails. The DA D_1 like receptors are classified into DA D_1 and D_5. In the DA D_1 and D_5 receptor third intracellular loop and the carboxy terminus are similar in size but divergent in their sequence. In contrast, the small cytoplasmic loops 1 and 2 are highly conserved so that any difference in the biology of these receptors can be probably related to the third cytoplasmic loop and the carboxy terminal tail (Civelli *et al.*, 1993, Gingrich & Canon, 1993; O'Dowd, 1993). The external loop between transmembrane domain (TM) TM4 and TM5 is considerably different in the two receptor subtypes, being shorter (27 amino acids) in the D_1 receptor than in the D_5 receptor (41 amino acids). The amino acid sequence of this loop is divergent in the DA D_5 receptor (Marc *et al.*, 1998).

Dopamine D_1 receptor

DA D_1 receptors are found at high levels in the typical dopamine regions of brain such as the neostriatum, substantia nigra, nucleus accumbens and olfactory tubercles. DA D_1 receptor seems to mediate important actions of dopamine to control movement, cognitive function and cardiovascular function. The DA D_1 receptor gene, which lacks introns, encodes a protein that extends for 446 amino acids (Dohlman *et al.*, 1991). In humans DA D_1 receptor gene has been localized to chromosome 5 (Sunahara *et al.*, 1990). The DA D_1 receptors show characteristic ability to stimulate adenylyl cyclase and generate inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol *via*

The activation of phospholipase C (Sibley *et al.*, 1990; Monsma *et al.*, 1990). DA D_1 receptors are highly expressed in basal ganglia followed by cerebral cortex, hypothalamus and thalamus. DA D_1 receptors messenger ribonucleic acid

(mRNA) is colocalized in striatal neurons of the basal ganglia with mRNA for DA receptor phosphor protein (DARPP-32; KD) which is a dopamine and cAMP-regulated phosphoprotein. DA Receptor Phosphor Protein contributes to the actions of DA D_1 receptor (Hemmings & Greengard, 1986; Greengard *et al.*, 1987). The DA D_1 receptors in the brain are linked to episodic memory, emotion, and cognition.

Dopamine D_5 receptors

The DA D_5 receptor gene is intronless and encodes a protein that extends for 47 amino acids (George *et al.*, 1991). This protein has an overall 50% homology with DA D_1 receptor and 80% if only the seven transmembrane segments are considered. The gene encoding the human DA D_5 protein is located at the short arm of chromosome 4, the same region where the Huntington disease gene has been located (Gusella, 1989). Two DA D_5 receptor pseudogenes having 154 amino acids have been identified with 90% homology (Gusella, 1989). These pseudogenes, however, contain stop codons in their coding regions that prevent them from expressing functional receptors. The functions of these pseudogenes, which appear so far to be specific to humans, are not yet known (Allen *et al.*, 1991).

DA D_5 receptor mRNA expression is unique and limited to the hippocampus and parafascicular nucleus of the thalamus (Civelli *et al.*, 1992). It is involved in the thalamic processing of painful stimuli (Giesler *et al.*, 1979). DA D_5 receptors appear to interact with G-proteins and can stimulate adenylyl cyclase, with relatively high affinity for DA and DA D_1-selective agonists (George *et al.*, 1991).

Dopamine D_2 like family

DA D_2 like receptors belong to the G-protein coupled receptors and has 400 amino acid residues. DAD_2-like receptors are characterized by a long extracellular amino terminus which has several glycosylation sites and a shorter carboxy terminal tail with putative phosphorylation sites. The function of sugar moieties is unclear (Marc *et al.*, 1998; Sibley, 1999). It is generally believed that the membrane enclosed part of the amino-acid chain of G-protein coupled receptors is folded into seven α-helices. The transmembrane helices consist primarily of hydrophobic amino-acid residues. The unique feature of DA D_2 like receptors family is that they posses a bigger third cytoplasmic (intracellular) loop in common, which is thought to be the site where the G-protein couples (Marc *et al.*, 1998). Between the different dopamine receptors, the third loop also displays the greatest variability in amino-acid sequence. This may have consequences for their respective second messenger systems. The DA D_2-like receptors are coupled to Gi-protein and inhibit the formation of cAMP. The DA D_2 receptors tertiary structure is stabilized by two cysteine disulphide bridges.

Dopamine D_2 receptors

The DA D_2 receptor gene encodes a protein that extends for 415 amino acids. Similar to other G-protein coupled receptors, the DA D_2 receptor has seven transmembrane segments, but in contrast to DA D_1-like receptors, the third cytoplasmic domain is long and the carboxy terminus is short. Unlike the DA D_1-like receptor genes, the DA D_2 receptor gene contains seven introns that are spliced out during mRNA transcription (Fischer *et al.*, 1989). The gene encoding this receptor was found to reside on q22-q23 of human chromosome 11 (Makam *et al.*, 1989). The DA D_2 receptor was the first receptor to be cloned (Chrisre *et al.*, 1988). The DA D_2 receptors are involved in several signal transduction cascades, including inhibition of

cAMP production (Vallar & Meldolesi, 1989), inhibition of phosphoinositide turnover (Epelbaum *et al.,* 1986), activation of potassium channels and potentiation of arachidonic acid release (Axelrod *et al.*, 1991). The DA D_2 receptors are highly expressed in basal ganglia, nucleus accumbens septi and ventral tegmental area (Schwartz *et al.,* 1992).

The DA D_2 receptor exists as two alternatively spliced isoforms differing in the insertion of a stretch of 29 amino acids in the third intracellular loop and are designated as DA D_{2S} and DA D_{2L} (Seeburg *et al.*,1989; Marc *et al.*, 1998). Because this loop seems to play a central role in receptor coupling, the existence of a splicing mechanism at this level could imply functional diversity. However, in spite of the efforts of several groups, no obvious differences have emerged so far between the two DA D_2 receptor isoforms. The two isoforms derived from the same gene by alternative RNA splicing which occurs during the maturation of the DA D_2 receptor pre-mRNA (Schwartz *et al.*, 1989). DA D_2 receptor isoforms (DA D_{2L} and DA D_{2S}) vary within each species by the presence or absence of a 29-amino acid sequence in the third cytoplasmic domain of the DA D_2 receptor peptide chain. Both variants share the same distribution pattern; with the shorter form less abundantly transcribed in addition they appear to differ in their mode of regulation (Marc *et al.*, 1998). Pharmacologically, both isoforms exhibit nearly similar profiles in terms of their affinities to different DA D_2-selective agents, and inhibit adenylyl cyclase activity. However, these isoforms display an opposite regulatory effect (Sibley *et al.*, 1993). These isoforms have the same pharmacological profile, even though a marginal difference in the affinity of some substituted response to dopamine treatment is reported: Dopamine induces the up-regulation of DA D_{2L} isoform of DA D_2 receptors (Castro & Strange, 1993). When expressed in host cell lines, both isoforms inhibited adenylyl cyclase (Marc *et al.*, 1998; Sibley, 1999). However, the DA D_{2S} receptor

isoform displayed higher affinity than the DA D_{2L} in this effect (Seeburg *et al.*, 1993). The isoforms of DA D_2 mediate a phosphatidylinositol-linked mobilization of intracellular calcium in mouse Ltk [-] fibroblasts. Protein kinase C (PKC), however, differentially modulates DA D_{2S} and D_{2L}-activated transmembrane signalling in this system with a selective inhibitory effect on the dopamine D_{2S}-mediated response.

Dopamine D_3 receptors

Dopamine D_3 receptor gene contains five introns and encodes a 446 amino acid protein (Schwartz *et al.*, 1992). The gene encoding this receptor resides on chromosome 3 (Giros *et al.*, 1990). The DA D_3 receptors bear close structural and pharmacological similarities to the DA D_2 receptors. DA D_3 mRNA occurs in longer and shorter spliced forms generated from the same gene (Schwartz *et al.*, 1992). Distribution of DA D_3 receptor mRNA are distributed and expressed mainly in subcortical limbic regions including islands of Calleja, nucleus accumbens septi and olfactory tubercle, with low levels of expression in the basal ganglia. D_3 receptor mRNA has also been found in neurons of the cerebellum, which regulate eye-movements (Levesque *et al.*, 1992). The status of the DA D_3 molecular entity as a functional receptor remains uncertain since it neither couples to G-proteins nor consistently transduces an effector mechanism. However, the structural similarity with DA D_2 receptor raises the possibility that DA D_3 receptor also inhibit adenylyl cyclase activity in its normal cellular setting. More recent studies reported that DA D_3 receptors mediate positive regulatory influences of DA on production of the peptide neurotensin (Schwartz *et al.*, 1992; Sokoloff *et al.*, 1990).

Dopamine D_4 receptors

DA D_4 receptor gene contains four introns and encodes a 387 amino acid protein (Van Tol *et al.*, 1991). The overall homology of the DA D_4 receptor to the DA D_2 and D_3 receptors is about 41% and 39% respectively, but this homology increases to 56% for both receptors when only the transmembrane spanning segments are considered. The gene encoding the human DA D_4 protein is located at the tip of the short arm of chromosome 11 (Civelli & Bunzow, 1993; Missale *et al.*, 1998). DA D_4 receptor gene has been localized in brain regions like hippocampus and frontal cortex using specific histoprobes. The stimulation of DA D_4 receptor inhibits adenylyl cyclase activity and release arachidonic acid in brain neurons (Huff *et al.*, 1994, Misalle *et al.*, 1998). In humans, DA D_4 receptor occurs in several genomic polymorphic variants that contain two to eleven repeats of a 48 base pair segment that is expressed in the third cytoplasmic domain (Van Tol *et al.*, 1992; Misalle *et al.*, 1998). These are called the DA D_4 alleles which are represented as DA $D_{4.2}$, $D_{4.4}$ and $D_{4.7}$. These may contribute to the pathophysiology of certain neuropsychiatric disorders (Jackson & Westlind, 1994).

GLUTAMATE RECEPTORS

Glutamate (Glu) functions as a fast excitatory transmitter in the mammalian brain. Glutamate triggers neuronal death when released in excessive concentrations by over excitation of its receptors (Vizi, 2000). The excitatory amino acid Glu is the most prevalent transmitter in the brain; its effect on postsynaptic receptors is limited by uptake process (Erecinska, 1997) and by diffusion of Glu from the cleft. The removal of Glu from the extracellular fluid, limitation of its action occurs by uptake and by diffusion (Tong & Jahr, 1994). This is accomplished by a transporter in the plasma

membrane of both neurons and astrocytes (Brooks-Kayal *et al.*, 1998; Gelagashvili & Schousboe, 1998). Electrophysiological evidence was obtained that the block of Glu transporters potentiates postsynaptic excitation of Glu receptors (Tong & Jahr, 1994). The cellular uptake of Glu is driven by the electrochemical gradients of Na^+ and K^+ and is accompanied by voltage and pH changes.

The majority of excitatory synapses are glutamergic, in which Glu transmits the signal through postsynaptic ionotropic [N-methyl-D-aspartic acid (NMDA), -amino-3-hydroxy-5-methysoxazole-4-propionic acid (AMPA), and kainate (KA)] and metabotropic receptors (Bettler & Mulle, 1995). Glu is a fast excitatory transmitter in the CNS and has been shown, with GABA, to interact primarily with receptors in the synaptic cleft (Dingledine *et al.*, 1999). The extracellular accumulation of glutamate results in neuronal death by activating ionotropic glutamate receptors sensitive to NMDA or AMPA–kainate (Choi, 1988). The presence of G protein-coupled glutamate receptors (metabotropic Glu receptors) has been described, and since 1991 (Conn & Pin, 1997), eight receptors have been discovered and classified into three groups based on their linkage to second messenger systems and their pharmacology: group I acts *via* the phosphoinositol system, and groups II and III inhibit adenylyl cyclase. In addition, the stimulation of receptors of these three groups directly influences voltage-gated Ca^{2+} and K^+ channels through their G proteins, but their physiological correlate has not yet defined.

There are several reports of presynaptic localization of Glu receptors and their involvement in transmitter release. The fact that NMDA releases Glu (Pittaluga *et al.*, 1996), DA (Kuo *et al.*, 1998) and NE (Pittaluga & Raiteri, 1992) from axon terminals indicates that Glu released is able to facilitate transmitter release *via* NMDA receptors (Barnes *et al.*, 1994; Desai *et al.*, 1994). Montague *et al.* (1994) suggested that Glu and NE release from cortical synaptosomes was in correlation with NMDA-induced

production of nitric oxide (NO), an endogenous chemical that is able to inhibit basal membrane transporters, thereby increasing the concentration and life-span of transmitters (e.g., Glu and NE) released into the extracellular space. The inhibition of neuronal NO synthase by 7-nitroindazole protects against NMDA mediated excitotoxic lesions but not against those evoked by AMPA or KA (Schulz *et al.*, 1995).

NMDA receptors

NMDA sensitive ionotropic glutamate receptors probably consist of tetrameric and heteromeric subunit assemblies that have different physiological and pharmacological properties. They are differentially distributed throughout the CNS (Seeburg, 1993; Hollmann and Heinemann, 1994; McBain and Mayer, 1994; Danysz *et al.*, 1995; Parsons *et al.*, 1998). NMDA receptors are probably heteromeric assemblies of four subunits. Each subunit has four hydrophobic regions, although only three form membrane-spanning domains (TM1, TM2, and TM4). TM2 makes a hairpin bend within the membrane and forms the channel pore; the "TM" terminology is therefore inappropriate. Functional NMDA receptor complexes are formed by combinations of NR1 and NR2 subunits, which contain glutamate recognition sites. Alternative splicing at three exons, one in the amino-terminal domain (N1) and two in the carboxyl-terminal domain (C1 and C2), generates eight isoforms for the NR1 subfamily. All heteromeric and homomeric NMDA receptor subtype complexes are permeant to Ca^{2+}, Na^{+}, and K^{+}. The open NMDA channel is blocked by Mg^{2+} and uncompetitive NMDA receptor antagonists, such as memantine and (+)MK-801, in a voltage-dependent manner. The speed and voltage observed in this effect depend on the antagonist affinity and the subunit composition. In addition, most NMDA

receptors are influenced by Zn^{2+} ions in a voltage-dependent manner, as well as by oxidation/reduction and pH.

The NMDA channel is blocked in a use- and voltage-dependent manner by Mg^{2+}. This means that NMDA receptors are activated only after depolarization of the postsynaptic membrane by, for example, AMPA receptor activation, which relieves the voltagedependent blockade by Mg^{2+}. This biophysical property and their high Ca^{2+} permeability render NMDA receptors inherently suitable for their role in mediating synaptic plasticity underlying learning processes and development (Collingridge & Singer, 1990; Danysz *et al.*, 1995). Similar to Mg^{2+}, uncompetitive NMDA receptor antagonists such as ketamine, dextromethorphan, memantine, phencyclidine (PCP), and (+)MK-801 [(+)5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine maleate] block the NMDA channel in the open state, although the blocking kinetics and voltage of this effect depend on the antagonist (Rogawski, 1993; Parsons *et al.*, 1995).

To date, two major subunit families, designated NR1 and NR2, have been cloned. Various heteromeric NMDA receptor channels formed by combinations of NR1 and NR2 subunits are known to differ in gating properties, magnesium sensitivity, and pharmacological profile (Sucher *et al.*, 1996; Parsons *et al.*, 1998). The heteromeric assembly of NR1 and NR2C subunits, for instance, has much lower sensitivity to Mg^{2+} but increased sensitivity to glycine and very restricted distribution in the brain. In situ hybridization has revealed overlapping but different expression profiles for NR2 mRNA. For example, NR2A mRNA is distributed ubiquitously like NR1, with the highest densities occuring in hippocampal regions and NR2B is expressed predominantly in forebrain but not in cerebellum, where NR2C

predominates; NR2D is localized mainly in the brainstem (Moriyoshi *et al.*, 1991; Monyer *et al.*, 1992; Nakanishi, 1992; McBain and Mayer, 1994).

In addition to NR1 and NR2, the NR3A subunit has recently been discovered. This receptor subunit, previously termed chi-1, or NMDAR-L, is a relatively recently identified member of a new class in the ionotropic glutamate receptor family. It attenuates NMDA receptor currents when coexpressed with NR1/NR2 subunits in *Xenopus* oocytes but has no effect when tested with non-NMDA receptors or when expressed alone (Ciabarra *et al.*, 1995; Sucher *et al.*, 1995; Das *et al.*, 1998). Highest levels are present in the spinal cord, brainstem, hypothalamus, thalamus, CA1 field of the hippocampus, and amygdala and this distribution remains the same throughout life. Genetic knockout of NR3A in mice results in enhanced NMDA responses and increased dendritic spines in early postnatal cortical neurons, suggesting that NR3A is involved in the development of synaptic elements by modulating NMDA receptor activity (Das *et al.*, 1998).

The highest levels of NR1 mRNA in the adult rat and mouse CNS are in the olfactory bulb and the lowest levels are expressed in the spinal cord. Intermediate levels were found in frontal cortex, hippocampus, cerebellum, and whole brain (Franklin *et al.*, 1993; Akazawa *et al.*, 1994). Similar findings have been reported with antibodies to NR1 subunits (Petralia *et al.*, 1994; Benke *et al.*, 1995). mRNA for double-splice variants in the C1/C2 regions, such as $NR1_{011}$ (NR1a), show an almost complementary pattern with respect to those lacking both of these inserts, such as $NR1_{100}$ (NR1b). NR1a mRNA are more concentrated in rostral structures such as cortex, caudate, and hippocampus, whereas NR1b mRNA are principally found in more caudal regions such as thalamus, colliculi, locus coeruleus, and cerebellum (Laurie and Seeburg, 1994; Paupard *et al.*, 1997). Others reported that the

predominant splice variants in cortex and hippocampus were NR1a without N1 insert, whereas in the cerebellum the major variant was NR1b, containing N1 (Zhong *et al.*, 1994). In the hippocampus, NR1a mRNA shows high levels in all regions and is expressed more intensely in CA3 pyramidal neurons (Paupard *et al.*, 1997). mRNA for NR1a and NR1b splice forms is found nearly homogeneously throughout the adult CNS, whereas NR1a and NR1b mRNA is scarce, being detected only at very low levels in postnatal cortex and hippocampus (Laurie and Seeburg, 1994; Paupard *et al.*, 1997). The predominant splice variant in cultured cortical neurons is NR1a (Zhong *et al.*, 1994).

In developing rats, NR1 mRNA levels in cortex and hippocampus increased nearly three-fold from postnatal day 3 to day 15 and approximately doubled from day 15 to day 67 (Franklin *et al.*, 1993; Nowicka & Kaczmarek, 1996). In contrast, cerebellum and brainstem showed no change in NMDAR1 mRNA levels between postnatal days 3 and 15 but levels also doubled from day 15 to day 67 (Franklin *et al.*, 1993). Similar results were reported by a different group, although levels in the hippocampus peaked at postnatal day 10 and declined thereafter (Pujic *et al.*, 1993). In the hippocampus, NR1a mRNAs dominate at birth and exhibit mature patterns of labeling, with high levels of expression in the CA1 and CA3 regions and the dentate gyrus. In contrast, NR1b mRNAs are initially expressed at lower uniform levels but levels increase more in the CA3 region than in the CA1 region or the dentate gyrus in the second and third postnatal weeks (Paupard *et al.*, 1997).

The NMDA receptor antagonists have potential therapeutic applications. NMDA receptors are involved in learning and other forms of plasticity, such as drug dependence and addiction, chronic pain, and CNS development, as well as in normal or disturbed synaptic transmission in some areas of the CNS. Activation of NMDA

receptors depends not only on the level of synaptic activity but also on other factors, such as agonist affinity, gating kinetics and Mg^{2+} sensitivity. The role of NMDA receptors in various processes depends on the subtype composition and area of the CNS involved. In animals, most NMDA receptor antagonists produce impairment of learning when given at sufficiently high doses before the association phase but not when administered after this phase or during retrieval (Danysz *et al.*, 1995, Meldrum, 1985; Rogawski, 1993; Leeson and Iversen, 1994; Danysz *et al.*, 1995; Besnard *et al.*, 1996; Ishimaru and Toru, 1997; Parsons *et al.*, 1998).

Brain neurotransmitters and diabetes

Diabetes mellitus is a metabolic disorder that either arrives during the early years of growth (Juvenille diabetes) or later in life called as maturity onset diabetes. It is observed as the body's inability to effectively regulate the sugar balance which leads to severe complications such as hyperglycaemia, obesity, neuropathy, nephropathy, retinopathy, cardiopathy, osteoporesis and coma leading to death. Pancreatic damage resulting in the dysfunction of α and β cells causes disordered glucose homeostasis. In diabetic individuals the regulation of glucose levels by insulin is defective, either due to defective insulin production which is called as Insulin Dependent Diabetes Mellitus (IDDM) or due to insulin resistance that is termed as Non Insulin Dependent Diabetes Mellitus (NIDDM).

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, serotonergic and DA D_2 receptor function alterations in the brain of diabetic rats (Abraham & Paulose, 1999; Padayatti & Paulose, 1999; Paulose *et al.*, 1999, Eswar *et al.*, 2007).

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. Strepozotocin -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).

Dopamine and its receptor alterations during diabetes

Dopamine is implicated in diabetes. Hyperglycaemia in rats is reported to decrease dopaminergic activity in the striata suggesting the up regulation of dopamine receptors possibly due to the decreased DA metabolism (Hio *et al.*, 1994). In experimental diabetes and insulin deficiency there is a rapid onset of detectable alterations in hypothalamic DA activity leading to secondary neuroendocrine abnormalities. Lim *et al.*, (1994) have described an increase in the striatal DA and decrease in its metabolites dihydroxyphenylacetic acid and HVA. Tyrosine hydroxylase is reported to be depleted in nigrostriatal neurons in the genetically diabetic rat causing marked reduction in mesolimbic dopamine system. Insulin treatment could not restore the decreased DA to controlled conditions, impairing the dopamine biosynthesis (Kamei & Saitoh, 1994). Dopamine uptake affinity and velocity in synaptosomes is decreased significantly during diabetes. The DA content was increased in cerebral cortex and hypothalamus of diabetic rats (Chen & Yang, 1991; Ohtani *et al.*, 1997; Tassava *et al.*, 1992; Shimizu, 1991). Diabetes is reported to cause increased DA release with altered turnover ratio of DA metabolites from the mesolimbic systems. This resulted in the enhanced spontaneous locomotor activity which is suggested to be due to the up regulation of δ-opioid receptor-mediated functions (Kamei *et al.*, 1994). The decrease in striatal DA transporter mRNA in experimental diabetes is suggested to be a possible cause for the disturbance in DA metabolism (Figlewicz *et al.*, 1996). The DA turnover ratio in the limbic forebrain and midbrain in diabetic mice were significantly greater than those in non-diabetic mice (Kamei & Saitoh, 1996). Yawning behaviour in streptozotocin induced diabetes was significantly lowered when compared with their age-matched normal controls as a result of altered DA metabolism and decreased turnover to its metabolites (Heaton & Varrin, 1993).

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). Intracerebroventricular application of alloxan and streptozotocin in rat striatum is reported to have caused 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). 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).

Diabetes mellitus causes a condition called as neurocytoglucopenia where the increased glucose results in an increased sympathetic outflow into the liver, pancreas, adrenal medulla, adipose tissue and the circulation. This causes an increased hepatic glucose production, inhibition of insulin secretion and free fatty acid mobilization from the adipose tissue. Participation of dopaminergic tone in the control of insulin secretion and hyperglycaemia has been given little focus. Recent studies have shown that dopamine agonists play an important role in lowering the elevated shift in the sympathetic tone as a result of increased glucose levels and stimulate the parasympathetic tone which increases the insulin response (Oliveira *et al.*, 1998).

Brain neurotransmitters and hypoglycaemia

Glucose in brain supplies energy essential for maintenance of the nervous system. During hypoglycaemia, energy dependent mechanisms for restoring normal transmembrane gradients of sodium and calcium cannot operate because of the depletion of ATP and phosphocreatine associated with hypoglycaemia. Excess calcium influx activates cellular phospholipases and proteases, alters mitochondrial metabolism, triggers free radical formation, changes patterns of synaptic transmission, and eventually may result in selective neuronal necrosis (Jane *et al.*, 1999). Deficiency in glucose that results from hypoglycaemic insults can trigger neuronal injuries. Balance in ion homeostasis is disturbed, which in turn results in membrane depolarization and massive release of neurotransmitters, including glutamate (Siesjo, 1978; Erecinska & Silver, 1989). The extracellular accumulation of glutamate results in neuronal death by activating ionotropic glutamate receptors sensitive to NMDA or AMPA-kainate (Choi, 1988). In addition, neurons impaired of energy metabolism appear to be highly sensitive to excitotoxicity (Simon *et al.*, 1984; Wieloch, 1985; Monyer *et al.*, 1992; Cebers *et al.*, 1998).

Hypoglycaemia causes several-fold elevations in brain extracellular glutamate and aspartate concentrations, and ablation of presynaptic glutamergic terminals can prevent hypoglycaemic neuronal death (Wieloch *et al.*, 1985; Butcher *et al.*, 1987). Pretreatment with glutamate receptor antagonists can also reduce hypoglycaemic neuronal death (Wieloch, 1985), but these agents are less effective when administered after hypoglycaemia has occurred (Nellgard & Wieloch, 1992). An additional limitation to the use of glutamate receptor antagonists in clinical settings is that these agents are themselves neurotoxic (Olney *et al.*, 1989). Hypoglycaemic neuronal death

is not a direct and immediate consequence of low-energy substrate but results instead from a cascade of events precipitated by the lack of substrate. Sustained activation of glutamate receptors has been established as a necessary upstream event in this cascade (Auer & Siesjo, 1993). Because of the extensive neuronal loss, one of the neurological sequalae associated with hypoglycaemia is cognitive decline. According to clinical studies, significant learning and memory deficits correlate with the frequency of hypoglycaemia not only in patients with type 1 diabetes, but also in the relatively younger group among the population with type 2 diabetes (Dey *et al.*, 1997; Sang *et al.*, 2005). It is reported that moderate prolonged hypoglycaemia results in reduced cardiac vagal outflow in both diabetic patients and nondiabetic subjects (Minna *et al.*, 2005).

Clinically, hypoglycaemia results in depression of CNS function, with rostral brain regions being affected before more caudally situated regions. For example, in severe hypoglycaemia associated with isoelectric EEG tracings, cerebral cortical activity is absent but medullary function persists, as indicated by the maintenance of effective respiratory and cardiovascular activity. Reduced synthesis of neurotransmitters rather than a global cerebral energy deficit explains the neurological symptoms and EEG changes in moderate hypoglycaemia (Butterworth, 1983, 1999). The physiologic disturbances associated with acute hypoglycaemia result in a stress response, with release of catecholamines and glucagon and subsequent lipolysis and glycogenolysis in an attempt to increase substrate availability for normal metabolic processes (Jane, 1999). Protection against epinephrine defects, both without and with antecedent hypoglycaemia in diabetes, is associated with enhancement of adrenal catecholamine-synthesizing enzyme levels. Karen *et al.*, (2006) reported increased phenylethanolamine N-methyltransferase, tyrosine hydroxylase (TH), DBH protein in

diabetic rats exposed to hyperinsulinemic-hypoglycaemia. It is reported that 2-D Glucose augmented the turnover of NE, DA and 5-HT under the fasted condition. Insulin perfusion within the medial hypothalamic sites evoked a significant increase in the synthesis and release of DA from the sated rat, but did not alter its turnover. However, in the interval following insulin perfusion, DA and 5-HT turnover were enhanced while the efflux of 5-HT was suppressed (Minano *et al.*, 1982).

Pyruvate derived from glucose is the major precursor of the acetyl group of. Inhibition of pyruvate oxidation results in reduced ACh synthesis both *in vitro* and *in vivo.* Incorporation of [^{14}C]choline into ACh in brain *in vivo* is decreased in rats with insulin-induced hypoglycaemia. Hypoglycaemia results in decreased synthesis of the neurotransmitter pool of ACh are supported by the observation that administration of the CNS cholinesterase inhibitor physostigmine to hypoglycaemic animals delays the onset of seizures and coma (Gibson & Blass, 1976). It is also reported that the extracelluar concentrations of acethylcholine both in the hippocampus and striatum did not change during hypoglycaemia. Changes of hippocampal cholinergic release is not involved in the mechanism of cognitive impairment during hypoglycaemia (Hiroyuki *et al.*, 2006).

Similar findings of an adverse effect of hypoglycaemia on the synthesis of the amino acid neurotransmitters GABA and glutamate have also been reported. Utilization of amino acids such as glutamate and glutamine as alternative energy substrates in moderate to severe hypoglycaemia results in accumulation of aspartate and ammonia in the brain. Hypoglycaemia also produces a transient but substantial increase in extracellular concentrations of glutamate, GABA and dopamine, as measured using *in vivo* cerebral microdialysis (Butcher *et al.*, 1987; Butterworth,

1983, 1999). Studies reported that modulation of the GABAergic system in the ventromedial hypothalamus (VMH) alters both glucagon and sympathoadrenal, but not corticosterone, responses to hypoglycaemia. GABAergic inhibitory tone within the VMH modulates glucose counterregulatory responses (Owen *et al.*, 2006). Alterations of neurotransmission mediated by ACh, Glu, GABA and/or DA contribute to the neurological signs and symptoms that characterize moderate hypoglycaemia.

Hypoglycaemia results in cognitive dysfunction. Wredling *et al.*, (1990) reported permanent neuropsychological impairment after recurrent episodes of severe hypoglycaemia in diabetic patients. Severe deterioration in cognitive function and personality in patients with long-standing diabetes as a complication of a consequence of insulin treatment is reported. IDDM patients with hypoglycaemia unawareness exhibited more profound cognitive dysfunction during acute hypoglycaemia which persisted for longer following blood glucose recovery (Gold *et al.*, 1995). Severe hypoglycaemia with cognitive dysfunction is three times more common in intensively, rather than conventionally, treated IDDM (Maran *et al.*, 1995). Severe hypoglycaemia causes pronounced neuroglycopenia that results in a profound degree of cognitive dysfunction and rarely can cause permanent neurological impairment (Mark *et al.*, 2000). In the insulin treated diabetic patients exposed to a spontaneous episode of severe hypoglycaemia, the cognitive decrements and altered mood states noted is persistent and is the consequence of previous exposure to recurrent episodes of severe hypoglycaemia (Strachan *et al.*, 2000). Recurrent hypoglycaemia significantly diminished cognitive performance in both diabetic and nondiabetic animals. The diabetic hippocampus adapt to high circulating glucose, with increased susceptibility to reductions in glucose availability. RH diminishes ability to meet the demands of a relatively demanding cognitive challenge during hypoglycaemia (McNay, 2005).

Recurrent hypoglycaemia markedly affects hippocampally dependent spatial working memory task. This is accompanied by alterations within the hippocampus, including both ECF glucose and lactate levels during cognitive testing and electrophysiological function. The impact of recurrent hypoglycaemia on cognition is multifaceted and includes both metabolic and electrophysiological components (McNay *et al.*, 2006).

Exposure to stress is known to precipitate or exacerbate many neuropsychiatric disorders such as depression, Parkinson's disease, schizophrenia, and others (Schwab & Zieper, 1965; Mazure, 1995). All these disorders involve a working memory deficit caused by prefrontal cortical (PFC) dysfunction (Mattes, 1980; Weinberger *et al.*, 1986; Deutch, 1993; Fibiger, 1995). Several antidepressants increase DA levels in the PFC (Tanda *et al.*, 1994), and raising the DA level in patients with Parkinson's disease with L-3,4-dihydroxyphenylalanine improves their working memory deficit (Lange *et al.*, 1992). These findings suggest that a reduced dopaminergic transmission in the PFC is responsible for the working memory deficits in the neuropsychiatric disorders.

Acute stress increases dopamine release and metabolism in a number of brain areas (Zangen *et al.*, 1999). Dopaminergic innervation of the medial and dorsolateral PFC appears to be particularly vulnerable to stress and relatively low intensity levels of stress are capable of promoting significant responses. The prefrontal dopaminergic neurons have a number of higher functions including attention and working memory and the acquisition of coping patterns in response to stress (Castellano *et al.*, 1999). Amphetamines and cocaine agonise these receptors and have a similar effect as stress, resulting in symptoms such as anxiety, panic, hypervigilence, exaggerated startle

reflexes and paranoia (Horger *et al.*, 1999). NMDA and opiate receptors are plentiful in this area and stress-induced innervation of the fronto-cortical neurons is prevented if these receptors are selectively blocked. This increase of DA from the dendrites of dopamine neurons may be due to an alteration in GABA regulation of the dopamine neurons. As with noradrenergic systems, single or repeated exposures to stress potentiates the capacity of a subsequent stressor to increase DA function in the forebrain without altering basal DA turnover, suggesting that the receptors have been hyper-sensitized (Basso *et al.*, 1999). DA neurons are vulnerable to metabolic stress (Callahan *et al.*, 1998).

Although the mechanism responsible for cognitive deficits in stress-related neuropsychiatric disorders has been obscure, PFC dopaminergic dysfunction is thought to be involved. In animals, the mesoprefrontal dopaminergic system is particularly vulnerable to stress and chronic stress induces working memory impairment. Chronic stress induces working memory impairment through a D_1 receptor-mediated hypodopaminergic mechanism in the PFC (Mizoguchi *et al.*, 2000). The neurochemical studies on the dopaminergic neuronal activity in the PFC of the stressed rats revealed that the hyperdopaminergic mechanism is behind the acute stress-induced cognitive deficits (Arnsten & Goldman-Rakic, 1998; Mizoguchi *et al.*, 2000).

Effect of glucose on brain dopamine and its receptors

Dopamine has two distinct pathways that connect the striatum to the basal ganglia output nuclei - a direct pathway originating from neurons bearing DA D_1 receptors and an indirect pathway originating from neurons expressing DA D_2

receptors. Intrastriatal injection of selective DA D_1, DA D_2 or general DA agonists, in freely-moving rats reduced glucose utilization (Orzi *et al.*, 2001). Glucose modulates substantia nigra (SN) DA neuronal activity and its release by acting on an ATP-sensitive potassium channel (K_{ATP}) (Levin, 2000). Changing SN glucose levels is reported to affect activities of (K_{ATP}) channel and DA neurons. Glucose modulates the motor activity involved in food intake. In experimental rats food deprivation cause a decrease in the activity of striatal DAT (Figlewicz *et al.*, 1998). DA D_1 receptor binding significantly increased in the accumbens and DA D_2 binding decreased in the dorsal striatum as a result of excessive intake of sugar because palatable food stimulates the neural system. DA antagonists are reported to effectively modulate brain energy metabolism and release of DA thus effecting cerebral glucose utilisation (Walker *et al.*, 1999). Stimulation or blockade of DA D_3 receptors in cerebral cortex alters local glucose utilsation producing a unique pattern suggestive of potential antipsychotic activity (Levant *et al.*, 1998).

Effect of dopamine on blood glucose levels

DA and its agonists have been reported to affect the blood glucose levels. Increase in glucose level has been suggested to be due to sympathoadernal activation. Plasma glucose levels are reported to be under separate serotonergic and dopaminergic control exerted *via* 5-HT $_{1A}$ and DA D_3 receptors respectively (Hillegaart *et al.*, 1996). DA D_3 receptor agonist, 7-OH DPAT, injection caused an increase in blood glucose level and decreased plasma insulin content showing the involvement of this receptor in glucose homeostasis. Evidences show that DA D_2 receptor-mediated increase in plasma glucose is via sympathoadrenal activation (Saller & Kreamer, 1991). DA analogues like lergotrile, pergolide, bromocriptine (BRC), d-amphetamine and apomorphine when injected has reported to cause

hyperglycaemia in rats (Fischer *et al.*, 1984). In contrary obese diabetic rats treated with a combination of dopaminergic receptor agonists SKF/38393 and BRC is reported to reduce hyperglycaemia (Cincotta *et al.*, 1999).

Glutamate receptors in diabetes and hypoglycaemia

Neurodegeneration results from over activation of NMDA receptors (Rothman & Olney 1995), causing excitotoxicity proposed to be responsible for certain neurological diseases. 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. NMDA antagonists were screened against animal models of epilepsy (Avoli & Oliver, 1987), ischemia (Aitken *et al.*, 1988; Ford *et al.*, 1989; Rod and Auer, 1989), and hypoglycaemia (Wieloch, 1985).

Diabetes mellitus induces cognitive impairment and defects of long-term potentiation in the hippocampus as indicated by behavioural and electrophysiological analysis. Considered to be an important mechanism of learning and memory in mammals, long-term potentiation is known to require regulation of the glutamate receptor properties. According to many studies, defects of long-term potentiation in the hippocampus of diabetic animals are due to abnormal glutamate receptors. Earlier studies explained that changes in glutamate receptors account for modifications of long-term potentiation in various models of diabetes mellitus. 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). Previous studies demonstrated that disruption of glutamate homeostasis occurs in the diabetic retina (Qing & Donald, 2002). Binding properties of brain glutamate receptors of STZ

induced rats and the possible role of AMPA receptors in cognitive deficits during diabetes is reported by Gagne *et al.*, (1997). Altered glutamergic neurotransmission and calcium homeostasis contribute to retinal neural cell dysfunction and apoptosis in diabetic retinopathy. Elevated Glucose is reported to change the expression of ionotropic Glutamate receptor subunits and impairs calcium homeostasis in retinal neural cells (Ana *et al.*, 2006). It is suggested that enzymes of the glutamate system respond differently towards diabetes or deprivation of food and diabetes affect the glutamate uptake system in glial cells (Galanopoulos *et al.*, 1988). Recent studies suggest that glutamate plays a pivotal role in the processing of sensory information in the spinal cords of patients with diabetic neuropathy. Abnormal expression of multiple glutamate receptors is involved in the development of diabetic neuropathy (Tomiyama *et al.*, 2005).

Studies reported that neurons impaired of energy metabolism are highly sensitive to excitotoxicity (Simon *et al.*, 1984; Wieloch, 1985; Monyer *et al.*, 1989; Cebers *et al*, 1998). Pathophysiological mechanisms responsible for neuronal cell death in hypoglycaemia include the involvement of glutamate excitotoxicity. Hypoglycaemia specifically increases the sensitivity of NMDA receptors to activation by glutamate, which may result in a lower threshold for glutamate induced excitotoxicity (Jane, 1999). Severe and prolonged hypoglycaemia results in increased release of glutamate in the brain, leading to membrane depolarization. This is followed by cerebral energy failure and neuronal cell death. Glutamate neurotoxicity is thus implicated in the pathogenesis of hypoglycaemia induced neuronal death and Ca^{2+} calmodulin-dependent protein kinase II appears to be one of the intracellular targets for glutamate neurotoxicity in hypoglycaemia (Hu *et al.*, 1995). Hypoglycaemia causes several-fold elevations in brain extracellular glutamate

concentrations and pretreatment with glutamate receptor antagonists prevent hypoglycaemic neuronal death (Nellgard &Wieloch, 1992; Sandberg *et al.*, 1986; Wieloch, 1985).

MDH and GDH in diabetes and hypoglycaemia

Diabetes is characterized by chronic hyperglycaemia that produces dysregulation of cellular metabolism. The molecular and functional basis of hypoglycaemia and hyperglycaemia to a certain extend can be elucidated by studying the changes in the metabolic enzymes. There is increasing evidence that specific changes in mitochondrial function may play a significant role in the early events leading to hypoglycaemic encephalopathy. Decreased fluxes of substrate through the tricarboxylic acid cycle results in decreased availability of reducing equivalents in mitochondria. As a result, there is incomplete reduction of molecular oxygen within mitochondria and increased formation of oxygen free radicals, which damage both mitochondrial membranes and mitochondrial DNA. Fragmentation of mitochondrial DNA interferes with synthesis of electron transport chain enzymes, such as subunits of cytochrome oxidase and nicotinamide adenine dinucleotide (NADH)-dehydrogenase that are coded for by the mitochondrial genome. Thus, the ability of the cell to restore ATP levels is impaired (Jane, 1999).

MDH is an enzyme directly involved in glucose metabolism. It catalyses the interconversion of L- malate and oxaloacetate using nicotinamide adenine dinucleotide (NAD) as coenzyme. Since MDH has been shown to play a role in the regulation of cytosolic [NAD^+] / [NADH] redox state, it is possible that differences in this enzyme could cause differences in metabolic pathway. The mitochondrial enzyme

in addition to its role in the other half of the malate shuttle, it is also a necessary component of the TCA cycle. The simple Malate dehydrogenases occur in virtually all eukaryotic cells as isoenzynes identified as mitochondrial (m-MDH) and soluble or cytoplasmic (s-MDH) according to their cellular location. (Delbruk, 1959). In hypoglycaemia, gluconeogenesis is altered. Experimentally induced diabetes has shown to cause changes in the activity of metabolic enzymes altering the glucose metabolism (Chang *et al.*, 1977; Kazmi *et al.*, 1985; Tanaka *et al.*, 1988; Belfiore *et al.*, 1974).

GDH catalyzes reversible oxidative deamination of L-glutamate to α-ketoglutarate. Enzyme activity is regulated by several allosteric effectors. Transamination between this α- amino acid and α- keto acid determines the amount of this amino acid in the brain (Wurdig & Kugler, 1991). The importance of GDH in glucose homeostasis is also evident from recent findings that mutations in the GLUD1 gene, which encodes GDH, cause hyperinsulinism /hyperammonemia (HI/HA) syndrome (Smith *et al.*, 2001; Stanley *et al.*, 1998 & 2000; Tanizawa *et al.*, 2002; Weinzimer *et al.*, 1997; Zammarchi *et al.*, 1996). Insulin induced hypoglycaemic coma in animals was associated with inhibition of glycolysis and glycogenolysis and decreased activities of succinate dehydrogenase and GDH in the cerebral hemispheres and brainstem (Telushkin *et al.*, 2006).

Glucose uptake by pancreatic islets

Signal transduction in the pancreatic β-cell and thereby the insulin secretory process is regulated by a sophisticated interplay between glucose and a plethora of additional factors including other nutrients, neurotransmitters, islet generated factors

and systemic growth factors. The coupling of glucose metabolism to electrical activity remains central in all models of β-cell stimulus-secretion coupling. The resting membrane potential of the β-cell is set by the ATP-sensitive potassium (K_{ATP}) channel (Ashcroft & Rorsman, 1990). Incubation of the pancreatic β-cell with stimulatory glucose concentrations leads to the activation of a cascade of reactions, which ends in the exocytosis of stored insulin. This complex of processes starts with the uptake of glucose by the β-cell high-K_m/low affinity glucose transporter GLUT2 and proceeds with the conversion of glucose into glucose-6-phosphate by the β-cell isoform of glucokinase (Matschinsky, 1996). Metabolism of glucose in glycolysis and the Krebs cycle results in the generation of ATP. Elevation in the ATP/ADP ratio leads to closure of the K_{ATP}, which in turn results in depolarization of the plasma membrane. The subsequent opening of voltage-gated L-type Ca^{2+} channels leads to an increase in the cytoplasmic free Ca^{2+} concentration, $[Ca^{2+}]_i$, which promotes insulin secretion (Berggren & Larsson, 1994).

Glucose is transported into the β-cell cell by facilitated diffusion through a glucose transporter; elevated concentrations of glucose in extracellular fluid lead to elevated concentrations of glucose within the β-cell. Elevated concentrations of glucose within the β-cell cell ultimately leads to membrane depolarization and an influx of extracellular calcium. The resulting increase in intracellular calcium is thought to be one of the primary triggers for exocytosis of insulin-containing secretory granules. The mechanisms by which elevated glucose levels within the β-cell cell cause depolarization is not clearly established, but seems to result from metabolism of glucose and other fuel molecules within the cell, perhaps sensed as an alteration of ATP:ADP ratio and transduced into alterations in membrane conductance. An increased level of glucose within β cells also appears to activate calcium-independent pathways that participate in insulin secretion.

Factors affecting insulin regulation from pancreatic β-cells

D-Glucose is the major physiological stimulus for insulin secretion. The mechanism of glucose induced insulin release is not completely understood. Phosphorylation of glucose to glucose-6-phosphate serves as the rate limiting step in glucose oxidation (Schuit, 1996). Glucokinase acts as a glucose sensor during this process. Glucokinase is also linked to the phosphate potential, [ATP]/([ADP] [Pi]) (Sweet *et al.*, 1996). An increased ATP/ADP ratio is believed to close K^+-ATP channel at the plasma membrane, resulting in decreased K^+ efflux and subsequent depolarisation of the β-cell (Dunne, 1991). Depolarisation activates voltage-dependent Ca^{2+} channels, causing an influx of extracellular Ca^{2+} (Liu *et al.*, 1996). Although intracellular Ca^{2+} activates protein kinases such as Ca^{2+} and calmodulin dependent protein kinase (Breen & Aschcroft, 1997), it remains unclear how increase in intracellular Ca^{2+} leads to insulin release. Intracellular Ca^{2+}stores appear to regulate a novel plasma membrane current [Ca^{2+} release activated non-selective cation current], whose activity may control glucose activated secretion. Lesions in these pathways lead to the pathogenesis of diabetes mellitus (Dukes *et al.*, 1997). Glucose induced insulin secretion is also partly dependent upon the activation of typical isoforms of protein kinase C (PKC) within the β-cell (Harris *et al.*, 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 *et al.*, 1994).

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. Studies have shown that preproinsulin mRNA levels rise 4-

10 fold 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).

ROLE OF NEUROTRANSMITTERS IN INSULIN REGULATION

Epinephrine and Norepinephrine

Epinephrine and norepinephrine has an antagonistic effect on insulin secretion and glucose uptake (Renstrom *et al.*, 1996; Porte, 1967). 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 phosphofructokinase. EPI and NE at low concentrations can bind and activate β-adrenergic receptors which in turn stimulate the insulin secretion from pancreatic islets and at high concentration they can bind to α_{2A} receptors and inhibit insulin secretion. Previous studies had 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}-adrenoceptors (Filipponi *et al.*, 1986) which are linked to adenylyl cyclase inhibiting insulin secretion. β_3 adrenoreceptors stimulation also results in enhanced insulin secretion (Alef *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. In humans, adrenaline stimulates lipolysis, ketogenesis, thermogenesis and glycolysis and raises plasma glucose concentrations by stimulating both glycogenolysis and gluconeogenesis. It is already known that, when used in high doses *in vivo* or *in vitro*, EPI 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 α_2-adrenergic receptors are blocked, it enhances islet cell proliferation and insulin secretion. Our previous studies demonstrated the role of α and β-adrenergic receptors in the insulin secretion (Ani *et al.*, 2006 a, b, c). We also reported the effect of NE in DA mediated insulin secretion (Eswar *et al.*, 2006).

Acetylcholine

Acetylcholine is the neurotransmitter of the parasympathetic system. Cholinergic receptors are classified as ionotropic nicotinic receptor and metabotropic muscarinic receptor. Acetylcholine increases insulin secretion through muscarinic receptors in pancreatic islet cells (Tassava *et al.*, 1992). Muscarinic receptors are classified as M_1, M_2, M_3, M_4 and M_5. They are G protein coupled receptors. They are characterized by having seven hydrophobic transmembrane-spanning regions that interacts with G-proteins and other effector molecules to mediate the physiological and neurochemical effects. Expression studies have revealed the presence of M_1 and M_3 receptors in the pancreas. Acetylcholine is reported to be involved in the activation of glucose transport in the chromaffin cells. The cholinergic activation affecting this process is coupled with calmodulin and protein kinase C (Serck-Hanssen *et al.*, 2002). It is reported that the role of acetylcholine in insulin secretion is mediated through M_1 and M_3 receptors (Paulose, 2004; Renuka *et al.*, 2004, 2005 & 2006).

γ-Aminobutyric acid

Gamma aminobutyric acid (GABA) is the major inhibitory neurotransmitter in central nervous system. GABA is reported to be 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). The brain GABAergic mechanisms also play an important role in glucose homeostasis. Recent studies reported the regulatory role of GABA during pancreatic regeneration (Kaimal *et al.*, 2007). Also, we reported the role of GABA in hepatocyte proliferation (Biju & Paulose, 2002). 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 is co-released with insulin from β-cells in response to glucose (Reetz *et al.*, 1991). GABA inhibits islet α and δ-cell hormonal secretion in a paracrine manner. GABA release is decreased in diabetes resulting in the enhancement of glucagon secretion from α-cells leading to hyperglycaemia. GABA is involved in the maintenance of glucose homeostasis and inhibition of central $GABA_A$ receptors increasing the plasma glucose concentration (Lang, 1995). Thus, any impairment in the GABAergic mechanism in central nervous system and/or pancreatic islets is important in the pathogenesis of metabolic stress.

Serotonin

Brain serotonin content decreased during diabetes (Jackson & Paulose, 1999). This decrease is reported to be due to a decrease in uptake of tryptophan through the blood brain barrier (BBB) (Madras *et al.*, 1974) and a decrease in rate of 5-HT synthesis (Carndall *et al.*, 1981). The turnover rate of 5-HT to 5-HIAA in diabetic rats was also reported to be lower (Sandrini *et al.*, 1997; Kwok & Juorio, 1987). A decrease in brain 5-HT will lead to an up regulation of 5-HT_{2A} receptors of cerebral cortex and brain stem which in turn can inhibit insulin secretion due to increased sympathetic activity (Jackson & Paulose, 1999). Our ealrier studies reported the role of serotonin in cell proliferation (Sudha & Paulose, 1998). The functional regulation of brain 5-HT during pancreatic regeneration is also reported. (Mohan *et al.*, 2005 a, b & 2005).

Dopamine in Pancreatic glucose uptake and Insulin Secretion

Glucose uptake is the initial step in glucose-stimulated insulin secretion (GSIS) by pancreatic β-cells (Guillam *et al.*, 2000). In the pancreatic islets of Langerhans, glucose uptake by β -cells initiates a cascade of cellular events resulting in insulin secretion. A key response leading to insulin release is the change in transmembrane potential associated with the opening and closing of ion channels. Glucose uptake and metabolism increases the ratio of ATP/ADP, leading to the blockade of ATP-sensitive potassium (K^+-ATP) channels. Inhibition of these channels results in cell membrane depolarization and subsequent activation of voltage-gated Ca^{2+} (CaV) channels. Influx of extracellular Ca^{2+} causes through (CaV) channels oscillatory elevations in $[Ca^{2+}]i$, fusion of insulin-containing vesicles with the cell membrane, and insulin release (Rorsman & Renstrom, 2003). This entire process is

suppressed or terminated by the opening of voltage-gated K^+ (KV) channels (MacDonald *et al.*, 2001). The integrated process of channel gating is critical for the coordination of insulin release and thus the consequent maintenance of proper plasma glucose levels.

Insulin is involved in glucose disposal into skeletal muscles, inhibition of hepatic glucose production and inhibition of lipolysis in adipocytes. Intracerebroventricular infusion of insulin results in an increase in mRNA levels for the DA reuptake transporter (Figlewicz *et al.*, 1998). Dopamine analogues are reported to inhibit glucose-stimulated insulin release from the endocrine pancreas (Fischer *et al.*, 1984). Islets cells have been shown to contain the putative enzymes that synthesis dopamine like tyrosine hydroxylase and di-hydroxy phenylalanine decarboxylase. DA and increased glucose stimulus reduced the insulin release from the pancreatic islets with no change in calcium efflux (Nogueira *et al.*, 1994). Acute L-DOPA-induced dopamine accumulation in pancreatic islets is reported to cause an inhibitory effect on glucose-stimulated insulin response resulting in an increased MAO activity (Lundquist *et al.*, 1991, Lundquist, 1985). Our recent studies reported the role of DA in insulin secretion mediated through DA D_2 receptors (Eswar *et al.*, 2006).

DA is involved in the control of food intake, energy expenditure, glucose and lipid metabolism, blood pressure and insulin release. DA D_2 receptor neurotransmission is diminished in the brains of obese animal models and humans. DA D_2 receptor activation facilitates glucose metabolism, lowers blood pressure and stimulates resting energy expenditure in non-diabetic obese individuals. Long term treatment with DA D_2 receptor agonists improves metabolic control in obese humans with type 2 diabetes. Effects of DA on insulin secretion in general and on pancreatic

β-cell function in particular have been poorly studied. Insulin exocytosis from the β-cell is primarily controlled by metabolism-secretion coupling. First, glucose equilibrates across the plasma membrane and is phosphorylated by glucokinase, initiating glycolysis (Matschinsky, 1996). Subsequently, mitochondrial metabolism generates ATP, which promotes the closure of ATP-sensitive potassium channels and as a consequence, depolarization of the plasma membrane (Rorsman *et al.*, 1996). This leads to calcium influx through voltage-gated calcium channels and a rise in cytosolic calcium, triggering insulin exocytosis (Lang, 1999). Additional signals participating in the amplifying pathway (Henquin, 2000) are necessary to reproduce the sustained secretion elicited by glucose. Insulin secretion evoked by glucose metabolism can be further modulated by parasympathetic and sympathetic neurotransmitters (Ahren, 2000).

The role and the peripheral mechanism of action of central dopamine on basal pancreatic exocrine secretion in conscious rats revealed that central dopamine inhibited pancreatic exocrine secretion *via* DA D_1 like receptors and that the inhibitory effect is mediated *via* sympathetic nerves, especially α-adrenoceptors. Presence of dopamine is reported in peripheral tissues (Hakanson *et al.*, 1989). Dihydroxy phenyl acetic acid decarboxylase (DDC), DBH and aromatic L-amino decarboxylase (AAD) are present in endocrine cells of adult rats (Gagliardino *et al.* 1997; Yamada *et al.*, 1999; Kampe *et al.*, 1995). As dihydroxy phenyl acetic acid decarboxylase and DBH are enzymes specifically involved in catecholamine synthesis and insular cells are reported to possess the capacity to synthesise these amines. Thus, endogenously synthesised islet catecholamines have been suggested to participate in paracrine regulation of insulin secretion. Secretory granules of pancreatic β-cells have the ability to store (Ahren & Lundquist, 1985) substantial amounts of calcium, dopamine

and serotonin. L-3, 4-dihydroxyphenylalanine is rapidly converted in islet beta-cells to dopamine. Acute L-DOPA-induced dopamine accumulation in pancreatic islets is accompanied by rapid changes in MAO activity, concomitant with an inhibitory effect on glucose-stimulated insulin response (Ahren & Lundquist, 1985). It is reported that increased hydrogen peroxide production, following increased MAO activity, augment the inhibitory effect of dopamine accumulation on insulin release (Lundquist *et al.*, 1991). Dopamine is reported to suppress the somatostatin secretion predominantly through activation of dopaminergic receptors, whereas it suppresses insulin release through an alpha adrenergic mechanism and stimulates glucagon release through a β-adrenergic mechanism (Malaisse *et al.*, 1992). There has not been any detailed study on the distribution of dopamine receptor subtypes in the pancreatic islets or the pancreas except for these studies.

Dysfunction of pancreatic islets plays an important role in the etiology of diabetes as chronic hyperglycaemia impairs islet function. It has been proposed that chronic hyperglycaemia resulting from peripheral insulin resistance impair secretogogue-induced insulin release. Dopamine agonists influence central circadian neuroendocrine activities regulating metabolism to reduce insulin secretion (Lang *et al.*, 1998). Timed dopaminergic stimulation is reported to normalize the circardian rhythm of corticosterone release in obese insulin resistant animals (Lang *et al.*, 1998). It has been reported that administration of dopamine receptor agonists, bromocriptine and/or SKF38393 in diabetic rats decreased insulin resistance, increased secretion of insulin from the islet cells and normalized the daily corticosterone rhythm. Dopamine receptor agonists are suggested to improve the decreased regulatory mechanisms in the hypothalamic-neuroendocrine system during diabetes and reduce β-cell toxicity.

Hyperglycaemia causes functional deficits in the CNS aminergic neurons which are too subtle and take a longer time to manifest. Reports emphasized that treatment of gastric stasis in diabetic patients using dopamine blocker metoclopramide resulted in increased frequency and severity of dopamine associated tardive dyskinesia (Casey *et al.*, 1991). Tardive dyskinesia due to DA supersensitivity in antiphsycotic drug treated animals can be corrected by Prolyl-leucyl-glycinamide (PLG) (Chiu *et al.*, 1981). Also, diabetes caused a shift in the CNS resulting in an increased sympathetic tone that resulted in a decreased insulin secretion. Recently the presence of DA in the adrenal medulla is being stated to draw importance as it is necessary to control secretions of NE and EPI. Both dopamine (Salamone *et al.*, 2003) and insulin (Oomura& Kita, 1981; Havrankova *et al.*, 1981; Bruning *et al.*, 2000) actions in the brain modulate appetite and feeding behaviours. Interestingly, treatment with L-dopa alters insulin secretion in patients with Parkinson disease (Rosati, 1976). Moreover, antipsychotic (neuroleptic) drugs blocking dopamine receptors may cause hyperinsulinemia (Sowell *et al.*, 2002), hypoglycaemia (Budman & Gayer, 2001), increase appetite, and obesity (Pijl, 2003, Ananth *et al.*, 2004) and are associated with diabetes (Pijl, 2003; Marder *et al.*, 2004; Citrome, 2004). Dopamine action on beta cells have relevant implications for the study of obesity and diabetes, in particular in situations where dopamine transmission is altered (Blanca *et al.*, 2005). Dopamine regulates pancreatic insulin secretion in a concentration dependent manner (Eswar *et al.*, 2006). But the molecular mechanism is not well studied in detail.

Glutamate in Pancreatic Glucose uptake and Insulin secretion

Although the role of glutamate as a signaling molecule is well established in the central nervous system, a similar role in the periphery has only recently been

suggested. Weaver *et al.*, (1996) and Inagaki *et al.*, (1995) have detected functional glutamate receptors in the pancreatic islets of Langerhans. Pancreas is composed of four major cell types: the insulin-secreting β-cell, the glucagon-secreting α-cell, the pancreatic polypeptide-secreting PP cell, and the somatostatin-secreting delta cell. The electrically excitable β-cells are stimulated to secrete insulin in response to changes in serum glucose concentrations. Secretion of insulin, and the three other major peptide hormones found in islets, is also believed to be affected by other metabolic and neuronal signals (Boyd, 1992 & Ashcroft *et al.*, 1994). Bertrand *et al.*, (1992 & 1993) have shown that AMPA receptor agonists can potentiate both insulin and glucagon secretion from a perfused pancreas preparation and that oral or intravenous glutamate can increase insulin secretion and glucose tolerance *in vivo* (Bertrand *et al.*, 1995).

The precise role of a glutamergic signaling system in islet physiology or pathology is not completely understood. Glutamate also subserves communication between islets and the central nervous system. Glucose-stimulated insulin release is Ca^{2+}-dependent, perhaps because Ca^{2+} couples the process of stimulus recognition to that of insulin discharge (Douglas, 1968; Malaisse, 1973; Malaisse & Pipeleers, 1974). Although several studies have indicated that glucose alters the state of Ca^{2+} in the pancreatic islets, the nature of the changes and the mechanisms by which they occur are poorly understood (Taljedal, 1976).

Electrophysiological changes during diabetes and hypoglycaemia

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. 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. 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 degree of metabolic control had no effect on the electroencephalographic findings during the early years of diabetes, but previous severe hypoglycaemia, young age, and early onset seem to be important risk factors for electroencephalographic abnormalities (Soltesz & Acsadi, 1989).

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 as a result of hypoglycaemia. (Tupola *et al.*, 1998). As blood glucose concentrations fall below 2 mm, EEG initially shows increased amplitude and decreased frequency, followed by decreased amplitude and frequency as blood glucose concentrations approach 1 mm. Below 1 mm blood glucose concentrations, brain ATP levels become depleted (Siesjo, 1978). As hypoglycaemia progresses below 1mm, the EEG becomes isoelectric and neuronal cell death ensues (Butterworth, 1999). Hypoglycaemia only causes neuronal death when the EEG becomes flat. This usually occurs after glucose levels have fallen below 1 mM (18 mg/dl) for some period, depending on body glycogen reserves. At

the time that abrupt brain energy failure occurs, the excitatory amino acid aspartate is massively released into the limited brain extracellular space and floods the excitatory amino acid receptors located on neuronal dendrites. Calcium fluxes occur and membrane breaks in the cell lead rapidly to neuronal necrosis (Auer, 2004). 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).

Hypoglycaemic brain injury is a common and serious complication of insulin therapy and occurs most frequently in patients attempting tight glucose control (Davis *et al.*, 1998). Neuronal death resulting from hypoglycaemia is the result of a series of events triggered by reduced glucose availability, and the normalization of blood glucose levels does not necessarily block or reverse this cell death process once it has begun. Glutamate receptor activation and excitotoxicity has long been recognized as an upstream event in this cascade. Elimination of hypoglycaemia from the lives of people with diabetes and long term maintenance of euglycaemia will undoubtedly require glucose-regulated insulin replacement or secretion. Pending that ultimate goal, there is a critical need to develop therapeutic approaches that minimize both hyper- and hypoglycaemia. The only treatment for hypoglycaemia is blood glucose repletion, and there is no currently available intervention for preventing the neuronal death that develops after hypoglycaemia is corrected. Recurrent hypoglycaemia in IDDM has become even more a major focus of research and clinical interest. The brain regions most vulnerable to hypoglycaemia are important for learning and memory. Accordingly, patients who recover from severe hypoglycaemia are left with difficulties in cognition, particularly short-term memory, out of proportion to gross motor disability (Langan *et al.*, 1991). The preservation of neuron cell bodies is not

always accompanied by normal synaptic activity and function (Li *et al.*, 2003). Several lines of evidence suggest that dopamine is associated with mechanisms underlying the neurobiologic response to metabolic stress.

Studies on the functional regulation of DA through DA D_1 and DA D_2 receptors during hyperglycaemia and hypoglycaemia will lead to a better understanding of the cognitive and memory function due to neuronal damage in the brain. The present study will be carried out to elucidate hypoglycaemic and hyperglycaemic effect on brain cellular function of dopamine through DA D_1 and DA D_2 receptors and glutamate through NMDA receptors. EEG recording in hypoglycaemic and hyperglycaemic will be carried out to measure brain activity. *In vitro* studies will be done to confirm the receptor subtypes functional regulation on glucose uptake and insulin secretion.

Materials and Methods

Chemicals used and their sources

Biochemicals

Dopamine, homovanillic acid, sodium octyl sulfonic acid, ethylene diamine tetra acetic acid-EDTA, HEPES - [n' (2-hydroxy ethyl)] piperazine-n'-[2-ethanesulfonic acid], ascorbic acid, streptozotocin, pargyline, D-glucose, calcium chloride, butaclamol, (-) sulpiride, SCH 23390, (+)MK-801 [(+)5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine maleate, collagenase type XI and bovine serum albumin fraction V. (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 Sigma, India.

Radiochemicals

[^{3}H]Dopamine (Sp. activity- 45.1Ci/mmol), [^{3}H]SCH 23390 (Sp. activity 83Ci/mmol) and [^{3}H]YM-09151-2 (*cis–N-(1-benzyl-2-methylpyrrolidine-3-yl)-5-chloro-2-methoxy-4-methylaminobenzamide* Sp. activity - 85.0Ci/mmol) were purchased from NEN Life Sciences Products, Inc. Boston, USA.

[^{14}C]D-Glucose and Radioimmunoassay kit for insulin assay was purchased from Bhabha Atomic Research Centre (BARC), Mumbai, India.

Molecular Biology Chemicals

Random hexamers, Taq DNA polymerase, human placental RNAse inhibitor & dNTPS were purchased from Bangalore Genei, India. Reverse transcriptase enzyme MuMLV, was obtained from Amersham Life Science, UK. Tri-reagent kit was purchased from MRC, USA. Primers and Taqman probes for Real-Time PCR were purchased from Applied Biosystems, USA.

Animals

Adult male Wistar rats of 200-250g body weight 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 and hypoglycaemia

Animals were divided into the following groups as i) Control ii) Diabetic iii) Insulin induced hypoglycaemia in diabetic rats (diabetic+IIH) and iv) Insulin induced hypoglycaemia in control rats (control+IIH). Each group consisted of 4-6 animals.

Diabetes was induced by a single intrafemoral dose (55 mg/kg body weight) of STZ prepared in citrate buffer, pH 4.5 (Hohenegger & Rudas, 1971; Arison *et al.*, 1967). The diabetic+IIH group received daily 2 doses (10Unit/Kg body weight) of regular human insulin (Actrapid) and received daily 2 doses (1.5Unit/Kg body weight) of regular insulin (Flanagan *et al.*, 2003). Diabetic+IIH and control+IIH group had daily two episodes of insulin-induced hypoglycaemia for 10days. Control rats were injected with citrate buffer.

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 of diabetic+IIH group and control+IIH group was determined 180 and 60 min after insulin injection respectively. Food was removed from the cages after insulin (or saline) injection and returned after the glucose estimation. Blood glucose in the rats exposed to hypoglycaemia ranged from less than 40–50 mg/dL (Herlein *et al.*, 2006). 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 505nm in (Milton Roy Genesys 5 Spectronic) spectrophotometer.

Tissue preparation

Rats were sacrificed by decapitation on the 10^{th} day of the experiment. The cerebral cortex, corpus striatum, brain stem, and hippocampus were dissected out quickly over ice according to the procedure of Glowinski & Iversen, (1966). The tissues were 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 tissues from brain regions were homogenised in 0.4N perchloric acid. The homogenate was centrifuged at 5000xg for 10 minutes at 4°C (Sigma refrigerated centrifuge) and the clear supernatant was filtered through 0.45 μm HPLC grade filters and used for HPLC analysis.

Dopamine and Homovanillic acid 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 + 0.8 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.

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

Dopamine DA receptor binding studies using [^{3}H]Dopamine

Dopamine DA receptor assay was done using [^{3}H]DA as per Madras *et al.*, (1988) and Hamblin & Creese, (1982). Brain tissues were homogenised in a polytron homogeniser with 20 volumes of cold 50mM Tris-HCl buffer, along with EDTA 1mM, ascorbic acid 0.01%, $MgCl_2$ 4mM, $CaCl_2$ 1.5 mM pH.7.4 and centrifuged at 38,000xg for 30min. at 4°C. The pellet was washed twice by rehomogenization and centrifuged twice at 38,000 g for 30min. at 4°C. This was resuspended in appropriate volume of the buffer containing the above mentioned composition.

Binding assays were done using different concentrations i.e., 0.25nM-1.5nM of [^{3}H]DA in 50mM Tris-HCl buffer, along with EDTA 1mM ascorbic acid 0.01%, $MgCl_2$ 1mM, $CaCl_2$ 2 mM, NaCl 120mM, 5mM KCl pH.7.4 in a total incubation volume of 250µl containing 200-300 µg of proteins. Specific binding was determined using 100µM unlabelled dopamine. Competition studies were carried out with 0.25nM [^{3}H]DA in each tube with unlabelled ligand concentrations varying from 10^{-9} - 10^{-4}M of DA.

Tubes were incubated at 25^{0}C for 60 min. and filtered rapidly through GF/Bfilters (Whatman). The filters were washed quickly by three successive washing with 5.0 ml of ice cold 50mM Tris buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

Dopamine D_1 receptor binding studies using [^{3}H]SCH 23390

Dopamine D_1 receptor binding assay using [^{3}H]SCH 23390 in the brain regions were done according to the modified procedure of Mizoguchi *et al.*, (2000). The tissues were weighed and homogenized in 10 volumes of ice cold 50mM Tris-HCl buffer, along with 1mM EDTA, 4mM $MgCl_2$, 1.5mM $CaCl_2$, 5mM KCl pH.7.4. The homogenate was centrifuged at 40,000xg for 30 min. The pellet was washed and recentrifuged with 50 volumes of the buffer at 40,000xg for 30 min. This was suspended in appropriate volume of the buffer containing the above mentioned composition.

Binding assays were done using different concentrations i.e., 0.5 - 5.0nM of [^{3}H]SCH 23390 in 50mM Tris-HCl buffer, along with 1mM EDTA, 4mM $MgCl_2$, 1.5 mM $CaCl_2$ 5mM KCl with 12µM pargyline and 0.1% ascorbic acid in a total incubation volume of 250µl containing 150-200 µg proteins. Specific binding was determined using 50µM unlabelled SCH 23390. Competition studies were carried out with 1.0nM [^{3}H]SCH 23390 in each tube with unlabelled ligand concentrations varying from 10^{-9}- 10^{-4} M of SCH 23390.

Tubes were incubated at 25^{0}C for 60 min. and filtered rapidly through GF/B filters. The filters were washed quickly by three successive washing with 5.0 ml of ice cold 50mM Tris buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

Dopamine D_2 receptor binding studies using [^{3}H]YM-09151-2

Dopamine D_2 receptor binding assay was done according to the modified procedure of Unis *et al.*, (1998) and Madras *et al.*, (1988). The dissected brain tissues were weighed and homogenized in 10 volumes of ice cold 50mM Tris-HCl buffer, along with 1mM EDTA, 5mM $MgCl_2$, 1.5mM $CaCl_2$, 120mM NaCl, 5mM KCl pH.7.4. The homogenate was centrifuged at 40,000xg for 30 min. The pellet was washed and recentrifuged with 50 volumes of the buffer at 40,000xg for 30 min. This was suspended in appropriate volume of the buffer containing the above mentioned composition.

Binding assays were done using different concentrations i.e., 0.1 -2.0nM of [^{3}H]YM-09151-2 in 50mM Tris-HCl buffer, along with 1mM EDTA, 5mM $MgCl_2$, 1.5mM $CaCl_2$, 120mM NaCl, 5mM KCl with 10μM pargyline and 0.1% ascorbic acid in a total incubation volume of 300μl containing 200-300μg of protein. Specific binding was determined using 5.0 μM unlabelled sulpiride. Competition studies were carried out with 0.5nM [^{3}H]YM-09151-2 in each tube with unlabelled ligand concentrations varying from 10^{-9}- 10^{-4}M of sulpiride.

Tubes were incubated at 25^{0}C for 60 min. and filtered rapidly through GF/B filters (Whatman). The filters were washed quickly by three successive washing with 5.0 ml of ice cold 50mM Tris buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

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 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 DOPAMINE D_1 AND D_2 RECEPTOR IN DIFFERENT BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS.

Preparation of RNA

RNA was isolated from the different brain regions of control and xperimental rats using the Tri reagent from Sigma Aldrich.

Isolation of RNA

Tissue (25-50 mg) homogenates were made in 0.5 ml 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. RNA precipitate forms a pellet on the sides and bottom of the tube. 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. 2 µl of RNA was made up to 1 ml and absorbance were measured at 260nm and 280nm. For pure RNA preparation the ratio of absorbance at 260/280 was $\geq$ 1.7. The concentration of RNA was calculated as one absorbance $_{260}$ = 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, Fosterity, 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 a 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 Tag 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 Dopamine DA-D1 and DA-D2) gene, glutamergic- NMDAR1 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. Each run contained both negative (no template) and positive controls.

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 ($2^{-\Delta\Delta CT}$).

Glutamate dehydrogenase assay

GDH activity was measured in the crude extract of brain and liver (Gyan & Kanungo, 1970). Sample extracts were prepared by making 5% homogenate of the tissue in cold distilled after and the supernatant fluid was collected after centrifugation at 10,000xg for 20 minutes. The enzyme activity was measured in supernatant fluid as

follows. The reaction mixture in the experimental and reference cuvettes contain 0.04 M triethanolamine buffer pH 8.0, 2.6mM EDTA, 105Mm Ammonium acetate and 100µl of the enzyme sample extract of appropriate concentrations. The reaction mixture of 1ml volume was assayed at 366nm using Milton Roy Genesis spectrophotometer by adding saturating concentrations of α-ketoglutarate and 10mm NADH. Decrease in optical density (O.D) due to the oxidation of NADH was measured at 15 second intervals for two minutes at room temperature. The decrease in absorbance was linear during the course of the assays. One unit of enzyme activity is equal to the change in O.D of 0.1 in 100 seconds at 366nm. Activity of enzyme was expressed as specific activity represented by Units/mg protein. Kinetic parameters V_{max} and K_m, were calculated from the data of GDH activity measured at substrate concentrations of 0.5mM, 1Mm, 2mM and 4mM of α-ketoglutarate.

Malate dehydrogenase Assay

Malate dehydrogenase was assayed in the brain regions – brainstem and cerebral cortex according to Mehler *et al.*, (1948). Crude sample was prepared by making a 5% homogenate of the brain tissue in phosphate buffer, pH 7.4 using polytron homogenizer. The homogenate was centrifuged at 1000xg for 10 minutes. The supernatant was collected and centrifuged at 10,000xg for 20 minutes. The reaction mixture in the experimental and reference cuvette contain phosphate buffer, pH 7.4, NADH, oxaloacetate and enzyme. The reaction mixture of 1ml was assayed at 340nm in the spectrophotometer (Milton Roy Genesis spectrophotometer) by measuring the decrease in optical density due to oxidation of NADH measured at 15 seconds interval for 1 minute at room temperature. The decrease in absorbance was linear during the course of all assays. One unit of enzyme activity was equal to the change in OD of 0.1 for 100 seconds at 340nm. Activity of enzyme was expressed or

specific activity represented by units/mg of protein. Kinetic parameters - V_{max} and K_m were calculated from the data of MDH activity measured at substrate concentration 0.01- 0.07 mM.

EEG analysis

Brain activity changes in the frontal region of the control and experimental rats were studied using EEG recorder. The electroencephalograph were 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 is 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 frontal region of the scalp of experimental rats, reference electrodes were placed on the ear and ground reference on the trunk. Each electrode were placed 10-20 percent away from the neighbouring electrodes. Brain wave activity were analysed from the EEG recorded data of control and experimental rats.

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. The pancreas was aseptically transferred to a sterile glass vial containing 2.0ml 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 500 μm 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 was chosen for cell culture and other experiments.

[^{14}C]Glucose uptake studies by pancreatic islets *in vitro*

Glucose uptake was measured using D-[^{14}C]glucose by the modified procedure of Crane & Mandelstan, (1960). The islets isolated by the above mentioned method were resuspended in HEPES buffered HBSS with 4mM glucose and pre-incubated for 1hour at 37^{0}C (Howell & Taylor, 1968). The islet suspension was centrifuged at 4^{0}C at 500xg to remove inherent insulin. The pre-incubated islets were then washed thrice with cold 10mM Tris HCl buffer, pH 7.4 and finally resuspended in HBSS without glucose. The islets (100islets/tube) were incubated in the presence of three different glucose concentrations - 1, 4 and 20mM (D-[^{14}C]glucose - 10000dpm/tube). The final incubation volume was made up to 0.25ml. The tubes were incubated for 30 min, 60 min and 120 minutes at 37^{0}C in a shaking water bath.

The tubes were centrifuged after incubated for one hour at 1,500xg for 10min at 4^{0}C. The supernatant was aspirated out and pellet washed superficially with 0.2ml of HBSS twice to remove free [^{14}C]Glucose. The pellet was digested with 100μl of 1M KOH overnight and counted in a liquid scintillation counter with Cocktail-T to

measure the [^{14}C]Glucose uptake. ^{14}C glucose uptake was expressed as nmoles/mg protein.

Similarly the islets were incubated with combinations of DA, glutamate and their and antagonists to study the effect of DA, glutamate and their receptors on uptake by pancreatic islets.

***In vitro* insulin secretion study in the pancreatic islets**

Pancreatic islets were isolated by collagenase digestion method and islets were suspended in Krebs Ringer Bicarbonate buffer, pH 7.3 (KRB), 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^{0}C. The cells were then harvested and resuspended in fresh KRB (100islets/ml medium) with glucose - 1mM, 4mM and 20mM and dopamine - 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 different concentrations of dopamine, glutamate, their agonist and antagonist**

The isolated islets were incubated for 1hour at 37^{0}C with 10^{-8} M, 10^{-7} M , 10^{-6} M, 10^{-5} M, 10^{-4} M concentrations of DA, glutamate and three different concentrations of glucose i.e., (i) 1mM glucose, (ii) 4mM glucose and (iii) 20mM glucose. Cells after incubation and centrifugation at 1,500xg for 10 min at 4^{0}C, the supernatant were transferred to fresh tubes for insulin assay by radioimmunoassay.

Similarly the islets were incubated with combinations of DA, glutamate and their agonists and antagonists to study the effect of DA and its receptors on glucose induced insulin secretion using radioimmunoassay.

Radioimmuno assay of insulin

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 are 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 200 μU/ml, insulin free serum and insulin antiserum (25μl each) were added together and the volume was made up to 250μl with assay buffer. Samples of appropriate concentration from the experiments from the secretion studies were used for the assay. They were incubated overnight at 2°C. Then [^{125}I]insulin (25μl) was added and incubated at room temperature for 3hours. The second antibody was added (25μl) along with 250μl of PEG. The tubes were then vortexed and incubated for 20minutes and they were centrifuged at 1500xg for 20minutes. 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 were determined from the standard curve plotted using MultiCalc™ software (Wallac, Finland).

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 were 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

Streptozotocin induced diabetic rats showed a significant decrease ($p<0.001$) in body weight after 10 days compared to control. Insulin induced hypoglycaemia in diabetes (Diabetic+IIH) and control (control+IIH) rats showed no significant change in the body weight compared to control (Table-1).

Diabetic rats showed a significant increase ($p<0.001$) in blood glucose compared to control. There was a significant decrease ($p<0.001$) in blood glucose level, diabetic+IIH and control+IIH rats when compared to diabetic and control. Insulin administration to diabetic rats decreased blood glucose level significantly ($p<0.001$) below 50mg/dL after 3 hours and in control after 1hour. The decreased glucose level reversed to diabetic level after 5 hours and control level after 2 hours respectively (Table-1, Fig-1).

DOPAMINE AND HOMOVANILLIC ACID CONTENTS IN DIFFERENT BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

Hippocampus

DA content in the hippocampus was significantly decreased ($p<0.001$) in diabetic, diabetic+IIH and control+IIH rats compared to control. There was a significant decrease in DA content ($p<0.001$) in diabetic+IIH and control+IIH compared to diabetic. HVA, the metabolite of DA showed no significant change in the

diabetic and control+IIH groups. In diabetic+IIH, HVA content decreased significantly ($p<0.001$) compared to control and diabetic. The HVA/DA turnover rate showed a significant increase ($p<0.001$) in the hippocampus of diabetic, diabetic+IIH and control+IIH rats compared to control. Control+IIH showed a significant increase ($p<0.01$) in HVA/DA turnover rate of HVA/DA compared to diabetic and diabetic+IIH whereas diabetic+IIH showed no significant change compared to diabetic (Table-2).

Brainstem

DA content in the brainstem decreased significantly ($p<0.001$) in diabetic, diabetic+IIH and control+IIH rats when compared to control. There was a significant decrease in DA content in diabetic+IIH ($p<0.01$) and control+IIH ($p<0.001$) compared to diabetic. Control+IIH also showed a significant decrease ($p<0.01$) compared to diabetic+IIH. HVA content of diabetic, diabetic+IIH and control+IIH rats showed a significant increase ($p<0.05$) compared to control. The HVA/DA turnover rate was significantly increased in diabetic ($p<0.01$), diabetic+IIH ($p<0.01$) and control+IIH ($p<0.001$) rats when compared to control. There was a significant increase ($p<0.01$) in HVA/DA turnover rate of diabetic+IIH and control+IIH compared to diabetic. Also, there was significant increase in HVA/DA turnover rate of control+IIH ($p<0.01$) compared to diabetic+IIH (Table-3).

Cerebral cortex

DA content decreased significantly ($p<0.001$) in the cerebral cortex of diabetic, diabetic+IIH and control+IIH rats when compared to control. Diabetic + IIH and control+IIH group showed a significant decrease ($p<0.01$) in DA content compared to diabetic. Control+IIH also showed a significant decrease ($p<0.05$)

compared to diabetic+IIH. HVA content also showed a significant decrease ($p<0.05$) in all the experimental groups compared to control. The HVA/DA turnover rate showed no change in diabetic while it significantly increased ($p<0.05$) in diabetic+IIH and control+IIH when compared to control. There was a significant increase in HVA/DA turnover rate of diabetic+IIH ($p<0.05$) and control+IIH ($p<0.01$) compared to diabetic. Also, there was significant increase in HVA/DA turnover rate in control+IIH ($p<0.05$) compared to diabetic+IIH (Table-4).

Corpus striatum

There was a significant decrease in DA content of corpus striatum in diabetic ($p<0.05$), diabetic+IIH ($p<0.001$) and control+IIH rats ($p<0.001$) when compared to control. Diabetic + IIH ($p<0.05$) and control+IIH ($p<0.01$) showed a significant decrease in DA content compared to diabetic. Control+IIH also showed a significant decrease ($p<0.01$) compared to diabetic+IIH. HVA was found to be significantly increased ($p<0.001$) in all the experimental group of rats compared to control. Diabetic + IIH ($p<0.05$) and control+IIH ($p<0.01$) showed a significant increase in HVA content compared to diabetic. Control+IIH also showed a significant increase ($p<0.05$) compared to diabetic+IIH. The HVA/DA turnover rate was significantly increased in diabetic ($p<0.01$), diabetic+IIH ($p<0.001$) and control+IIH rats ($p<0.001$) when compared to control. There was a significant increase in HVA/DA turnover rate of diabetic+IIH ($p<0.05$) and control+IIH ($p<0.01$) compared to diabetic. Also, there was significant increase in HVA/DA turnover rate in control+IIH ($p<0.01$) compared to diabetic+IIH (Table-5).

BRAIN DOPAMINE RECEPTOR CHANGES IN CONTROL AND EXPERIMENTAL RATS

Hippocampus

Scatchard analysis using [^{3}H]Dopamine against dopamine

Scatchard analysis of [^{3}H]DA against DA in hippocampus of diabetic, diabetic+IIH and control+IIH groups of rats showed a significant increase ($p<0.001$) in B_{max} and K_d when compared to control. Diabetic + IIH and control+IIH showed a significant increase ($p<0.001$) in B_{max} compared to diabetic. Control+IIH also showed a significant increase ($p<0.01$) in B_{max} compared to diabetic+IIH. There was no significant change in K_d between diabetic, diabetic+IIH and control+IIH groups (Table-6, Fig-2).

Displacement analysis of [^{3}H]Dopamine against Dopamine

The competition curve for [^{3}H]DA fitted for a single- sited model in control, diabetic, diabetic+IIH and control+IIH groups with Hill slope values near unity. In all experimental groups, Log (EC_{50}) decreased and Ki increased showing a shift in affinity from higher to lower state (Table-7; Fig-3).

Real-Time PCR analysis of DA D_1 receptors

The Real-Time PCR analysis in the hippocampus showed a significant increase ($p<0.001$) in the expression of DA D_1 receptor mRNA in diabetic, diabetic+IIH and control+IIH rats when compared to control. There was an increased expression ($p<0.001$) of DA D_1 receptor mRNA in diabetic+IIH and control+IIH compared to diabetic rats. Also, the expression of DA D_1 receptors increased significantly ($p<0.05$) in control+IIH compared to diabetic+IIH (Table-8; Fig-4).

Real-Time PCR analysis of DA D_2 receptors

Real-Time PCR analysis revealed an increased expression ($p<0.001$) of DA D_2 receptor mRNA in diabetic, diabetic+IIH and control+IIH rats in the hippocampus compared to control. In diabetic+IIH and control+IIH groups, there was an increased expression ($p<0.001$) of DA D_2 receptor mRNA when compared to diabetic. Also, the expression of DA D_2 receptors increased significantly ($p<0.05$) in control+IIH compared to diabetic+IIH (Table-9; Fig-5).

Brainstem

Scatchard analysis using [^{3}H]Dopamine against dopamine

Scatchard analysis of [^{3}H]DA against DA in brainstem showed a significant increase in B_{max} and K_d of diabetic ($p<0.01$), diabetic+IIH ($p<0.001$) and control+IIH ($p<0.001$) rats when compared to control. Diabetic+IIH and control+IIH showed a significant increase in B_{max} ($p<0.01$, $p<0.001$) and K_d ($p<0.05$, $p<0.01$) respectively when compared to diabetic. Also, Control+IIH showed a significant increase in B_{max} and K_d ($p<0.05$) compared to diabetic+IIH (Table-10; Fig-6).

Displacement analysis of [^{3}H]Dopamine against Dopamine

The competition curve for [^{3}H]DA fitted for a single- sited model in control, diabetic, diabetic+IIH and control+IIH groups with Hill slope values near unity. In all experimental groups Log (EC_{50}) decreased and K_i increased with a shift in affinity from higher to lower state (Table-11; Fig-7).

Scatchard analysis using [^{3}H]SCH 23390 against SCH 23390

Scatchard analysis of [^{3}H]SCH 23390 against SCH 23390 showed a significant increase in B_{max} in diabetic ($p<0.01$), diabetic+IIH ($p<0.001$) and control+IIH ($p<0.001$) group without any significant change in K_d compared to control. There was a significant increase in B_{max} of diabetic+IIH ($p<0.01$) and control+IIH ($p<0.001$) group compared to diabetic. Also, there was a significant increase ($p<0.01$) in B_{max} of control+IIH compared to diabetic+IIH (Table-12; Fig-8).

Displacement analysis of [^{3}H]SCH 23390 against SCH 23390

The competition curve for [^{3}H]SCH 23390 fitted to a two site model in all groups. The Hill slope values were away from unity, which confirmed the two site model. In all experimental groups both Log (EC_{50}) and Ki did not show any change (Table-13; Fig-9).

Real-Time PCR analysis of DA D_1 receptors

Real-Time PCR analysis showed an increased expression of DA D_1 receptor mRNA ($p<0.001$) in the brainstem of diabetic, diabetic+IIH and control+IIH group when compared to control. There was an increased expression of DA D_1 receptor mRNA in diabetic+IIH ($p<0.05$) and control+IIH ($p<0.01$) compared to diabetic group (Table-14; Fig-10).

Scatchard analysis using [^{3}H]YM-09151-2 against sulpiride

Scatchard analysis of DA D_2 receptors in the brainstem of diabetic showed a significant decrease ($p<0.01$) in B_{max} with no change in its affinity compared to control. Diabetic+IIH showed a significant decrease in B_{max} ($p<0.001$) and K_d ($p<0.001$, $p<0.01$) compared to control and diabetic respectively. In control+IIH

group, B_{max} and K_d increased significantly ($p<0.001$) compared to control, diabetic and diabetic+IIH (Table-15;Fig-11).

Displacement analysis of [^{3}H]YM-09151-2 against sulpiride

The competition curve for [^{3}H]YM-09151-2 fitted to a single site model in control, diabetic, diabetic+IIH and control+IIH groups with Hill slope values near unity. In diabetic and diabetic+IIH, Log (EC_{50}) increased compared to control and control+IIH. Ki showed a decrease in diabetic and diabetic+IIH compared to control and control+IIH. Also, Ki of control+IIH showed an increase compared to control (Table-16; Fig-12).

Real-Time PCR analysis of DA D_2 receptors

Real-Time PCR analysis revealed a decreased expression of DA D_2 receptor mRNA in the brainstem of diabetic and diabetic+IIH groups while in control+IIH rats the expression was significantly increased ($p<0.001$) when compared to control. There was a significant decrease in the expression of DA D_2 receptor mRNA in diabetic+IIH ($p<0.01$) and a significant increase in control+IIH ($p<0.001$) compared to diabetic group. Control+IIH showed a significant increase ($p<0.001$) in DA D_2 expression compared to diabetic+IIH (Table-17; Fig-13).

Cerebral cortex

Scatchard analysis using [^{3}H]Dopamine against dopamine

Scatchard analysis of [^{3}H]DA against DA in cerebral cortex of diabetic, diabetic+IIH and control+IIH groups showed a significant increase ($p<0.001$) in B_{max} and K_d ($p<0.001$) compared to control. Significant increase in B_{max} ($p<0.001$)and K_d ($p<0.01$) of diabetic+IIH and control+IIH was observed compared to diabetic.

Control+IIH showed a significant increase ($p<0.05$) in B_{max} and K_d compared to diabetic+IIH (Table-18; Fig-14).

Displacement analysis of [^{3}H]Dopamine against Dopamine

Displacement analysis in cerebral cortex of diabetic, diabetic+IIH and control+IIH groups fitting the equation to a single-site model as seen in control. This was confirmed by the Hill slope value which was near unity in all groups. In all experimental groups, Log (EC_{50}) decreased and Ki increased showing a shift in affinity from higher to lower state (Table-19; Fig-15).

Scatchard analysis using [^{3}H]SCH 23390 against SCH 23390

Scatchard analysis of [^{3}H]SCH 23390 against SCH 23390 in cerebral cortex of diabetic, diabetic+IIH and control+IIH rats showed a significant increase in B_{max} ($p<0.001$) and K_d ($p<0.05$) compared to control. There was a significant decrease in B_{max} of diabetic+IIH ($p<0.001$) and control+IIH ($p<0.05$) compared to diabetic group with no change in K_d . Also, there was a significant increase ($p<0.01$) in B_{max} of control+IIH compared to diabetic+IIH with no significant change in K_d (Table-20; Fig-16).

Displacement analysis of [^{3}H]SCH 23390 against SCH 23390

The competition curve for [^{3}H]SCH 23390 fitted to a two site model in all groups. The Hill slope values were away from unity, which confirmed the two site model. In all experimental groups Log (EC_{50}) decreased and Ki_H and Ki_L increased compared to control (Table-21; Fig-17).

Real-Time PCR analysis of DA D_1 receptors

The Real-Time PCR analysis in the cerebral cortex showed an increased expression ($p<0.001$) of DA D_1 receptor mRNA in diabetic, diabetic+IIH and control+IIH rats compared to control. There was a significant decrease in the expression of DA D_1 receptors in diabetic+IIH ($p<0.01$) and control+IIH ($p<0.05$) compared to diabetic group. Control+IIH showed a significant increase ($p<0.01$) in DA D_1 receptor mRNA compared to diabetic+IIH (Table-22; Fig-18).

Scatchard analysis using [^{3}H]YM-09151-2 against sulpiride

Scatchard analysis of [^{3}H]YM-09151-2 against sulpiride in cerebral cortex of diabetic ($p<0.01$), diabetic+IIH ($p<0.001$) and control+IIH ($p<0.001$) groups showed a significant increase in B_{max} with no change in K_d compared to control. The B_{max} of diabetic+IIH and increased significantly ($p<0.001$) compared to diabetic with no change in K_d. In control+IIH, there was a significant increase ($p<0.01$) in B_{max} compared to diabetic+IIH (Table-23; Fig.-19).

Displacement analysis of [^{3}H]YM-09151-2 against sulpiride

The competition curve for [^{3}H]YM-09151-2 fitted for a single site model in all groups with Hill slope values near unity. In all experimental groups both Log (EC_{50}) and Ki showed no significant change (Table-24; Fig-20).

Real-Time PCR analysis of DA D_2 receptors

The Real-Time PCR analysis in the cerebral cortex showed an increased expression of DA D_2 receptor mRNA in diabetic ($p<0.01$), diabetic+IIH ($p<0.001$) and control+IIH ($p<0.001$) groups compared to control. There was an increased expression of DA D_2 receptor mRNA in diabetic+IIH ($p<0.001$) and control+IIH

($p<0.001$) compared to diabetic. Also, a significant increase in DA D_2 receptor expression was observed in control+IIH ($p<0.05$) compared to diabetic+IIH (Table-25; Fig-21).

Corpus striatum

Scatchard analysis using [^{3}H]Dopamine against dopamine

Scatchard analysis in corpus striatum of diabetic group showed significant decrease in B_{max} ($p<0.05$) and K_d ($p<0.001$) when compared to control. There was a significant increase in B_{max} of diabetic+IIH ($p<0.01$) and control+IIH ($p<0.001$) compared to control with no significant change in K_d. B_{max} and K_d increased significantly in diabetic+IIH ($p<0.001$) and control+IIH ($p<0.001$) compared to diabetic. No significant change in B_{max} and K_d was observed in control+IIH compared to diabetic+IIH (Table-26; Fig-22).

Displacement analysis of [^{3}H]Dopamine against Dopamine

The competition curve for [^{3}H]DA fitted for a single site model in control, diabetic, diabetic+IIH and control+IIH groups with Hill slope values near unity. In diabetic group Log (EC_{50}) decreased and Ki increased compared to control, diabetic+IIH and control+IIH groups. Log (EC_{50}) and Ki showed no change in diabetic+IIH and control+IIH groups compared to control (Table-27; Fig-23).

Scatchard analysis using [^{3}H]SCH 23390 against SCH 23390

Scatchard analysis of DA D_1 receptors using [^{3}H]SCH 23390 in the corpus striatum of diabetic group showed a significant decrease ($p<0.01$) in B_{max} and K_d compared to control. In diabetic+IIH and control+IIH, there was a significant increase in B_{max} ($p<0.001$) and K_d ($p<0.01$) when compared to control and diabetic.

Control+IIH showed a significant increase in B_{max} ($p<0.05$) compared to diabetic+IIH with no significant change in K_d (Table-28, Fig-24).

Displacement analysis of [^{3}H]SCH 23390 against SCH 23390

The competition curve for [^{3}H]SCH 23390 in corpus striatum fitted to a two site model in all groups. The Hill slope values were away from unity, which confirmed the two site model. Ki_H and Ki_L in diabetic decreased whereas it showed an increase in diabetic+IIH and control+IIH groups compared to control. There was no significant change in Log (EC_{50}) in all groups (Table-29; Fig-25).

Real-Time PCR analysis of DA D_1 receptors

Real-Time PCR analysis of DA D_1 receptor mRNA expression showed a decrease ($p<0.001$) in the corpus striatum during diabetes whereas the expression increased significantly ($p<0.001$) in diabetic+IIH and control+IIH compared to control. There was a significant increase ($p<0.001$) in DA D_1 receptor mRNA expression in diabetic+IIH and control+IIH compared to diabetic. There was no significant change in DA D_1 receptor mRNA expression between diabetic+IIH and control+IIH (Table-30; Fig-26).

Scatchard analysis using [^{3}H]YM-09151-2 against sulpiride

In diabetic rats, B_{max} and K_d increased significantly ($p<0.01$) when compared to control. B_{max} ($p<0.001$) and K_d ($p<0.01$) of diabetic+IIH and control+IIH significantly decreased when compared to control. B_{max} and K_d ($p<0.001$) of diabetic+IIH and control+IIH significantly decreased when compared to diabetic. There was no significant difference in B_{max} and K_d of control+IIH and diabetic+IIH (Table-31; Fig-27).

Displacement analysis of [^{3}H]YM-09151-2 against sulpiride

All the experimental groups fitted to one site model. This was confirmed by the Hill slope value that was unity in all the groups. The Ki increased and decreased Log (EC_{50}) in diabetic group compared to control. Diabetic+IIH and control+IIH groups showed significant decrease in Ki with increased Log (EC_{50}) compared to control and diabetic (Table-32; Fig.-28).

Real-Time PCR analysis of DA D_2 receptors

Real-Time PCR analysis showed that DA D_2 receptor mRNA expression increased significantly ($p<0.001$) in the corpus striatum during diabetes whereas the expression decreased significantly ($p<0.001$) in diabetic+IIH and control+IIH group compared to control. There was a significant decrease in DA D_2 receptor expression in diabetic+IIH ($p<0.001$) and control+IIH ($p<0.001$) compared to diabetic. Control+IIH showed a significant decrease ($p<0.01$) in DA D_2 receptor expression compared to diabetic+IIH (Table-33; Fig.-29).

GLUTAMATE DEHYDROGENASE ACTIVITY IN THE BRAINSTEM AND CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL RATS

Brainstem

Glutamate dehydrogenase activity in the brainstem showed a significant increase ($p<0.001$) in the diabetic and diabetic+IIH groups whereas the enzyme activity decreased significantly ($p<0.001$) in control+IIH groups compared to control. In diabetic+IIH group the V_{max} increased significantly ($p<0.01$) when compared to diabetic. V_{max} of control+IIH decreased significantly compared to diabetic ($p<0.001$) and diabetic+IIH ($p<0.001$)

groups. K_m showed no significant change in all the experimental groups when compared to control (Table-34; Fig-30).

Cerebral cortex

Glutamate dehydrogenase activity in the cerebral cortex showed a significant increase ($p<0.001$) in the diabetic and diabetic+IIH groups whereas the enzyme activity decreased significantly ($p<0.001$) in control+IIH groups compared to control. In diabetic+IIH group, V_{max} increased significantly ($p<0.05$) when compared to diabetic. V_{max} of control+IIH decreased significantly compared to diabetic ($p<0.001$) and diabetic+IIH ($p<0.001$) groups. K_m showed no significant change in all the experimental groups when compared to control (Table-35; Fig-31).

MALATE DEHYDROGENASE ACTIVITY IN THE BRAINSTEM AND CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL RATS.

Brainstem

Malate dehydrogenase activity in the brainstem showed a significant decrease ($p<0.001$) in diabetic, diabetic+IIH and control+IIH groups without any significant change in K_m when compared to control. V_{max} decreased significantly in diabetic+IIH ($p<0.01$) and control+IIH ($p<0.001$) groups compared to diabetic (Table-36; Fig-32).

Cerebral cortex

Malate dehydrogenase activity decreased significantly in the cerebral cortex of diabetic ($p<0.01$), diabetic+IIH ($p<0.001$) and control+IIH ($p<0.001$) groups without any significant change in K_m when compared to control. V_{max} decreased significantly in diabetic+IIH ($p<0.05$) and control+IIH ($p<0.001$) groups compared to

diabetic. In control+IIH group, V_{max} decreased significantly compared to diabetic+IIH ($p<0.001$) group (Table-37; Fig-33).

GENE EXPRESSION STUDIES OF GLUTAMATE RECEPTOR (NMDAR1) IN DIFFERENT BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

Hippocampus

Real-Time PCR analysis in the hippocampus showed an increased expression ($p<0.001$) of NMDAR1 subunit of glutamate receptor mRNA in diabetic, diabetic+IIH and control+IIH groups compared to control. In diabetic+IIH ($p<0.01$) and control+IIH ($p<0.001$) group, there was an increased expression of NMDAR1 receptor mRNA compared to diabetic. NMDAR1 receptor expression increased significantly ($p<0.05$) in contol+IIH compared to diabetic+IIH (Table-38; Fig-34).

Brainstem

Real-Time PCR analysis in the brainstem showed an increased expression ($p<0.001$) of NMDAR1 receptor mRNA in diabetic, diabetic+IIH and control+IIH groups compared to control. In diabetic+IIH and control+IIH group, there was an increased expression ($p<0.001$) of NMDAR1 receptor mRNA compared to diabetic. NMDAR1 receptor expression increased significantly ($p<0.05$) in control+IIH compared to diabetic+IIH (Table-39; Fig-35).

Cerebral cortex

Real-Time PCR analysis in the cerebral cortex showed that the NMDAR1 receptor mRNA decreased significantly ($p<0.001$) in diabetic group when compared to control. In diabetic+IIH and control+IIH groups, there was an increased ($p<0.01$) expression of NMDAR1 receptor mRNA when compared to control. In diabetic+IIH and control+IIH group, NMDAR1 receptor expression increased significantly ($p<0.001$) compared to diabetic. NMDAR1 receptor expression increased significantly ($p<0.05$) in control+IIH compared to diabetic+IIH (Table-40; Fig-36).

Corpus striatum

Real-Time PCR analysis in the corpus striatum showed that NMDAR1 receptor mRNA decreased significantly ($p<0.001$) in diabetic group when compared to control. In diabetic+IIH ($p<0.01$) and control+IIH ($p<0.01$) groups, there was an increased expression of NMDAR1 receptor mRNA when compared to control. In diabetic+IIH and control+IIH group, NMDAR1 receptor expression increased significantly ($p<0.001$) compared to diabetic (Table-41; Fig-37).

EEG analysis of brain activity in the frontal region of control and experimental rats

EEG recording analysis in the frontal region of the brain of 10 days diabetic rats showed a significant difference in the wave pattern compared to control. In diabetic+IIH (3hours) and control+IIH (1hour) after insulin administration EEG showed a significant change brain wave activity difference compared to control and diabetic (Fig-38).

GLUCOSE UPTAKE STUDIES IN PANCREATIC ISLETS

Glucose uptake by pancreatic islets was studied *in vitro* using [^{14}C]Glucose. Pancreatic islets were incubated for different time periods - 30, 60 and 120min in the presence of three different glucose concentrations - 1, 4 and 20mM. 1mM glucose in the incubation medium represents hypoglycaemic, 4mM physiological concentration and 20mM glucose hyperglycaemic respectively. The effect of incubation time on glucose uptake by pancreatic islets was determined. There was a significant decrease in ^{14}C glucose uptake in 1mM when compared to 4mM after 30 minutes ($p<0.001$), 60min ($p<0.001$) and 120 min incubation ($p<0.001$). In 20 mM glucose, we observed a significant increase ($p<0.001$) in glucose uptake by pancreatic islets when compared to 4mM glucose concentration in all the time periods (Fig-39). The maximum glucose uptake was observed at 60 minutes in all three glucose concentrations which were chosen for all *in vitro* glucose uptake studies.

Effect of dopamine on glucose uptake *in vitro*

There was a significant decrease ($p<0.001$) of glucose uptake in control and 10^{-8} to 10^{-4}M DA in hypoglycaemic condition, 1mM glucose compared to physiological concentration (4mM glucose). There was a significant inhibition ($p<0.01$) of glucose uptake in hypoglycaemic condition at 10^{-8} M DA compared to control. We observed a significant progressive glucose uptake with increased concentrations of DA (10^{-7} to 10^{-4}M) with maximum uptake ($p<0.01$) at 10^{-4}M DA in hypoglycaemic condition (Fig-40).

In hyperglycaemic condition, 20mM glucose, a significant increase ($p<0.001$) of glucose uptake in control and 10^{-8} to 10^{-4}M was observed compared to physiological concentration. There was a significant inhibition ($p<0.001$) of glucose uptake in hyperglycaemic condition at 10^{-8} M DA compared to control. We observed a

significant progressive glucose uptake with increased concentrations of DA (10^{-7} to 10^{-4}M) with maximum uptake ($p<0.001$) at 10^{-4}M DA in hyperglycaemic condition (Fig-41).

Effect of dopamine antagonists on glucose uptake *in vitro*

Dopamine action on [^{14}C]glucose uptake through its receptors showed that butaclamol, a general antagonist of dopamine, at 10^{-4} M concentration blocked the inhibitory effect of 10^{-8} M DA concentration and stimulatory effect of 10^{-4} M DA concentration in hypoglycaemic, physiological and hyperglycaemic condition ($p<0.001$, Fig-42,43).

In hypoglycaemic condition, DA D_1 receptor antagonist SCH 23390 at 10^{-4} M concentration significantly blocked the inhibitory effect ($p<0.001$) of 10^{-8} M DA and stimulatory effect ($p<0.001$) of 10^{-4} M DA on [^{14}C]glucose uptake in hypoglycaemic and hyperglycaemic condition (Fig-42, 43).

Sulpiride, DA D_2 receptor antagonist, at 10^{-4} M concentration significantly blocked the inhibitory effect ($p<0.001$) of 10^{-8} M DA on [^{14}C]glucose uptake and stimulatory effect ($p<0.001$) of 10^{-4} M DA in hypoglycaemic condition and in hyperglycaemic condition. The inhibitory and stimulatory effect of DA, blocked by sulpiride at 10^{-4} M concentration was significantly higher ($p<0.001$), on glucose uptake compared to 10^{-4} M SCH 23390 in both hypoglycaemic and hyperglycaemic condition (Fig-42, 43).

Effect of glutamate and its antagonist on glucose uptake *in vitro*

There was a significant decrease ($p<0.001$) of glucose uptake in control and 10^{-8} to 10^{-4}M Glu in hypoglycaemic condition compared to physiological concentration. There was a significant decrease ($p<0.01$) of glucose uptake in hypoglycaemic condition at 10^{-8} M Glu compared to control. We observed a significant progressive glucose uptake with increased concentrations of Glu (10^{-7} to 10^{-4}M) with maximum uptake ($p<0.001$) at 10^{-4}M Glu in hypoglycaemic condition (Fig-44).

In 20mM glucose concentration, we observed a significant decrease ($p<0.001$) of glucose uptake at 10^{-8} M Glu compared to control. There was concentration dependant (10^{-4} to 10^{-7} M) significant decrease in glucose uptake ($p<0.001$) compared to hyperglycaemic control. A significant increase ($p<0.001$) of glucose uptake in control and 10^{-8} to 10^{-4}M in hyperglycaemic condition was observed compared to physiological concentration (Fig-45).

MK-801, antagonist of NMDA receptor subtype of glutamate, at 10^{-4} M concentration blocked the inhibitory effect ($p<0.001$) of 10^{-8}M Glu and stimulatory effect ($p<0.001$) of 10^{-4}M Glu on glucose uptake in both hypoglycaemic and hyperglycaemic condition (Fig-46, 47).

INSULIN SECRETION STUDIES IN PANCREATIC ISLETS

Effect of dopamine on glucose induced insulin secretion *in vitro*

There was a significant reduction of glucose induced insulin secretion in control ($p<0.001$), 10^{-8} ($p<0.001$) and 10^{-7} to 10^{-4}M ($p<0.05$) in hypoglycaemic condition compared to physiological concentration. Dopamine showed a dose

dependent decrease on insulin secretion in hypoglycaemic condition, 10^{-8} to 10^{-6}M ($p<0.05$), 10^{-5}M ($p<0.01$) with maximum ($p<0.001$) at 10^{-4} M DA compared to control (Fig-48).

In hyperglycaemic condition (20mM glucose), we observed a significant stimulation ($p<0.001$) of insulin secretion at 10^{-8} M DA compared to control. There was a significant progressive inhibition ($p<0.001$) of insulin secretion with increased concentration of DA (10^{-7} to 10^{-4}M) with maximum at 10^{-4}M DA compared to hyperglycaemic control. In hyperglycaemic condition, a significant decrease ($p<0.01$) of insulin secretion in 10^{-7} to 10^{-4}M DA was observed compared to physiological concentration (Fig-49).

Effect of dopamine antagonists on glucose induced insulin secretion

Dopamine action on glucose induced insulin secretion through its receptors, showed that butaclamol, a general antagonist of dopamine, at 10^{-4} M concentration significantly blocked the inhibitory effect ($p<0.001$) of 10^{-4} M DA in hypoglycaemic condition. SCH 23390, a potent dopamine D_1 receptor antagonist, at 10^{-4} M concentration significantly ($p<0.001$) blocked the inhibitory effect of 10^{-4} M DA in hypoglycaemic condition. Sulpiride, a potent dopamine D_2 receptor antagonist, at 10^{-4} M concentration significantly ($p<0.001$) blocked the inhibitory effect of 10^{-4} M of DA in hypoglycaemic condition. The inhibitory of effect of DA, blocked by sulpiride at 10^{-4} M concentration was significantly higher ($p<0.001$), on insulin secretion compared to 10^{-4} M SCH 23390 in hypoglycaemic condition (Fig-50).

In hyperglycaemic condition, 10^{-4} M butaclamol significantly blocked the stimulatory effect ($p<0.001$) of 10^{-8} M DA and inhibitory effect ($p<0.001$) of 10^{-4} M

DA. SCH 23390 and sulpiride at 10^{-4} M concentration significantly ($p<0.001$) blocked the stimulatory effect of 10^{-8} M DA. Also the inhibitory effect of 10^{-4} M DA was blocked significantly by 10^{-4} M SCH 23390 ($p<0.05$) and sulpiride ($p<0.001$). The blocking effect of SCH 23390 at 10^{-8} M DA is significantly higher ($p<0.001$) compared to the effect of 10^{-4} M sulpiride. The inhibitory effect of DA, blocked by sulpiride at 10^{-4} M concentration was significantly higher ($p<0.001$), on insulin secretion compared to 10^{-4} M SCH 23390 in hyperglycaemic condition (Fig-51).

Effect of glutamate and its antagonist on glucose induced insulin secretion *in vitro*

Glutamate showed a dose dependent decrease on glucose induced insulin secretion in hypoglycaemic condition. We observed a significant inhibition ($p<0.001$) of insulin secretion at 10^{-8} M Glu compared to control. There was significant progressive decrease ($p<0.001$) of insulin secretion with decreased concentrations of Glu (10^{-4} to 10^{-8}M). There was a significant reduction ($p<0.001$) of insulin secretion in control and 10^{-8} to 10^{-4}M in hypoglycaemic condition compared to physiological concentration (Fig-52).

In hyperglycaemic condition, we observed a dose dependent decrease on glucose induced insulin secretion. 10^{-4} M Glu showed maximum inhibition ($p<0.001$) of glucose induced insulin secretion compared to control. There was significant progressive inhibition ($p<0.05$) of insulin secretion with increased concentrations of Glu (10^{-8} to 10^{-4}M). We observed a significant increase ($p<0.05$) of insulin secretion in control and 10^{-8} to 10^{-4}M Glu in hyperglycaemic condition compared to physiological concentration (Fig-53).

MK-801, NMDA antagonist at 10^{-4} M concentration significantly blocked the inhibitory effect ($p<0.001$) of 10^{-8} M Glu in hypoglycaemic condition and the inhibitory effect of 10^{-4} M Glu ($p<0.001$) in hyperglycaemic condition (Fig-54,55).

Discussion

Hypoglycaemia constitutes a unique metabolic brain insult. Hypoglycaemic brain injury is a common and serious complication of insulin therapy and occurs most frequently in patients attempting tight glucose control (Davis *et al.*, 1998). Several experimental models have been described which provide information on the etiology of IDDM. 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). 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). 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. 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). During diabetes there is decrease in body weight as a result of altered metabolic function.

Administration of 1.5 U/Kg of regular insulin produced a fall in glucose level below 50mg/dL after 1hour in control+IIH rats. The minimum required dose to produce irreversible severe hypoglycaemia was 0.5 units/kg (Abdul-Ghani *et al.*, 1989). In diabetic+IIH rats, administration of 10U/Kg of insulin decreased the blood glucose level below 50mg/dL after 3hours. It is well recognized that the glucose level is the primary determinant of the hormonal and metabolic counter regulatory

responses to insulin induced hypoglycaemia. A single episode of very mild hypoglycaemia (56 mg/dL) causes a reduction of neuroendocrine counter regulation that is readily discernible about 24 h later. A similar effect of a single hypoglycaemic episode has been shown in healthy (Hvidberg *et al.*, 1996) and diabetic (Dagogo *et al.*, 1993) humans. The glycaemic levels during antecedent hypoglycaemia in those studies were (46–50 mg/dL). The plasma glucose level during antecedent hypoglycaemia has been shown to be a major determinant of the effects on subsequent counter regulation (Davis *et al.*, 1997). Heller and Cryer, (1991) reported a reduced counter regulatory response 18 h after one mild hypoglycaemic episode (plasma glucose, 54 mg/dL). Recurrent episodes of hypoglycaemia have been demonstrated to reduce subsequent endocrine counter regulation (Veneman *et al.*, 1993; Heller & Cryer, 1991; Davis *et al.*, 1992; Widom & Simonson, 1992; Davis *et al.*, 1997; George *et al.*, 1995; Davis & Shamoon, 1991). The prolonged effects of even mild hypoglycaemia on subsequent counter regulation underline the importance of scrupulously avoiding even mild hypoglycaemic episodes in patients with diabetes. The body weights of diabetic+IIH and control+IIH rats showed no significant change compared to control.

CENTRAL NERVOUS SYSTEM ALTERATIONS OF DOPAMINE AND HOMOVANILLIC ACID DURING HYPOGLYCAEMIA AND HYPERGLYCAEMIA

Diabetes mellitus is a metabolic disorder that not only causes a decrease in efficiency of the pancreatic β-cells to secrete insulin but also is accompanied by altered monoamine levels and their turnover rates in the CNS (Bhattacharya & Saraswathi, 1991; Garris, 1990; Lackovic *et al*, 1990). It is characterized by

hyperphagia, polydypsia and activation of the hypothalamic pituitary axis (HPA) producing a marked increase in food and water intake. Hyperglycaemia is reported to be a major factor that damages the CNS monoaminergic activity as a result of neuronal degeneration in different regions of the brain. Onset of diabetes has been reported to inhibit the firing of dopaminergic neurons (Saller, 1984) with alteration in its metabolism. The magnitude and duration of DA signalling during diabetes is reported to be altered as a result of decreased activity of DA transporter (DAT) causing a low clearance of DA (Galli *et al.*, 2002; Figlewicz *et al.*, 1996; 2003). Hyperglycaemia as a result of destruction in the pancreatic islets during diabetes is suggested to have an important role in the impairment of DA and other neurotransmitter functions.

Large alterations in the brain monoamine content of diabetic rats were reported (Baudrie & Chouloff, 1992). Our previous studies reported increased monoamine content in the plasma and platelet of diabetic patients (Jackson *et al.*, 1997). We reported increased NE in the brainstem of young diabetic rats while the NE content decreased in old diabetic rats (Abraham & Paulose, 1999). EPI content increased in adult diabetic rats without any change in NE. NE to EPI turn over showed a significant increase during diabetes (Pius & Paulose, 1999). Diabetes associated CNS abnormality was characterized by progressive alterations of neurotransmitters and of transduction G_i/G_o proteins (Seeman, 1980). There is a significant reduction in the cortical and brainstem 5-HT content of diabetic rats (Jackson & Paulose, 2000). Alterations of central neurotransmission and environmental factors 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). Neurotransmitter alterations in the brain of insulin induced hypoglycaemic rats are poorly studied.

Hippocampus

The effect of hypoglycaemic episodes is visible in brain regions associated with memory, especially the hippocampus. Earlier studies reported higher levels of DA in the ventral hippocampus (Hortnagl *et al.*, 1991) and the role of hippocampal DA in synapse specific enhancement of LTP (Jay, 2003). Our results showed a decreased DA content in the hippocampus of diabetic, diabetic+IIH and control+IIH rats compared to control. Recent studies suggest that children with type I diabetes who experience hypoglycaemia exhibit impairment of hippocampal-dependent memory (Hershey *et al.*, 1999 & 2003). HVA, the metabolite of DA, decreased significantly in hypoglycaemia induced diabetic rats. Nitric oxide (NO) modulates the levels of various neurotransmitters in the CNS. NO exerts a negative control over the levels of HVA and DA in the hippocampus (Wegener *et al.*, 2000). HVA is implicated as an important marker for DA metabolism in the central and peripheral nervous system (Eisenhofer *et al.*, 1992; Sternberg *et al.*, 1983). Dopamine metabolites DOPAC and HVA accumulation was decreased in STZ treated diabetic rats (Trulson & Himmel, 1983). Yamato *et al.*, (2004) and Kino *et al.*, (2004) reported that diabetes mellitus decreases the DA release from the hippocampus in both experimentally and spontaneously diabetic rats. The turnover rate of DA to HVA showed a significant increase in the hippocampus of diabetic, diabetic+IIH and control+IIH rats which indicates an increase in the metabolism of DA. This decreased DA in the hippocampus with increased DA metabolism account for the impairment in hippocampal synaptic plasticity due to diabetes and insulin induced hypoglycaemia.

Brainstem

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 and a dopaminergic input from paraventricular nucleus of hypothalamus (Lowcy *et al.*, 1994). Lesions in these brain regions are reported to affect the pancreatic islet cell population and growth. DA content in the brainstem decreased significantly in diabetic, diabetic+IIH and control+IIH rats with a significant increase in the HVA. Our previous studies also reported a decreased DA content in the brainstem of 14 day diabetic rats (Eswar *et al.*, 2007). The turnover ratio from DA to HVA also increased significantly in diabetic, diabetic+IIH and control+IIH rats, which show an increased DA metabolism in the brainstem of diabetic and insulin induced diabetic rats.

Cerebral cortex

DA plays important role in the function of primate PFC (Sawaguchi & Goldman-Rakic, 1991, 1994; Goldman-Rakic, 1995; William & Goldman-Rakic, 1995) and the rodent medial frontal cortex (Glowinski *et al.*, 1984), which is believed to be the functional counterpart of primate dorsal/lateral prefrontal cortex (particularly the prelimbic area of the medial frontal cortex) (Floresco *et al.*, 1997; Justin *et al.*, 1997; Birrell & Brown, 2000). Extracellular DA originates from DA and NE neurons in the PFC. Recent reports suggested that extracellular DA release in the cortex depend on NE rather than DA innervation. In the cortex DA acts not only as NE precursor but also as co-transmitter (Gessa *et al.*, 2001). The co-release of NE and DA seems to be controlled by α_2 adrenergic receptors located on NE nerve terminals.

In the cerebral cortex of diabetic rats, DA and HVA content decreased significantly without a significant change in the turn over ratio. Cortical DA metabolism is reported to decrease because increased glucose during diabetes (Kwok & Juorio, 1987) affects the dopaminergic activites such as working memory and stress response (Tam & Roth, 1997). D-glucose is reported to suppress the dopaminergic transmission and firing in the brain, lowering the DA metabolism decreasing the metabolite content. The enzymes involved in the synthesis and metabolism of DA are reported to be decreased during diabetes (Trulson & Himmel, 1983). During diabetes a lack of tyrosine affect markedly the physiology and functions of these DA neurons. The overall deficit in the availability of the precursor amino acid tyrosine which has been previously reported has an influence in the functioning of DA neurons. The plasma concentration of the DA is used as an indicator of central nervous system dopaminergic activity (Lambert *et al.*, 1991). Previous studies from our lab reported a decrease in plasma DA concentration which indicates that diabetes causes an alteration in the overall dopaminergic function and activity (Eswar *et al.*, 2007).

Glucose deprivation provides a method to measure the effects of metabolic stress on neurophysiology, including DA release. In preclinical studies, hypoglycaemia was associated with varied neurophysiological effects, including increased cerebral blood flow (Bryan *et al.*, 1994) and increased plasma HVA (Woolf *et al.*, 1983). Cortical DA and HVA content of the diabetic+IIH and control+IIH rats decreased with increased turnover rate of HVA from DA. This shows an increase in the metabolism of DA due to insulin induced hypoglycaemia. Various studies have reported that stress reduces DA transmission in the PFC (Mizoguchi *et al.*, 2000). Chronic exposure to cold stress exhibits a large increase in the PFC DA metabolism after subsequent exposure to acute stress (Gresch *et al.*, 1994). Thus decreased DA

with an increased turnover during hypoglycaemia in the cerebral cortex is associated with metabolic disturbance and behavioural changes.

Corpus striatum

In the corpus striatum there was a significant decrease in the dopamine content during diabetes. There was also a corresponding increase in the HVA content during diabetes. The turn over ratio of DA to HVA also increased in the striatum of diabetic rats. Brain tyrosine concentration is decreased in diabetes (Crandall & Fernstrom, 1983). Tyrosine is the precursor amino acid for the synthesis of dopamine. In diabetic condition the decrease in endogenous tyrosine concentration is reported to cause a reduction in the affinity of the enzyme tyrosine hydroxylase. The equilibrium in the tyrosine and tyrosine hydroxylase levels are reported to be much lower in diabetic rats. Diabetes is reported to cause a decrease in the accumulation of L-DOPA due to the inhibition of DOPA decarboxylase activity (Trulson & Himmel, 1983). The tyrosine hydroxylase enzyme mRNA expression in dopaminergic cells decreased during diabetes in the ventral tegmental area/substantia nigra compacta (VTA/SNc). There was a significant decrease in striatal DA content during diabetes which resulted in the notable reduction of HVA. Saller, (1984) studied the changes in the HVA content at various time periods in the striatum and found that four days after the alloxan administration there was an elevation of HVA content which decreased 21 days later and declined consistently after 42 days. Our results also showed an increase in the level of striatal HVA in 10 day diabetic rats which is suggested to be due to an increase in the metabolism of DA.

Our results showed a significant decrease in the striatal DA content of diabetic+IIH and control+IIH rats with corresponding increase in the HVA content. It

is reported that striatal DA decreased 75% during hypoglycaemia but returned to control values during recovery, while striatal HVA were increased more than two fold during both hypoglycaemia and recovery (Melvyn *et al.*, 1990). There is a significant increase in the turnover of DA to HVA which shows increased DA metabolism in the striatum of insulin induced hypoglycaemic rats.

BRAIN DOPAMINE RECEPTOR ALTERATIONS IN HYPOGLYCAEMIC AND HYPERGLYCAEMIC BRAIN

Hippocampus

The hippocampal formation receives a DA input from different midbrain groups and a more prominent DA input into the temporal pole of hippocampus (Hortnagl *et al.*, 1991). The hippocampus has long been known to be important for memory function. It is reported that profound hypoglycaemia selectively damages CA1 and the dentate gyrus of the hippocampus (Tasker *et al.*, 1992). The hippocampus is particularly sensitive to damage from hypoglycaemia (Auer & Siesjo, 1988; Fujioka *et al.*, 1997). The selectively greater reduction in hippocampal CBF indicate more severe impairment in glucose metabolism at more moderate levels of hypoglycaemia in these structures as compared with the remainder of the brain (Denise *et al.*, 2004). We observed a significant upregulation of DA receptors in the hippocampus of diabetic, diabetic +IIH and control+ IIH rats with decrease in affinity. Diabetic +IIH rats showed significantly increased DA receptors than diabetic rats. Our data suggest that the impairment in glucose metabolism causes upregulation of hippocampal dopamine receptors.

The characterizations of neuronal populations expressing DA receptor subtypes in the hippocampus have shown a prominent labeling of D_1 receptors in dentate gyrus and subicular complex (Fremeau *et al.*, 1991). Yokoyama (1995) demonstrated widespread distribution of D_2 like receptor in the hippocampus. DA D_2 receptors in the ventral hippocampus were shown to have important influences on spatial working memory (Wilkerson & Levin, 1999). DA D_2 receptor plays a role in hippocampal memory function (Hiroshige *et al.*, 2005). An intact mesocortical dopaminergic input to the PFC has been reported to be necessary for long-term potentiation to occur at hippocampal-prefrontal cortex synapses. Earlier studies suggest that DA D_1 but not DA D_2 receptors are crucial for the DA control of the NMDA receptor-mediated synaptic response on a specific excitatory input to the PFC. The interactions of these receptors play a crucial role in the storage and transfer of hippocampal information in the PFC. Real-Time PCR analysis showed an increased expression of DA D_1 and DA D_2 receptors in the hippocampus of diabetic, diabetic+IIH and control+IIH compared to control. There was a significant increase in the expression of DA D_1 and D_2 receptors in the hippocampus of diabetic+IIH rats when compared to diabetic rats.

Dopamine D_1 and D_2 receptors are generally considered to exert opposite effects at the cellular level, but many behavioural studies find an apparent cooperative effect of D_1 and D_2 receptors in the nucleus accumbens. Opposing influences of D_1 and D_2 receptor activation on cAMP-dependent signaling have been reported in many studies (Kebabian & Calne, 1979; Missale *et al.*, 1998), with D_1 receptors acting through the stimulatory G_s-like G_{olf}, and D_2 receptors acting through the inhibitory $G_{i/o}$ proteins. Hopf *et al.*, (2003) reported that cooperative action of D_1 and D_2 receptors in the brain could mediate DA-dependent behaviours. Recent studies explains that

stimulation of D_1 and D_2 DA receptors has the potential to give rise to different intracellular signals depending on whether D_1 or D_2 receptors are activated alone or together (Pollack, 2004). Thus our results suggest that the co activation of DA D_1 and DA D_2 receptors with DA depletion have particular relevance in the impairment of glucose metabolism and DA related functions. Also, co-activation of D_1 and D_2 receptors is reported to enhance glutamate mediated cellular excitation (Hopf *et al.*, 2003). The hippocampal cell populations in particular are important for learning and memory and impairment of cognitive abilities is the most common sequelae of insulin induced hypoglycaemic neuronal damage. Recurrent hypoglycaemia markedly affects hippocampally dependent spatial working memory task (McNay *et al.*, 2006). The increase in DA receptor sensitivity is a compensatory response to diminished firing of DA.

Brainstem

Brainstem is an important part of the brain in monitoring the glucose status and the regulation of feeding (Guillod *et al.*, 2003). When glucose levels were lowered to 2.8 mmol/l, however, brain function was impaired in nondiabetic rats as well. Recent studies suggest that moderate hypoglycaemia impairs brainstem function in normal humans and rats (McNay *et al.*, 2005). Our results showed an upregulation of DA receptors with decreased affinity in the brainstem of diabetic, diabetic+IIH and control+IIH rats compared to control. Mild hypoglycaemia causes a functional impairment in the IC region of the brainstem in both nondiabetic and diabetic rats. However, in the diabetic rats, this alteration occurs after a less pronounced hypoglycaemic stimulus. Chronic hyperglycaemia leads to metabolic adaptions that render the diabetic brain more susceptible to mild hypoglycaemia (Jacob *et al.*, 1995).

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). Brainstem DA D_1 receptors have never been reported previously. In diabetic and diabetic+IIH rats, DA D_1 receptors increased without change in affinity. We observed a significant increase in DA D_1 receptors of diabetic+IIH rats when compared to diabetic. Real-Time PCR analysis confirmed the receptor data.

Dopamine D_2 receptors significantly decreased in the brainstem of diabetic and diabetic+IIH rats. In diabetic+IIH rats, there was a significant decrease in DA D_2 receptors compared to diabetic. Gene expression studies using Real-Time PCR also showed a decreased expression of DA D_2 receptors. The decreased DA D_2 receptor in the brainstem of diabetic rats is concordant with our previous reports (Eswar *et al.*, 2007). From our data we suggest that the increased activation of sympathetic stimulation during diabetes as a result of increased NE and EPI (Tassava *et al,* 1992; Jackson *et al.*, 1997, 1999) is because of a decreased DA content in the brainstem with an up regulation of DA D_1 receptors and down regulation of DA D_2 receptors. In the brainstem there was a decrease in the expression of DA D_2 receptor mRNA as a result of diabetes. It has been reported that damages in the brain can cause alterations in the expression of the DA D_{2L} isoform which is expressed in the *in vivo* condition (Neve *et al.*, 1991; Snyder *et al.*, 1991).

Modest reductions in plasma glucose to 3mM produce marked alterations in brainstem responses to auditory stimuli. Adverse effects of mild hypoglycaemia on brain function are not limited to higher centers but also involve the brainstem (Jones *et al,* 1990). We observed an up regulation of DA D_1 receptors and down regulation of

DA D_2 receptors in the brainstem of control+IIH rats. Real-Time PCR analysis also showed an increased expression of DA D_1 receptors and decreased expression of DA D_2 receptors. These results indicate that the dopaminergic activity in the brainstem was altered in hyperglycaemic and hypoglycaemic rats impairing DA related functions of brainstem. Though some earlier studies reported that brainstem is universally spared in hypoglycaemic brain damage (Auer, 2004). Our result showed a prominent dopaminergic functional disturbance in the brainstem of hypoglycaemic rats than diabetic rats.

Cerebral cortex

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). Our results showed an up regulation of DA receptors accompanied with a decrease in its affinity in the cerebral cortex of diabetic, diabetic+IIH and control+IIH rats. The DA neurons projecting to the PFC are thought to be involved in various motor and behavioural functions (Tam & Roth, 1997). This increased number of DA receptors could account for the behavioural supersensitivity to DA agonist as a result of damage in the DA functions (Cresse *et al.*, 1977).

The mesoprefrontal dopaminergic system is particularly vulnerable to stress (Abercrombie *et al.*, 1989) and that an over stimulation of DA D_1 receptor in the PFC impairs the working memory (Zahrt *et al.*, 1997). 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. DA D_1 receptors are located postsynaptically on the cortical neurons (Tassin *et al.*, 1978, 1982), and the decreased DA level in the PFC induced by electrolytic lesion up regulates the DA D_1 receptor density in the PFC (Tassin *et al.*, 1982). We observed an increase in DA D_1 receptors in the cerebral cortex of diabetic, diabetic+IIH and control+IIH rats with decreased DA content. Real-Time PCR analysis also showed an increased expression of DA D_1 receptors. Excessive D_1 receptor stimulation is sufficient to produce marked PFC dysfunction. (Justin *et al.*, 1997). Stress impairs PFC cognitive function through a hyperdopaminergic mechanism. It is reported that chronic stress induced depressive state is caused by a DA D_1 receptor mediated hypodopaminergic mechanism in the PFC (Mizoguchi *et al.*, 2002). Thus excessive cortical DA D_1 receptor density with decreased DA is suggested to be the cause for cortical dysfunction during diabetes and insulin induced hypoglycaemia.

Cortical function during insulin induced hypoglycaemia was impaired in diabetics (Herold, 1985). However, the finding that D_1 receptor stimulation alone is sufficient to induce PFC dysfunction does not rule out an additional role for DA D_2 receptors. Cognitive deficits induced by either stress exposure or ketamine (Verma & Moghaddam, 1996) can be blocked by selective DA D_2 receptor antagonists. These findings suggest that both DA D_1 and DA D_2 receptor families contribute to the detrimental actions of DA in the PFC and that the two may synergize to take the PFC "off-line" during stress. We observed that DA D_2 receptors also increased significantly in the cerebral cortex of diabetic, diabetic+IIH and control+IIH rats. Real-Time PCR analysis also showed an enhanced expression of DA D_2 receptors. The increased binding of DA D_2 receptors in the cerebral cortex with no change in affinity during diabetes and insulin induced hypoglycaemia has a relevance to the alterations in

dopaminergic homeostasis affecting its function. Our previous studies also showed that DA D_2 receptor mRNA in the cerebral cortex increased during diabetes and remained high after the treatment with insulin (Eswar, 2003).

Dopaminergic neurotransmission is critically involved in many aspects of complex behaviour and cognition beyond reward/reinforcement and motor function. Cortical DA D_1 and DA D_2 receptor expression has never been previously reported in insulin induced hypoglycaemia. It is suggested that chronic treatment with selective DA D_1 or DA D_2 receptor blockers induces a receptor specific increase or decrease of DA receptors (Memo *et al.*, 1987). The co-activation of DA D_1 and DA D_2 receptors is reported to enhance glutamate mediated cellular excitation. It is reported that co-activation of DA D_1 and DA D_2 receptors produce opposite or competing intracellular signals through activation of separate DA D_1 and DA D_2 mediated signaling pathways (Hopf *et al.*, 2003). It has been suggested that phasic release of DA within a DA-depleted system over stimulate DA D_1 or DA D_2 receptors due to sensitization (Abi-Dargham & Moore 2003; Grace, 1991, 1993). Stimulation of DA D_1/D_2 receptors under DA depleted conditions causes a subtle impairment in spatial working memory performance (Ellis *et al.*, 2005). The neurochemical studies on the dopaminergic neuronal activity in the PFC of the stressed rats revealed that the hyperdopaminergic mechanism is behind the acute stress induced cognitive deficits (Arnsten & Goldman-Rakic, 1998; Mizoguchi *et al.*, 2000). Thus decreased DA with enhanced receptor activity in the cerebral cortex is suggested to cause impairment of DA related cortical functions during hypoglycaemia and hyperglycaemia.

Corpus striatum

Striatal DA receptors were markedly decreased with increased affinity during diabetes with the depletion of DA in the striatum and an increased HVA metabolism.

Striatal DA firing during diabetes is decreased affecting dopaminergic functions (Saller, 1984). The decreased DA receptor density during diabetes is related to the decreased locomotor activity in STZ-induced diabetic rats (Kobayashi *et al.*, 1990; Shimomura *et al.*, 1990). This finding correlates with our present data suggesting that the disturbances in the central dopaminergic receptors during STZ- induced diabetes affects DA related functions. The firing of DA neurons projecting from the substantia nigra to the striatum is reported to be rapidly suppressed by hyperglycaemia leading to the hypofunction of DA receptors (Saller, 1984). There are hypothesis that suggests activities related to the functional capacities of DA receptors like stereotypy, ambulation, behaviour are diminished due to hyperglycaemia (Lozovsky *et al.*, 1981). Also, a decrease in DA receptors during diabetes results in hyporesponsiveness (Saitoh *et al.*, 1998). It is suggested that in alloxan treated rats with the onset of diabetes causes metabolic changes such as weight loss and dehydration are reported to occur which modify the DA metabolism (Omar *et al.*, 1985). In diabetic+IIH and control+IIH rats we observed a significant increase in striatal DA receptors which can be a compensatory response to decreased DA content.

DA D_1 receptors decreased in the striatum during diabetes with an increased affinity. Real-Time PCR analysis also showed a decreased expression of DA D_1 receptors in the striatum of diabetic rats. This correlates with previous reports that D_1 receptor density decreased in the striatum of alloxan induced diabetic rats (Salkovic & Lackovic, 1992). DA D_1 stimulated cAMP production was markedly increased in diabetic rats, whereas ability of DA D_2 receptor action to reduce cAMP formation was almost abolished during diabetes (Gorio *et al.*, 1989). An imbalance between G_s - proteins and G_i/G_o protein mediated efficacy of G_s activity as a result of the loss of G_i/G_o inhibitory functions has been found in the striatum and other tissues of diabetic animals (Salkovic & Lackovic, 1992). But striatal DA D_1 receptors showed a

significant increase in diabetic+IIH and control+IIH rats. Gene expression studies using Real-Time PCR also showed an increased expression of DA D_1 receptors. Dopamine through its DA D_1 receptor stimulates adenylyl cyclase and inhibits adenylyl cyclase activity through its DA D_2 receptors. Increased DA D_1 receptors during insulin induced hypoglycaemia that we observed in the striatum is a major cause in affecting DA related functions.

Striatal DA D_2 receptor density was significantly increased during diabetes. Previously [^{3}H] spiroperidol binding to DA D_2 receptors have been reported to be increased during diabetes (Trulson & Himmel, 1983). DA D_2 receptors were increased significantly during diabetes and insulin treatment did not reverse the increased number of receptors to control levels. This shows that during diabetes the DA D_2 receptors were significantly increased in the striatum and insulin treatment had only a partial effect in normalising the altered levels (Eswar, 2003). Striatal DA D_2 receptor primarly represents a population of DA D_2 sites (Marzella *et al.*, 1997). Striatal DA D_2 receptors are reported to be involved in the modulation of morphine-induced antinocipception in diabetic mouse (Kamei & Saitoh, 1996). During diabetes it has been documented that the sensitization of these receptors and their increased number results in a decreased locomotory and ambulatory activity (Kobayashi *et al.*, 1990; Shimomura *et al.*, 1990). DA D_2 receptors exert their function activating G_i proteins in the brain. Chronic diabetes caused a marked increase of the striatal content of met-enkephalin, which is known to utilize G_i/G_o proteins for inhibition of adenylyl cyclase. DA D_2 receptor gene expression increased in the striatum during diabetes as a result of the decreased transmission of DA. Hyperglycaemia depresses the dopaminergic function. Therefore a decreased dopaminergic activity is always suggested to increase the DA D_2 receptors. An increase in the expression of DA D_2

receptors gene results in the increased number. Our Real-Time PCR data showed an increase in DA D_2 receptor gene expression during diabetes. A lesion in the striatum is reported to increase the expression of DA D_{2L} receptor gene (Zang *et al.*, 1994). Lesions in the corpus striatum is reported to cause an increased expression of long isoform of DA D_{2L} receptor mRNA (Higgins *et al.*, 1991; Sealfon *et al.*, 1991; Todd *et al.*, 1996).

In vivo release of DA from mesolimbic and neostriatal DA neurons appears to be modulated by DA D_2 but not by D_1 receptors, whereas both receptor types can modulate DA metabolism (Boyar & Altar, 1987). Our results showed that striatal DA D_2 receptors decreased with an increased DA metabolism in the diabetic+IIH and control+IIH 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). Insulin induced hypoglycaemia could damage the DA D_2 receptors, decreasing the DA related functions in the striatum and other brain regions. The two DA receptor subtypes interact in a synergistic way to adapt to the alterations in glucose metabolism.

GDH AND MDH ACTIVITY IN THE HYPOGLYCAEMIC AND HYPERGLYCAEMIC BRAIN

Glucose metabolism plays a pivotal role in many physiological and pathological conditions. The basic defects in diabetes mellitus are impaired glucose metabolism and basement membrane alterations (Alberti *et al.*, 1982; Cohen, 1989; Striker *et al.*, 1993, Jagannath, 2004). It has been reported from earlier studies that the glutamate dehydrogenase activity in the cerebellum of experimentally induced

diabetic rats showed a significant increase leading to an increased conversion of α-ketoglutarate to glutamate (Biju & Paulose, 1998; Nair *et al.*, 1996) leading to glutamate toxicity. We observed from our results that GDH activity increased significantly in the cerebral cortex and brainstem of diabetic and diabetic+IIH rats. Our previous studies also reported that GDH enzyme activity enhanced during diabetes and did not completely reverse even after insulin administration (Nair *et al.*, 1996; Aswathy *et al.*, 1998). Studies using young and old diabetic rats clearly revealed that GDH activity regulation is essential to avoid diabetic associated brain glutamate toxicity (Biju & Paulose, 1998).

In hypoglycaemia induced diabetic rats, GDH activity of cerebral cortex and brainstem increased significantly when compared to diabetic rats. Increased GDH enzyme activity leads to increased insulin release in the presence of amino acids. The glutamate dehydrogenase mutations that cause hyperinsulinism impair the sensitivity of the enzyme to inhibition (Charles *et al.*, 1998). The activity of GDH enzyme increased during insulin induced hypoglycaemic seizures (Abdul-Ghani *et al.*, 1989). Glucose uptake in the brain did not decrease during moderate hypoglycaemia in patients with IDDM who had nearly normal mean plasma glucose concentrations with insulin treatment. Recurrent hypoglycaemia in diabetics is probably the mechanism that leads to increased glucose uptake in the brain (Patrick *et al.*, 1995). Ultimately, there is a threshold at which the provision of glucose from the circulation will be insufficient to support neural activity, even with increased uptake. The plasma glucose concentration at which the metabolism of the brain is finally impaired causes serious neuroglycopenia that patients have limited opportunity to correct the situation. Therefore, increased glucose uptake in the brain is considered maladaptive (Patrick *et al.*, 1995). This increased brain glucose uptake during insulin induced hypoglycaemia

in diabetics is suggested to cause increased GDH activity. The increase in V_{max} of GDH observed in the diabetic and diabetic+IIH group can be the cause for increase in glutamate content (Nayeemunnisa & Nagraj, 1977).

Hypoglycaemic neuronal death is not a direct and immediate consequence of low energy substrate but results instead from a cascade of events precipitated by the lack of substrate. Sustained activation of glutamate receptors has been established as a necessary upstream event in this cascade (Auer & Siesjo, 1993). Our results showed a significant decrease of GDH activity in the cerebral cortex and brainstem during insulin induced hypoglycaemia in normal rats. Decreased glutamate catabolism results in an excess of glutamate in the nervous system and cause neuronal degeneration (Plaitakis *et al.*, 1982). Glucose deprivation results in ATP depletion which in turn triggers the cell death cascade (Yan *et al.*, 2003). The neuronal death that results from severe hypoglycaemia is not a direct and immediate consequence of low brain glucose availability, but results instead from a cascade of events precipitated by the lack of energy substrate. Decreased GDH activity during insulin induced hypoglycaemia in control rats is suggested to be due to ATP depletion and inhibition of glycolysis and glycogenolysis in the cerebral cortex and brainstem.

Malate dehydrogenase is involved in metabolic reactions for the production of energy and for the maintenance of homeostasis (Seema *et al.*, 1996). There is increasing evidence that specific changes in mitochondrial function play a significant role in the early events leading to hypoglycaemic encephalopathy. Decreased fluxes of substrate through the tricarboxylic acid cycle results in decreased availability of reducing equivalents in mitochondria (Jane, 1999). The decreased MDH activity in the cerebral cortex and brainstem indicates that there is a decrease in TCA cycle as MDH

is one of the intermediates in the cycle. Also, α-ketoglutarate once formed may be converted to glutamate which will induce neurotoxicity (Haces *et al.*, 2005; Klemens *et al.*, 1997). It is reported that addition of glucose metabolites, pyruvate and malate, attenuated neuronal death after exposure to glutamate or H_2O_2 (Desagher *et al.*, 1997; Ruiz *et al.*, 1998). The significant changes in the activity of the metabolic enzyme show the disturbances in the metabolic pathway of glucose affecting the brain activity during hypoglycaemia and hyperglycaemia.

GLUTAMATE RECEPTOR (NMDAR1) GENE EXPRESSION IN THE HYPOGLYCAEMIC AND HYPERGLYCAEMIC BRAIN

Hypoglycaemia is associated with increased glutamate release (Sandberg *et al,* 1986) and conversely, glutamate toxicity in neurons is augmented by hypoglycaemia (Novelli *et al*, 1988). Exposure to acute hypoglycaemia in the newborn piglets showed increased glutamate binding sites of cerebral NMDA receptors (McGowan *et al.*, 2002). We studied the NMDA mediated glutamate toxicity due to hypoglycaemia and hyperglycaemia in different brain regions by analyzing the NMDAR1 gene expression using Real-Time PCR. We observed an increased expression of NMDAR1 receptors in the hippocampus, brainstem, cerebral cortex and corpus striatum of diabetic+IIH and control+IIH rats. Previous studies reported that glutamate causes neuronal injury during hypoglycaemia *via* the NMDA type glutamate receptor. 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. Hypoglycaemia specifically increases the sensitivity of NMDA receptors to activation by glutamate, which results in a lower threshold for glutamate induced excitotoxicity (Jane, 1999). The hippocampus contains a high concentration of

NMDA receptors. These particular receptors are vulnerable to hypoglycaemic episodes. When hippocampal cultures were deprived of glucose, massive release of lactate dehydrogenase (LDH), an indicator of neuronal death, occurred *via* NMDA receptor activation (Geng *et al.*, 1997). 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). NMDA receptor activation in these brain regions can be detrimental to cognitive and memory deficits due to insulin induced hypoglycaemia.

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.

ELECTROPHYSIOLOGICAL CHANGES IN HYPOGLYCAEMIC AND 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. 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). 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 as a result of hypoglycaemia. (Tupola *et al.*, 1998). Our results showed significant changes in the brain activity in the frontal region of control+IIH and diabetic+IIH groups during hypoglycaemia. Hypoglycaemic effect on brain EEG in control+IIH was very prominent at 1 hour after insulin injection and in diabetic+IIH, it was at 3hours. The brain activity differences observed clearly shows that in hypoglycaemia the damage is much more than in hyperglycaemic state. 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). Reduced synthesis of neurotransmitters rather than a global cerebral energy deficit explains the neurological symptoms and EEG changes in moderate hypoglycaemia (Butterworth, 1999).

GLUCOSE UPTAKE BY PANCREATIC ISLETS *IN VITRO*

Effect of dopamine on glucose uptake by pancreatic islets *in vitro*

In the pancreatic islets of Langerhans, glucose uptake by β-cells initiates a cascade of cellular events resulting in insulin secretion. Glucose uptake is the initial step in glucose-stimulated insulin secretion (GSIS) by pancreatic β-cells (Guillam *et al.*, 2000). An increased level of glucose within β cells also appears to activate calcium-independent pathways that participate in insulin secretion. Insulin secretion evoked by glucose metabolism can be further modulated by parasympathetic and sympathetic neurotransmitters (Ahren, 2000). Effects of DA on glucose uptake by pancreatic islets in particular have been poorly studied. *In vitro* studies showed a significantly lower glucose uptake in hypoglycaemic condition, 1mMglucose and higher uptake in hyperglycaemic condition, 20mM glucose. High concentration of DA (10^{-4} M) enhanced glucose uptake by pancreatic islets, while low concentration of DA (10^{-8} M) inhibited glucose uptake. DA is involved in the control of food intake, energy expenditure, glucose and lipid metabolism, blood pressure and insulin release (Pijl *et al.*, 2006). In the β-cells, DA might be released from neurons innervating pancreatic islets and exocrine pancreas is an important source of DA (Mezey *et al.*, 1996; Ahren *et al.*, 1985). The function of islet β-cells is controlled by a glucose sensor that operates at physiological glucose concentrations and acts in synergy with signals originating from hypothalamic neurons. Evidence exists that the extra pancreatic cells producing and secreting these neuroendocrine signals also exhibit a glucose sensor activity and an ability to integrate nutrient and neuro-hormonal messages (Schuit *et al.*, 2001). It is reported that hypothalamic DA D_2 receptors have a regulatory role in pancreatic insulin secretion (Eswar *et al.*, 2007). Our studies in the pancreatic islets suggest that the DA exerts a differential regulatory role in glucose

uptake – its stimulatory effect is seen at increased concentrations and inhibitory its effect at lower concentrations.

To determine the modulation of glucose uptake by DA through its specific receptors, we used specific antagonists - butaclamol, SCH 23390 and sulpiride. Butaclamol, a general antagonist of DA receptors blocked the stimulatory effect on glucose uptake at 10^{-4} M DA and inhibitory effect at 10^{-8} M DA. DA D_1 receptor antagonist SCH 23390 and DA D_2 receptor antagonist, sulpiride effectively blocked the dopaminergic action on glucose uptake. The role of DA D_2 receptors on glucose uptake was found to be more prominent compared to DA D_1 receptors. DA D_2 receptor activation facilitates glucose metabolism and stimulates resting energy expenditure in non-diabetic obese individuals. Long-term treatment with DA D_2 receptor agonists improves metabolic control in obese humans with type 2 diabetes (Pijl *et al.*, 2006). The results suggest that DA binding to DA D_1 and DA D_2 receptors regulate the glucose uptake by pancreatic islets by appropriate stimulation of glucose transporters.

Effect of Glutamate on glucose uptake by pancreatic islets *in vitro*

Glutamate is an important signaling molecule in pancreas. Glucose stimulates glutamate generation, whereas branched-chain amino acids promote competitive glutamate expenditure (Broca *et al.*, 2003). In hypoglycaemic condition, we observed that low concentration of glutamate inhibited glucose uptake while high concentration stimulated glucose uptake by the islets. MK-801, NMDA receptor antagonist blocked the glutamergic action on glucose uptake by pancreatic cells. Thus glutamergic action on glucose uptake by pancreatic cells is mediated through NMDA receptors.

In hyperglycaemic condition, glutamate inhibited glucose uptake by pancreatic cells. The inhibitory action of glutamate on glucose uptake is effectively blocked by NMDA receptor antagonist. The decrease in cellular glutamate levels correlated with impaired insulin secretion stimulated by high glucose (Blanca *et al.*, 2001). Thus it is concluded that glutamate stimulates glucose uptake by pancreatic islets only at low glucose concentrations whereas it inhibits glucose uptake by islets at high concentration of glucose. The effect of glutamate on glucose uptake by islets is mediated through NMDA receptors.

INSULIN SECRETION BY PANCREATIC ISLETS *IN VITRO*

Effect of dopamine on glucose induced insulin secretion *in vitro*

The control of insulin secretion by the pancreatic β-cell is achieved through a complex metabolic cascade converting glucose and other nutrients into signals leading to appropriate insulin release (Wollheim, 2000; Calabresi *et al.*, 1992). Neurotransmitters especially catecholamines play an important role in insulin secretion. Dopamine is reported to modulate insulin secretion in the pancreatic islets (Nogueira *et al.*, 1994). DA in the islets is essential for maintaining the equilibrium of insulin secretion.

In hypoglycaemic condition, dopamine significantly inhibited insulin secretion by pancreatic islets. Dopamine at high concentration is reported to inhibit insulin secretion from the islets (Nogueira *et al.*, 1994). Our results also showed a maximum inhibition of insulin secretion at high concentration of DA in hypoglycaemic condition. In hyperglycaemic condition we observed a significant stimulation of insulin secretion at low concentration of DA and inhibition at high concentration. Thus the concentration of DA is very critical for glucose homeostasis.

Modulation of insulin secretion by dopamine depends on specific receptor-receptor interactions. Butaclamol significantly blocked the inhibitory effect of DA in hypoglycaemic condition. Both DA D_1 and DA D_2 receptor antagonists significantly blocked the inhibitory action of DA on insulin secretion. The role and the peripheral mechanism of action of central dopamine on basal pancreatic exocrine secretion in conscious rats revealed that central dopamine inhibited pancreatic exocrine secretion *via* DA D_1 like receptors and that the inhibitory effect is mediated *via* sympathetic nerves, especially α-adrenoceptors (Masao *et al.*, 1998). Recent studies reported that DA D_2 receptors are expressed in pancreatic cells and inhibit glucose induced insulin secretion (Blanc *et al.*, 2005, Eswar *et al.*, 2006, 2007). Thus our studies suggest that DA D_1 and DA D_2 receptors are involved in the DA regulation of insulin secretion during hypoglycaemia where DA D_2 receptors play a prominent role.

During hyperglycaemia, we observed that low concentrations of DA increased glucose induced insulin secretion while high concentration caused the maximum inhibition. This is concordant with our previous reports (Eswar *et al.*, 2006). Also, high concentrations of NE, DA and 5-HT in the pancreatic islets are reported to decrease glucose stimulated insulin secretion (Zern *et al.*, 1980).

In previous studies from our laboratory reported that addition of forskolin an activator of cAMP resulted in overcoming the effect of DA on insulin secretion (Abraham, 1998). It has been reported previously that BRC treatment in hyperglycaemic state had a strong stimulatory response to insulin secretion. The agonists of DA by acting through the neuroendocrine system improves peripheral energy metabolism and impaired islet function (Lang *et al.*, 1998). 7-OH DPAT showed an inhibitory effect on glucose induced insulin secretion. Previous reports

suggest that 7-OH DPAT induced hyperglycaemia decreased insulin secretion (Hillegaart *et al.*, 1996).

It was found that DA D_1 and DA D_2 receptor antagonists effectively blocked the stimulatory and inhibitory effect of DA on insulin secretion. DA D_1 receptors showed a prominent role in the stimulation of insulin secretion while DA D_2 receptors in inhibition of insulin secretion. DA differentially regulates the pancreatic islets insulin secretion mediated through its DA D_1 and DA D_2 receptors. Thus our results suggest that dopamine acting through DA D_1 and DA D_2 receptors regulate the glucose homeostasis. This has got immense clinical significance

Effect of glutamate on glucose induced insulin secretion *in vitro*

Glutamate acts as an intracellular messenger that couples glucose metabolism to insulin secretion (Maechler & Wollheim, 1999). Recent studies reported that insulin secretion is under the control of mGlu5 receptors (Marriana *et al.*, 2006). The role of NMDA receptor subunit of glutamate on glucose induced insulin secretion by pancreatic islets is poorly studied. In our *in vitro* studies, glutamate significantly inhibited insulin secretion in both hypoglycaemic and hyperglycaemic condition. Low concentration of glutamate showed maximum inhibiton on insulin secretion during hypoglycaemia where as glutamate at high concentration caused maximum inhibition in hyperglycaemia. Elevation of ATP is necessary for the membrane-dependant increase in cytosolic Ca^{2+}, the main trigger of insulin exocytosis (Maechler & Wollheim, 2000). Decreased ATP during hypoglycaemia could account for the inhibition of insulin secretion. NMDA receptor antagonist, MK-801, blocked the inhibitory action of glutamate in hypoglycaemic and hyperglycaemic conditions. Previous studies reported that an increased islet content of L-glutamate is necessary,

but not sufficient, to allow its net conversion into 2-oxoglutarate and its further metabolism in the Krebs cycle or the GABA shunt. This, and the subsequent stimulation of insulin secretion, requires activation of GDH by L-leucine (Sergio *et al.*, 2004). Our results suggest that glutamate regulation of insulin secretion is mediated through NMDA receptor which has therapeutic applications.

Our results showed that hypoglycaemic condition has more functional damage in brain than hyperglycaemia. The receptor mediated functional studies and *in vitro* studies using antagonists for the receptor subtypes confirmed the specific receptor mediated dopaminergic and glutamergic brain damage in hypoglycaemia and hyperglycaemia. Electrophysiological studies using EEG recording in hypoglycaemia showed a prominent brain activity difference compared to hyperglycaemia. Thus it is suggested that the corrective measures for the brain fuctional damage caused during diabetes and anti-diabetic treatment, through DA D_1, DA D_2 and glutamergic receptors, have clinical significance in the therapeutic management of hypoglycaemia and hyperglycaemia.

Summary

1. Insulin induced hypoglycaemia and streptozotocin induced diabetic rats were used as models to study alterations in brain dopaminergic function and glutamate receptor gene expression in hypoglycaemia and hyperglycaemia.

2. DA content decreased in the hippocampus, brainstem, cerebral cortex and corpus striatum of diabetic, diabetic+IIH and control+IIH groups with increased HVA/ DA turnover rate.

3. Dopaminergic receptor functional status was analysed by Scatchard and displacement analysis using specific [^{3}H]ligands. Receptor binding parameters were confirmed by studying the mRNA status of the corresponding receptor using Real-Time PCR. The total dopamine receptors in hippocampus, brainstem, cerebral cortex and corpus striatum of diabetic+IIH and control+IIH groups increased with decreased affinity. In diabetic group dopamine receptors increased in hippocampus, brainstem and cerebral cortex with decreased affinity whereas it decreased in the cerebral cortex with an increased affinity. Thus, a hyperdopaminergic function with DA depletion observed in different brain regions had a differential effect during hypoglycaemia and hyperglycaemia.

4. Dopamine mediates its action through DA D_1 and DA D_2 receptors. DA D_1 and DA D_2 receptors showed increased expression in hippocampus of diabetic, diabetic+IIH and control+IIH. This shows a co-actvation of DA D_1 and DA D_2

receptors with DA depletion in hippocampus affecting DA mediated functions. This co-activation of DA D_1 and DA D_2 receptors should produce opposite or competing intracellular signals through activation of separate DA D_1 and DA D_2 mediated signaling pathways.

5. In brainstem, DA D_1 receptors were up regulated in diabetic, diabetic+IIH and control+IIH groups. DA D_2 receptors decreased in diabetic and diabetic+IIH while it was increased in control+IIH group. Cortical DA D_1 and DA D_2 receptors were up regulated in diabetic, diabetic+IIH and control+IIH group. Corpus striatum of diabetic group showed a decrease of DA D_1 receptors with significant increase in DA D_2 receptors.

6. In diabetic+IIH and control+IIH group, DA D_1 receptors were up regulated with a down regulation of DA D_2 receptors. DA D_1 and DA D_2 receptors have differential regulatory role in different brain regions during hypoglycaemia and hyperglycaemia.

7. Glutamate dehydrogenase activity in the brainstem and cerebral cortex increased in diabetic and diabetic+IIH whereas the activity decreased in control+IIH. MDH activity showed a significant decrease in all the groups compared to control.

8. NMDAR1 receptor gene expression increased in hippocampus, brainstem and cerebral cortex of diabetic+IIH and control+IIH. In diabetic group, NMDAR1 receptor gene expression was increased in hippocampus and brainstem while it was decreased in cerebral cortex and corpus striatum.

9. A prominent brain activity was observed in diabetic+IIH and control+IIH rats compared to diabetic and control by EEG analysis.

10. *In vitro* glucose uptake studies showed that high concentration of DA (10^{-4} M) enhanced glucose uptake by pancreatic islets, while low concentration of DA (10^{-8} M) inhibited glucose uptake during hypoglycaemia and hyperglycaemia. DA D_2 receptors showed a prominent role on glucose uptake compared to DA D_1 receptors confirmed by using specific antagonists.

11. *In vitro* glucose uptake studies showed that low concentration of glutamate inhibited glucose uptake while higher concentration stimulated glucose uptake by the islets in hypoglycaemic condition. Glutamate inhibited glucose uptake by pancreatic islets in hyperglycaemic condition. This glutamergic action on glucose uptake by pancreatic islets was mediated through NMDA receptors confirmed by using specific antagonist.

12. *In vitro* insulin secretion studies showed inhibition of insulin secretion in hypoglycaemic condition with maximum inhibition at high concentration of DA. DA D_1 and DA D_2 receptors are involved in the DA regulation of insulin secretion during hypoglycaemia where DA D_2 receptors showed a prominent role as confirmed by using specific antagonist. We observed a significant stimulation of insulin secretion at low concentration of DA and inhibition at high concentration in hyperglycaemic condition. DA D_1 receptors showed a prominent role in the stimulation of insulin secretion while DA D_2 receptors in inhibition of insulin secretion confirmed by using specific antagonist.

Our studies showed hypoglycaemic and hyperglycaemic effect on brain function of dopamine through DA D_1 and DA D_2 receptors, glutamate through NMDA receptors, *in vitro* studies confirming the receptor subtypes functional regulation on glucose uptake and insulin secretion. Thus, it is suggested that the corrective measures for the brain fuctional damage caused during diabetes and anti-diabetic treatment, through DA D_1, DA D_2 and glutamergic receptors, have therapeutic role in the management of hypoglycaemia and hyperglycaemia.

Conclusion

Hypoglycaemia is the major obstacle to optimal blood glucose control in diabetic patients. Severe hypoglycaemia triggers a cascade of events in vulnerable neurons that culminate in cell death even after glucose normalization. Our findings demonstrated that dopaminergic system is impaired during hypoglycaemia and hyperglycaemia. The evaluations of these damages have important implications in understanding the molecular mechanism underlying cognitive deficits due to intensive insulin treatment in diabetics. DA content decreased during hypoglycaemia and hyperglycaemia. We observed a prominent significant decrease of DA content in the brain during hypoglycaemia compared to hyperglycaemia. This decreased brain DA content caused an increase in dopaminergic function. DA D_1 and DA D_2 receptor subtypes have differential regulatory role in different brain regions during hypoglycaemia and hyperglycaemia. The regional difference in receptor status is relevant to the role that dopamine plays during various physiological and behavioural activities. Dopamine functioning through DA D_1 and DA D_2 receptors regulate insulin secretion. *In vitro* studies on glucose uptake and insulin secretion using specific antagonist of DA D_1 and DA D_2 receptors have confirmed the role of these receptors in hypoglycaemic and hyperglycaemic conditions. The differential functional balance of these receptors control the glucose mediated insulin secretion. Also, glutamate receptor functional regulation has a control on glucose mediated insulin secretion. The binding parameters of DA D_1 and DA D_2 receptors and gene expression studies of DA D_1, DA D_2 and NMDAR1 receptors in diabetic, diabetic+ IIH and control + IIH showed a differential functional regulation during hypoglycaemia and

hyperglycaemia. Hypoglycaemic brain showed an increased glutamate toxicity mediated through NMDA than in hyperglycaemic brain. Thus our results showed that hypoglycaemic condition has more functional damage than hyperglycaemia. The receptor mediated functional studies and *in vitro* studies using antagonists for the receptor subtypes confirmed the specific receptor mediated dopaminergic and glutamergic brain damage in hypoglycaemia and hyperglycaemia. Thus, it is suggested that the corrective measures for the brain fuctional damage caused during diabetes and anti-diabetic treatment, through DA D_1, DA D_2 and glutamergic receptors, have therapeutic role in the management of hypoglycaemia and hyperglycaemia.

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Papers Published

1. T .R. Renuka, **Remya Robinson** and C. S. Paulose, Increased insulin secretion by muscarinic M1 and M3 receptor function from rat pancreatic islets *in vitro*. ***Neurochemical Research***; 31(3):313-20 (March, **2006**).

2. Ani Das.V, **Remya Robinson**, C. S. Paulose. Enhanced β- adrenergic receptors in the brain regions and pancreas during pancreatic regeneration in weanling rats. ***Molecular and Cellular Biochemistry***; 289(1-2):11-9 (September **2006**).

Awards

1. **IBRO 2007 Travel Award** to attend the 7th IBRO World Congress of Neuroscience being held at Melbourne, Australia, during July 12-17, 2007.
2. **IBS Award for the best original oral presentation in Medical Biotechnology** by Society for Biotechnologists (India) at National Seminar on Biotechnology and Economic Development – A Kerala Scenario held at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram (March, **2006**).

Abstracts Presented

1. **Remya Robinson** and C.S. Paulose, "Up regulation of Dopamine D1 receptors in the brain stem of insulin induced hypoglycaemic and streptozotocin induced diabetic rats" at the 7th IBRO World Congress of Neuroscience being held at Melbourne, Australia **(July, 2007– accepted).**

2. **Remya Robinson** and C.S. Paulose, "Enhanced dopamine D1 and D2 receptor gene expression in the cerebral cortex of hypoglycaemic and hyperglycemic rats" at National conference on Biotechnology in molecular medicine organised by AIMS, Kochi, Amrita School of Biotechnology, Amritapuri and Society for Biotechnologists, India **(January 2007).**

3. Amee Krishnakumar, **Remya Robinson** and C. S. Paulose, "Acetylcholinestrase activity in the cerebral cortex and muscle of insulin induced hypoglycaemic and Streptozotocin induced diabetic rats". XXV National Symposium on Reproductive Biology and Comparative Endocrinology. Translational endocrinology and reproductive biology. Organised by Society for Reproductive Biology and Comparative Endocrinology and Dept. of Zoology, University of Kerala **(January 2007).**

4. Anu Joseph, **Remya Robinson** and C. S. Paulose, "Glutamate dehydrogenase activity in the Cerebellum of Streptozotocin induced diabetic and insulin induced hypoglycaemic rats". 3rd International Symposium on Neurodegeneration and Neuroprotection and Society for Neurochemistry meeting. Organised by Indian Institute of Chemical Biology, Kolkatta, India **(January 2007).**

5. **Remya Robinson** and C.S. Paulose, "Enhanced dopamine receptor gene expression in the brain stem of hypoglycaemic and hyperglycaemic rats" at National Seminar on Biotechnology and Economic Development – A Kerala Scenario jointly organized by Rajiv Gandhi Centre for

Biotechnology, Thiruvananthapuram and Society for Biotechnologists (India) **(March, 2006).**

6. **Remya Robinson,** "Enhanced dopamine receptor gene expression in the cerebral cortex of hypoglycaemic and hyperglycaemic rats. XVIII Kerala Science Congress, CESS, Thiruvananthapuram **(January, 2006).**

7. Ani Das.V, **Remya Robinson,** C. S. Paulose, "Upregulation of β-adrenergic receptors in the brain regions and pancreas during pancreatic regeneration in weanling rats" International Conference on Biotechnology and Neuroscience jointly organized by CUSAT, Cochin and Society for Biotechnologists (India); **(December, 2004).**

8. Santhosh Thomas K, **Remya Robinson** and C. S. Paulose, "Dopamine D2 receptor functional regulation in hypothalamus and pancreas during pancreatic regeneration". Indian association of Biomedical Scientists Silver jubilee conference, IABMS, Chennai, India **(October, 2004).**

9. 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", International Neuroscience Conference, IAN, SNCI held at University of Hyderabad. **(May, 2004).**

Table-1

Body weight and blood glucose level of control and experimental rats

Animal status	Body weight (g)		Blood glucose (mg/dL)
	Day 0	Day 10	
Control	200 ± 20	240 ± 18	110 ± 5.77[a]
Diabetic	220 ± 8	170 ± 12***	251 ± 3.18 ***[b]
Diabetic+IIH	220 ± 10	210 ± 15	46 ± 3.05 ***¶¶¶[c]
Control+IIH	200 ± 12	240 ± 16	42 ± 1.45 ***¶¶¶[d]

Values are Mean ± S.E.M of 4-6 separate experiments

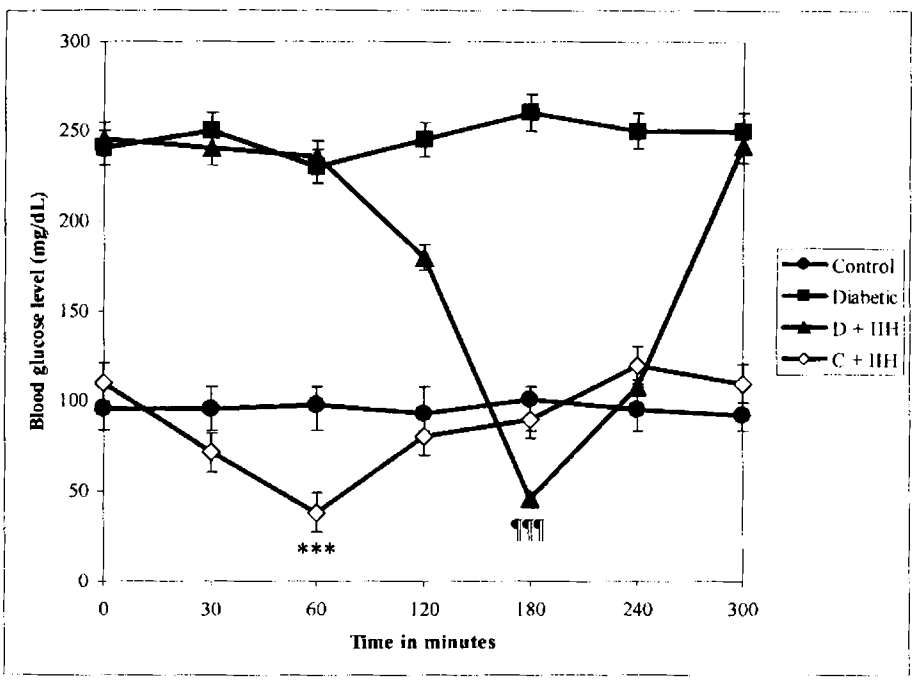
***p<0.001 when compared to control
¶¶¶p<0.001 when compared to diabetic

a and b values are of the 10th day of experiment
c and d values are 3 hours and 1 hour after insulin administration respectively

IIH – Insulin Induced Hypoglycaemia

Figure - 1

Glucose levels at different time intervals after insulin administration in control and experimental rats.



Values are Mean ± S.E.M. of 4-6 separate experiments

***p<0.001 when compared to control
¶¶¶p<0.001 when compared to diabetic

D+IIH- Diabetes + Insulin Induced Hypoglycaemia
C+IIH- Control + Insulin Induced Hypoglycaemia

Table-2

Dopamine and Homovanillic acid content in the hippocampus of control and experimental rats (nmoles/g wt.of tissue)

Animal status	DA	HVA	HVA/DA
Control	34.87 ± 0.65	1.09 ± 0.09	0.03 ± 0.01
Diabetic	9.90 ± 0.32***	1.11 ± 0.04	0.11 ± 0.05***
Diabetic+IIH	4.96 ± 0.14 ***¶¶¶	0.57 ± 0.03 ***¶¶¶	0.11 ± 0.04***
Control+IIH	5.08 ± 0.12***¶¶¶	1.11 ± 0.04	0.20 ± 0.07***¶¶††

Values are Mean ± S.E.M. of 4-6 separate experiments

***p<0.001 when compared to control
¶¶p<0.01, ¶¶¶ p<0.001 when compared to diabetic
††p<0.01 when compared to Diabetic+IIH

IIH – Insulin Induced Hypoglycaemia

Table-3

Dopamine and Homovanillic acid content in the brainstem of control and experimental rats (nmoles/g wt.of tissue)

Animal status	DA	HVA	HVA/DA
Control	7.90 ± 0.65	0.69 ± 0.29	0.09 ± 0.01
Diabetic	3.95 ± 0.32***	0.85 ± 0.18*	0.21 ± 0.01**
Diabetic+IIH	2.21 ± 0.14 ***¶¶	0.81± 0.72*	0.37± 0.01**¶¶
Control+IIH	1.30 ± 0.12***¶¶¶††	0.77 ± 0.59*	0.59 ± 0.04***¶¶††

Values are Mean ± S.E.M. of 4-6 separate experiments

*p<0.05, **p<0.01, ***p<0.001 when compared to control
¶¶ p<0.01, ¶¶¶ p<0.001 when compared to diabetic
††p<0.01 when compared to diabetic+IIH

IIH – Insulin Induced Hypoglycaemia

Table-4

Dopamine and Homovanillic acid content in the cerebral cortex of control and experimental rats (nmoles/g wt.of tissue)

Animal status	DA	HVA	HVA/DA
Control	35.10 ± 0.88	4.71 ± 0.23	0.13 ± 0.01
Diabetic	26.35 ± 0.88***	3.83 ± 0.06*	0.14 ± 0.01
Diabetic+IIH	20.43 ± 0.58***¶¶	3.57± 0.21*	0.17 ± 0.01*¶
Control+IIH	16.97 ± 0.58***¶¶†	3.56 ± 0.24*	0.21 ± 0.02*¶¶†

Values are Mean ± S.E.M. of 4-6 separate experiments

*p<0.05, ***p<0.001 when compared to control
¶p<0.05, ¶¶p<0.01 when compared to diabetic
†p<0.05 when compared to diabetic+IIH

IIH – Insulin Induced Hypoglycaemia

Table – 5

Dopamine and Homovanillic acid content in the corpus striatum of control and experimental rats (nmoles/g wt.of tissue)

Animal status	DA	HVA	HVA/DA
Control	27.06 ± 1.20	0.22 ± 0.11	0.01 ± 0.002
Diabetic	23.24 ± 1.45*	2.14 ± 0.09***	0.09 ± 0.001**
Diabetic+IIH	19.62 ± 0.88***¶	2.53 ± 0.13***¶	0.12 ± 0.031***¶
Control+IIH	12.47 ± 0.88***¶¶††	3.19 ± 0.14***¶¶†	0.25 ± 0.012***¶¶††

Values are Mean ± S.E.M. of 4-6 separate experiments

*p<0.05, **p<0.01, ***p<0.001 when compared to control
¶p<0.05, ¶¶p<0.01 when compared to diabetic
†p<0.05, ††p<0.01 when compared to diabetic+IIH

IIH – Insulin Induced Hypoglycaemia

Figure – 2

Scatchard analysis of [^{3}H]Dopamine against dopamine in the hippocampus of control and experimental rats

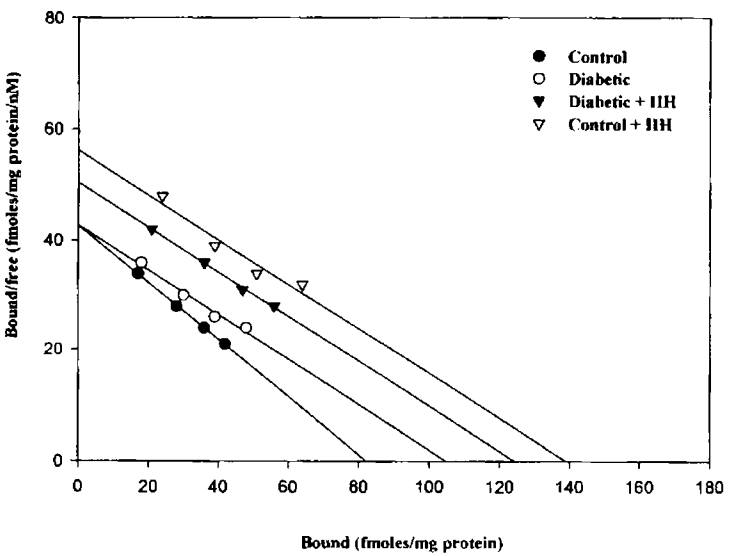


Table-6

Binding parameters of [^{3}H]Dopamine against dopamine in the hippocampus of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	82.00 ± 0.88	1.95 ± 0.07
Diabetic	105.00 ± 2.89 ***	2.50 ± 0.19 ***
Diabetic+IIH	125.00 ± 2.33 ***¶¶¶	2.50 ± 0.16 ***
Control+IIH	139.00 ± 0.58 ***¶¶¶††	2.53 ± 0.04 ***

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to control
¶¶¶p<0.001 when compared to diabetic
††p<0.01 when compared to diabetic+IIH
IIH- Insulin Induced Hypoglycaemia

Table-7

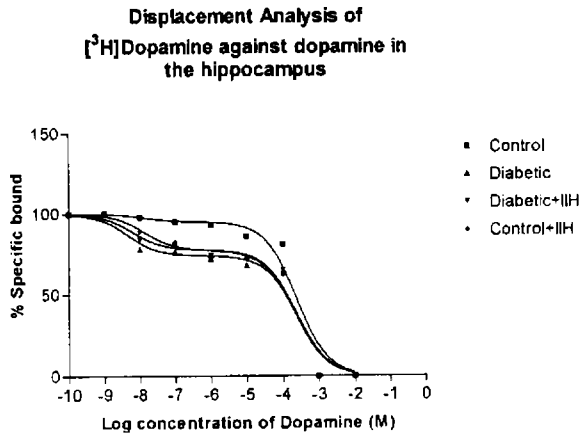
Binding parameters of [^{3}H]Dopamine against dopamine in the hippocampus of control and experimental rats

Animal status	Best-fit model	Log (EC_{50})	Ki	Hill slope
Control	One-site	-4.025	3.4090×10^{-5}	-0.9763
Diabetic	One-site	-4.174	4.0300×10^{-5}	-0.9373
Diabetic+IIH	One-site	-4.173	4.0150×10^{-5}	-0.9045
Control+IIH	One-site	-4.246	5.6700×10^{-5}	-0.9311

Values are mean of 4-6 separate experiments

IIH- Insulin Induced Hypoglycaemia

Figure – 3



Data were fitted with 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 – 4

Real-Time PCR amplification of Dopamine D_1 receptor mRNA from the hippocampus of control and experimental rats

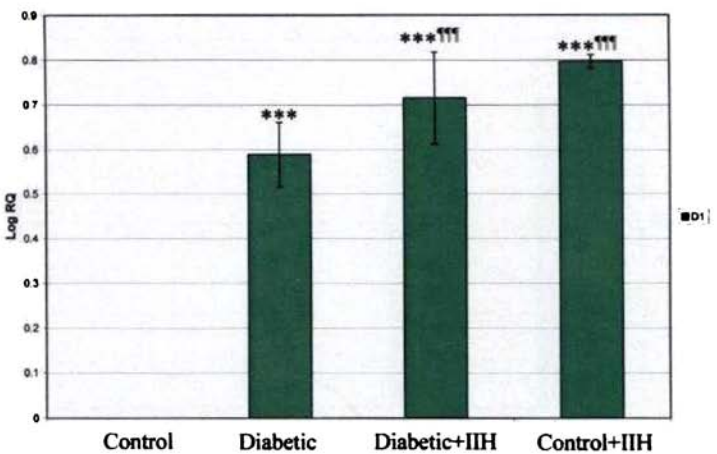


Table-8

Real-Time PCR amplification of Dopamine D_1 receptor mRNA from the hippocampus of control and experimental rats

Animal status	Log RQ
Control	0
Diabetic	$0.589 \pm 0.07^{***}$
Diabetic+IIH	$0.715 \pm 0.10^{***¶¶¶}$
Control+IIH	$0.796 \pm 0.01^{***¶¶¶}$

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to control
¶¶¶p<0.001 when compared to diabetic
IIH- Insulin Induced Hypoglycaemia

Figure – 5

Real-Time PCR amplification of Dopamine D_2 receptor mRNA from the hippocampus of control and experimental rats

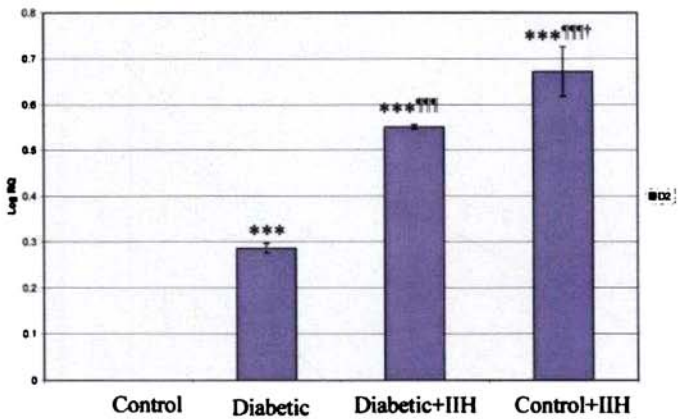


Table-9

Real-Time PCR amplification of Dopamine D_2 receptor mRNA from the hippocampus of control and experimental rats

Animal status	Log RQ
Control	0
Diabetic	$0.285 \pm 0.010^{***}$
Diabetic+IIH	$0.550 \pm 0.004^{***¶¶¶}$
Control+IIH	$0.671 \pm 0.053^{***¶¶¶†}$

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to control
¶¶¶ p<0.001 when compared to diabetic
†p<0.05 when compared to diabetic+IIH
IIH- Insulin Induced Hypoglycaemia

Figure – 6

Scatchard analysis of [^{3}H]Dopamine against dopamine in the brainstem of control and experimental rats

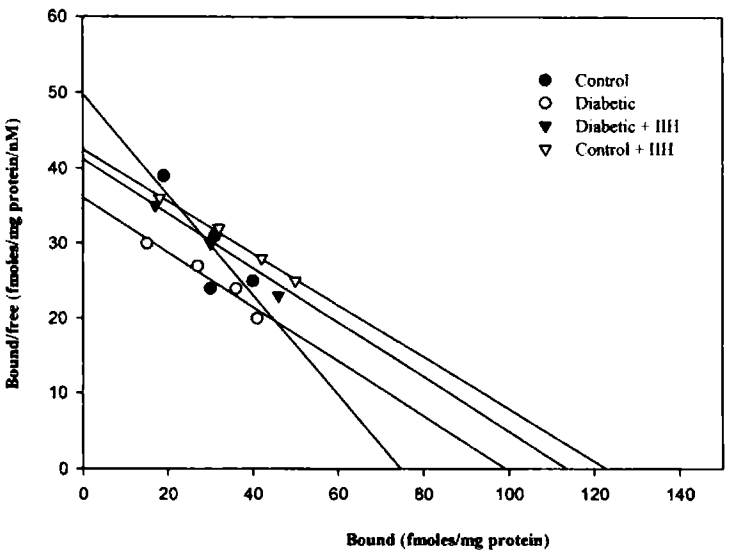


Table-10

Binding parameters of [^{3}H]Dopamine against dopamine in the brainstem of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	81 ± 2.1	1.40 ± 0.06
Diabetic	95 ± 2.6**	1.90 ± 0.06**
Diabetic+IIH	107 ± 1.7***¶¶	2.30 ± 0.12***¶
Control+IIH	123± 1.4***¶¶¶†	2.80 ± 0.11***¶¶†

Values are Mean ± S.E.M. of 4-6 separate experiments
**p<0.01, ***p<0.001 when compared to control
¶p<0.05, ¶¶p<0.01, ¶¶¶p<0.001 when compared to diabetic
†p<0.05 when compared to diabetic+IIH
IIH- Insulin Induced Hypoglycaemia

Table-11

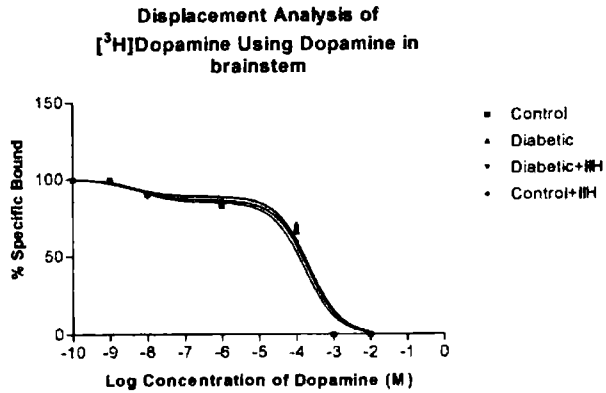
Binding parameters of [^{3}H]Dopamine against dopamine in the brainstem of control and experimental rats

Animal status	Best-fit model	Log (EC_{50})	Ki	Hill slope
Control	One-site	-4.162	3.0900×10^{-5}	-0.9532
Diabetic	One-site	-4.178	3.6390×10^{-5}	-0.9527
Diabetic+IIH	One-site	-4.217	3.9850×10^{-5}	-0.9579
Control+IIH	One-site	-4.288	4.1340×10^{-5}	-0.9540

Values are mean of 4-6 separate experiments

IIH- Insulin Induced Hypoglycaemia

Figure - 7



Data were fitted with 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 – 8

Scatchard analysis of DA D_1 receptor using [^{3}H]SCH 23390 against SCH 23390 in the brainstem of control and experimental rats

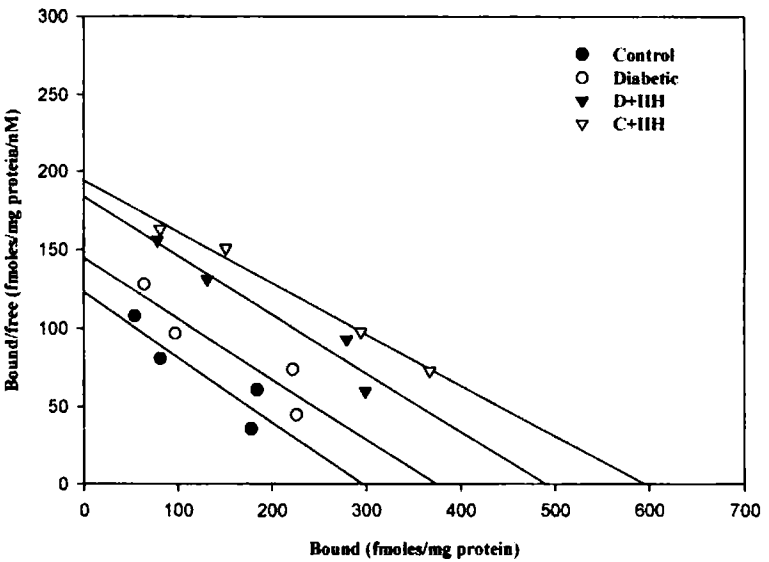


Table-12

Binding parameters of [^{3}H]SCH 23390 against SCH 23390 in the brainstem of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	283.33 ± 8.333	2.14 ± 0.08
Diabetic	374.33 ± 9.244**	2.32 ± 0.19
Diabetic+IIH	475.67 ± 10.55***¶¶	2.57 ± 0.19
Control+IIH	573.33 ± 8.02***¶¶¶††	2.61 ± 0.16

Values are Mean ± S.E.M. of 4-6 separate experiments
**p<0.01, ***p<0.001 when compared to control
¶¶p<0.01, ¶¶¶p<0.001 when compared to diabetic
††p<0.01 when compared to diabetic+IIH
IIH- Insulin Induced Hypoglycaemia

Table-13

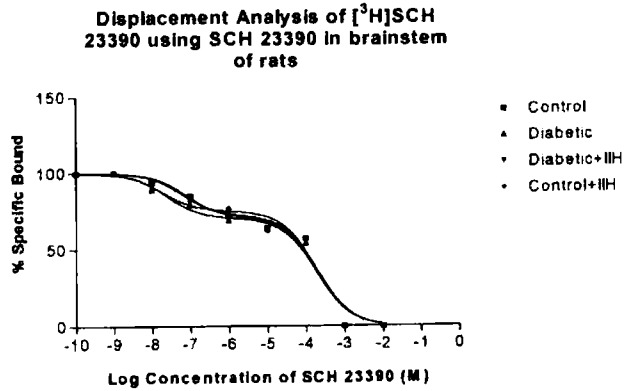
Binding parameters of [^{3}H]SCH 23390 against SCH 23390 in the brainstem of control and experimental rats

Animal status	Best fit model	Log(EC_{50})-1	Log(EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control	Two-site	-7.137	-4.166	1.6370×10^{-8}	2.4820×10^{-5}	-0.2906
Diabetic	Two-site	-7.241	-4.269	1.8830×10^{-8}	2.5210×10^{-5}	-0.2983
Diabetic+IIH	Two-site	-7.424	-4.297	1.8730×10^{-8}	2.5920×10^{-5}	-0.2809
Control+IIH	Two-site	-7.683	-4.424	1.6510×10^{-8}	2.4140×10^{-5}	-0.2511

Values are mean of 4-6 separate experiments

IIH- Insulin Induced Hypoglycaemia

Figure – 9



Data were fitted with iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki – The affinity of the receptor for the competing drug. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding.

Figure – 10

Real-Time PCR amplification of Dopamine D_1 receptor mRNA from the brainstem of control and experimental rats

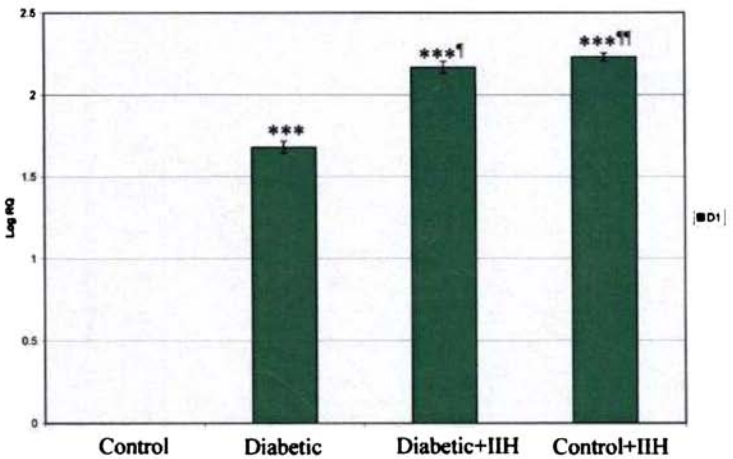


Table-14
Real-Time PCR amplification of Dopamine D_1 receptor mRNA from the brainstem of control and experimental rats

Animal status	Log RQ
Control	0
Diabetic	$1.679 \pm 0.037^{***}$
Diabetic+IIH	$2.166 \pm 0.037^{***¶}$
Control+IIH	$2.229 \pm 0.024^{***¶¶}$

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to control
¶p<0.05, ¶¶p<0.01 when compared to diabetic
IIH- Insulin Induced Hypoglycaemia

Figure – 11

Scatchard analysis of DA D_2 using [^{3}H]YM-09151-2 binding against sulpiride in the brainstem of control and experimental rats

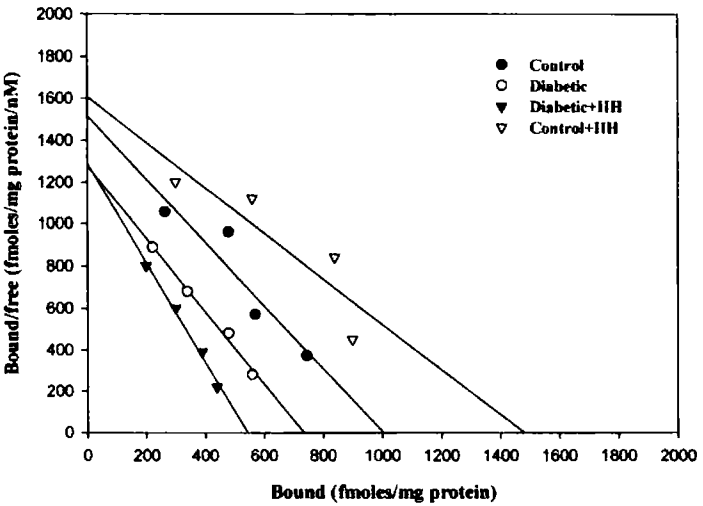


Table- 15

Binding parameters of [^{3}H]YM-09151-2 against sulpiride in the brainstem of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	1000.00 ± 18.333	0.64 ± 0.06
Diabetic	720.44 ± 19.244**	0.58 ± 0.08
Diabetic+IIH	520.85 ± 20.55***¶¶¶	0.42 ± 0.15***¶¶
Control+IIH	1490.00 ± 28.02***¶¶¶†††	0.93 ± 0.17***¶¶¶†††

Values are Mean ± S.E.M. of 4-6 separate experiments
**p<0.01, ***p<0.001 when compared to control
¶¶p<0.01, ¶¶¶p<0.001 when compared to diabetic
†††p<0.001 when compared to diabetic+IIH
IIH- Insulin Induced Hypoglycaemia

Table-16

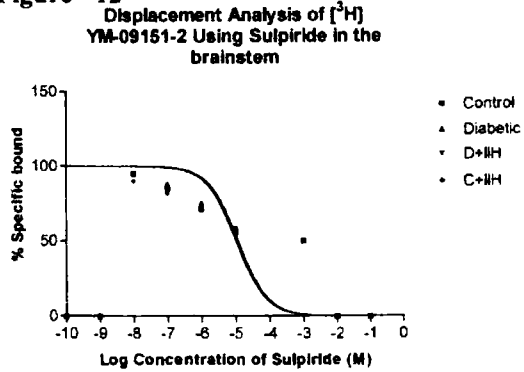
Binding parameters of [^{3}H]YM-09151-2 against sulpiride in the brainstem of control and experimental rats

Animal status	Best-fit model	Log (EC_{50})	Ki	Hill slope
Control	One-site	-4.401	3.8070×10^{-5}	-0.9455
Diabetic	One-site	-4.243	3.6490×10^{-5}	-0.9227
Diabetic+IIH	One-site	-4.205	2.5860×10^{-5}	-0.9281
Control+IIH	One-site	-4.411	4.1610×10^{-5}	-0.9116

Values are mean of 4-6 separate experiments

IIH- Insulin Induced Hypoglycaemia

Figure - 12



Data were fitted with 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 – 13

Real-Time PCR amplification of Dopamine D_2 receptor mRNA from the brainstem of control and experimental rats

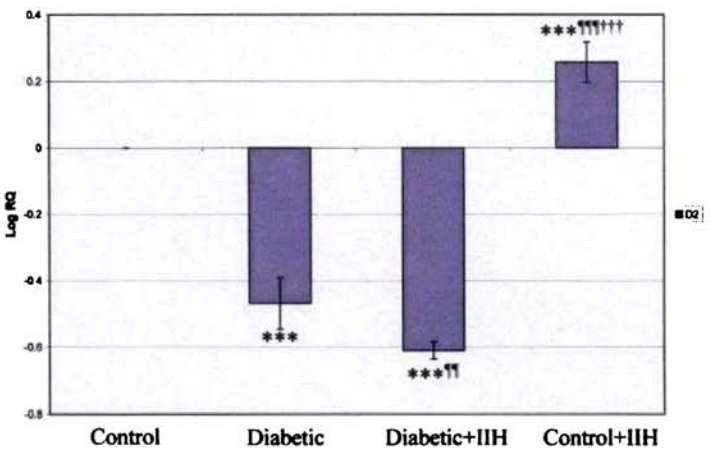


Table – 17

Real-Time PCR amplification of Dopamine D_2 receptor mRNA from the brainstem of control and experimental rats

Animal status	Log RQ
Control	0
Diabetic	-0.468 ± 0.076***
Diabetic+IIH	-0.610 ± 0.025***¶¶
Control+IIH	0.257 ± 0.060***¶¶†††

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to control
¶¶p<0.01, ¶¶¶p<0.001 when compared to diabetic
†††p<0.001 when compared to diabetic+IIH
IIH- Insulin Induced Hypoglycaemia

Figure – 14

Scatchard analysis of [^{3}H]Dopamine against dopamine in the cerebral cortex of control and experimental rats

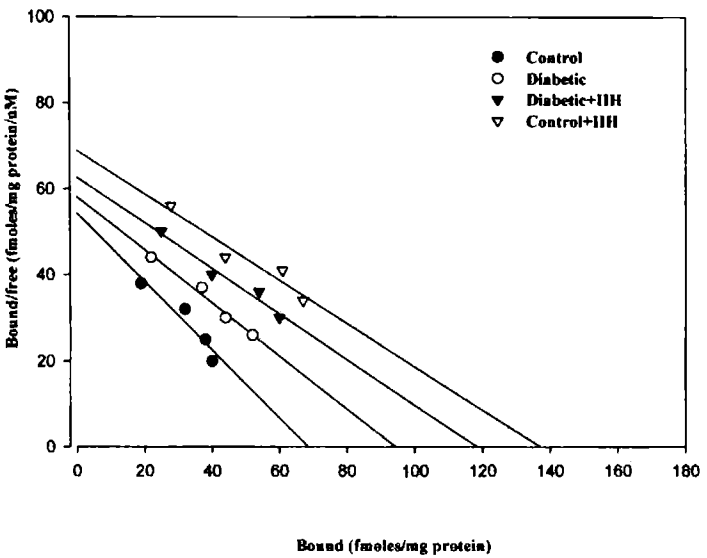


Table- 18

Binding parameters of [^{3}H]Dopamine against dopamine in the cerebral cortex of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	70.00 ± 1.00	1.11 ± 0.05
Diabetic	95.00 ± 0.67***	1.63 ± 0.07***
Diabetic+IIH	118.00 ± 2.46***¶¶¶	1.87 ± 0.15***¶¶¶
Control+IIH	136.00 ± 1.33***¶¶¶†	1.94 ± 0.11***¶¶¶†

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to control
¶¶p<0.01, ¶¶¶p<0.001 when compared to diabetic
†p<0.05 when compared to diabetic+IIH
IIH- Insulin Induced Hypoglycaemia

Table-19

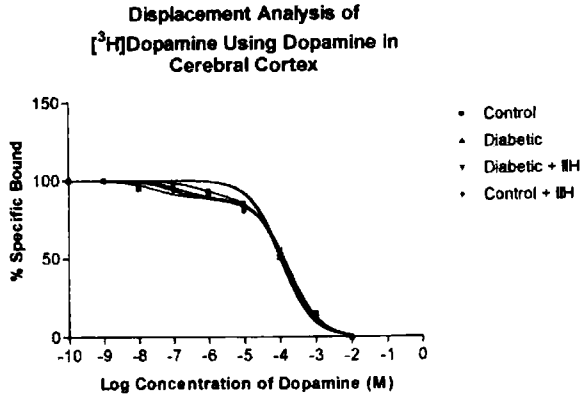
Binding parameters of [^{3}H]Dopamine against dopamine in the cerebral cortex of control and experimental rats

Animal status	Best-fit model	Log (EC_{50})	Ki	Hill slope
Control	One-site	-4.025	3.4090×10^{-5}	-0.9763
Diabetic	One-site	-4.169	4.0150×10^{-5}	-0.9373
Diabetic+IIH	One-site	-4.176	4.0300×10^{-5}	-0.9045
Control+IIH	One-site	-4.246	5.6700×10^{-5}	-0.9311

Values are mean of 4-6 separate experiments

IIH- Insulin Induced Hypoglycaemia

Figure – 15



Data were fitted with 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 – 16

Scatchard analysis of DA D_1 receptor using [^{3}H]SCH 23390 against SCH 23390 in the cerebral cortex of control and experimental rats

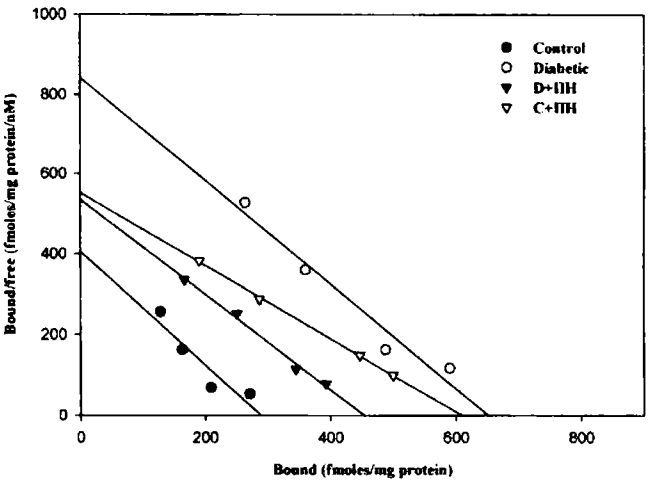


Table-20

Binding parameters of [^{3}H]SCH 23390 against SCH 23390 in the cerebral cortex of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	298.00 ± 4.41	1.03 ± 0.12
Diabetic	715.00 ± 7.63***	1.35 ± 0.15*
Diabetic+IIH	483.33 ± 6.0***¶¶¶	1.22 ± 0.14*
Control+IIH	601.67 ± 7.26***¶¶††	1.38 ± 0.11*

Values are Mean ± S.E.M. of 4-6 separate experiments
*p<0.05, ***p<0.001 when compared to control
¶p<0.05, ¶¶¶p<0.001 when compared to diabetic
††p<0.01 when compared to diabetic+IIH
IIH- Insulin Induced Hypoglycaemia

Table – 21

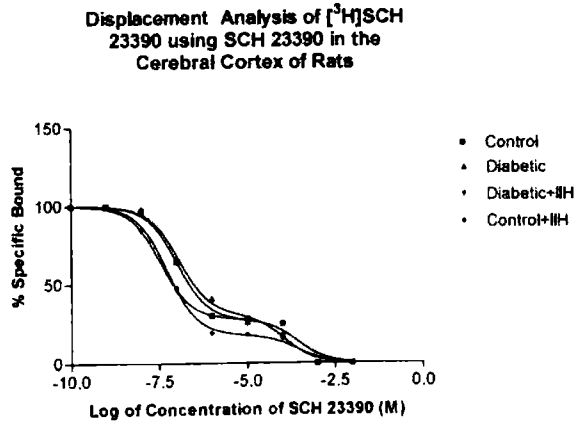
Binding parameters of [^{3}H]SCH 23390 using SCH 23390 in the cerebral cortex of control and experimental rats

Animal status	Best fit model	Log(EC_{50})-1	Log(EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control	Two-site	-7.137	-4.166	1.0370×10^{-8}	1.8820×10^{-5}	-0.2906
Diabetic	Two-site	-7.424	-4.297	2.8730×10^{-8}	2.6920×10^{-5}	-0.2983
Diabetic+IIH	Two-site	-7.241	-4.269	1.8830×10^{-8}	2.5210×10^{-5}	-0.2809
Control+IIH	Two-site	-7.683	-4.424	3.6510×10^{-8}	3.4140×10^{-5}	-0.2511

Values are mean of 4-6 separate experiments

IIH- Insulin Induced Hypoglycaemia

Figure – 17



Data were fitted with iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki – The affinity of the receptor for the competing drug. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding.

Figure – 18

Real-Time PCR amplification of Dopamine D_1 receptor mRNA from the cerebral cortex of control and experimental rats

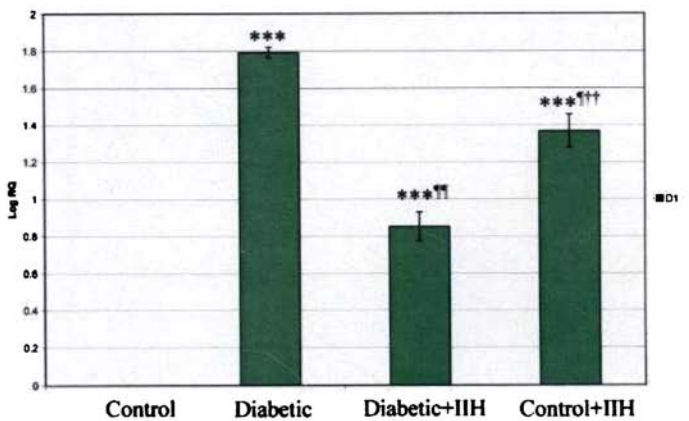


Table-22

Real-Time PCR amplification of Dopamine D_1 receptor mRNA from the cerebral cortex of control and experimental rats

Animal status	Log RQ
Control	0
Diabetic	$1.789 \pm 0.029^{***}$
Diabetic+IIH	$0.850 \pm 0.078^{***¶¶}$
Control+IIH	$1.366 \pm 0.09^{***¶††}$

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to control
¶p<0.05, ¶¶p<0.01 when compared to diabetic
††p<0.01 when compared to diabetic+IIH
IIH- Insulin Induced Hypoglycaemia

Figure – 19

Scatchard analysis of DA D_2 receptor using [^{3}H]YM-09151-2 against sulpiride in the cerebral cortex of control and experimental rats

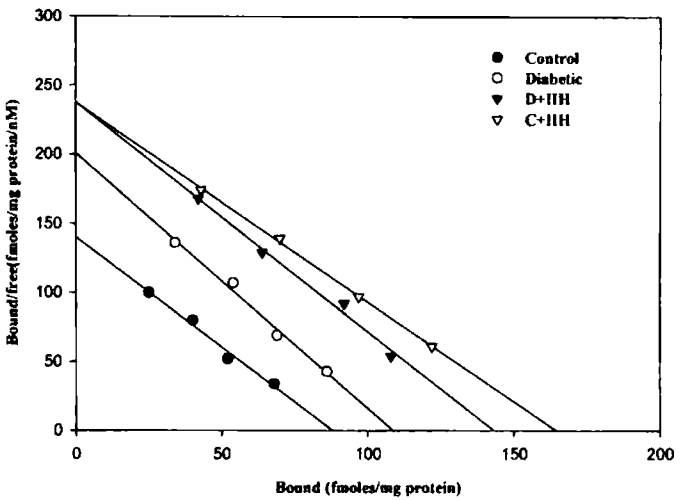


Table-23

Binding parameters of [^{3}H]YM-09151-2 against sulpiride in the cerebral cortex of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	85.00 ± 2.89	0.58 ± 0.15
Diabetic	115.00 ± 2.89**	0.57 ± 0.27
Diabetic+IIH	145.00 ± 4.41***¶¶¶	0.63 ± 0.19
Control+IIH	165.00 ± 4.41***¶¶¶††	0.67 ± 0.15

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 diabetic
††p<0.01 when compared to diabetic+IIH
IIH- Insulin Induced Hypoglycaemia

Table – 24

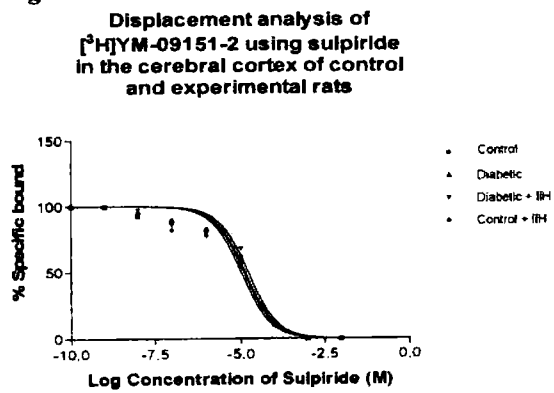
Binding parameters of [^{3}H]YM-09151-2 against sulpiride in the cerebral cortex of control and experimental rats

Animal status	Best-fit model	Log (EC_{50})	Ki	Hill slope
Control	One-site	-4.401	3.8070×10^{-5}	-0.9455
Diabetic	One-site	-4.205	2.5860×10^{-5}	-0.9227
Diabetic+IIH	One-site	-4.243	2.6490×10^{-5}	-0.9281
Control+IIH	One-site	-4.411	4.1610×10^{-5}	-0.9116

Values are mean of 4-6 separate experiments

IIH- Insulin Induced Hypoglycaemia

Figure – 20



Data were fitted with 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 – 21

Real-Time PCR amplification of Dopamine D_2 receptor mRNA from the cerebral cortex of control and experimental rats

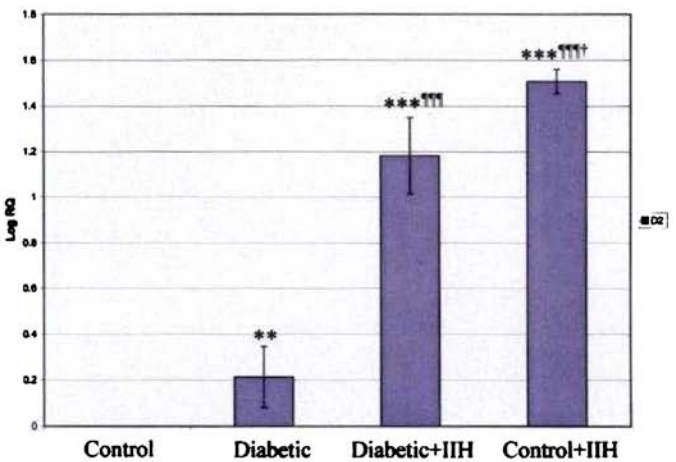


Table-25

Real-Time PCR amplification of Dopamine D_2 receptor mRNA from the cerebral cortex of control and experimental rats

Animal status	Log RQ
Control	0
Diabetic	$0.212 \pm 0.133^{**}$
Diabetic+IIH	$1.182 \pm 0.167^{***¶¶¶}$
Control+IIH	$1.507 \pm 0.053^{***¶¶¶†}$

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 diabetic
†p<0.05 when compared to diabetic+IIH
IIH- Insulin Induced Hypoglycaemia

Figure – 22

Scatchard analysis of [^{3}H]Dopamine against dopamine in the corpus striatum of control and experimental rats

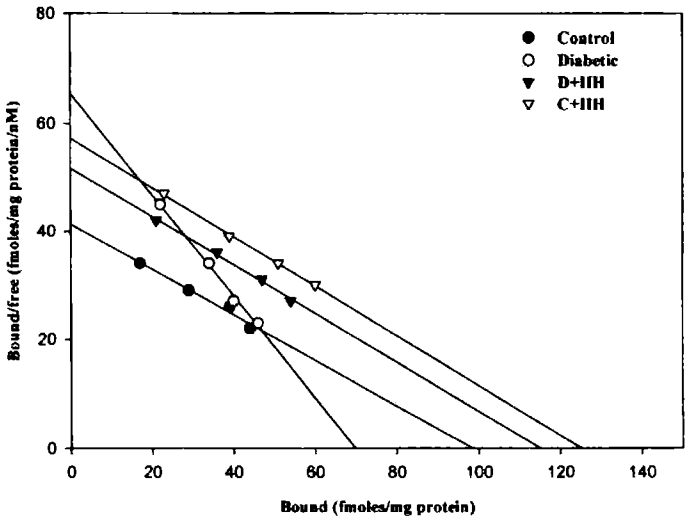


Table-26

Binding parameters of [^{3}H]Dopamine against dopamine in the corpus striatum of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	98.67 ± 3.18	2.067 ± 0.07
Diabetic	70.33 ± 6.01*	1.230 ± 0.07***
Diabetic+IIH	115.00 ± 2.65**¶¶¶	2.100 ± 0.07¶¶¶
Control+IIH	125.33 ± 5.78***¶¶¶	2.030 ± 0.05¶¶¶

Values are Mean ± S.E.M. of 4-6 separate experiments
*p<0.05, **p<0.01, ***p<0.001 when compared to control
¶¶¶p<0.001 when compared to diabetic
IIH- Insulin Induced Hypoglycaemia

Table 27

Binding parameters of [^{3}H]Dopamine against dopamine in the corpus striatum of control and experimental rats

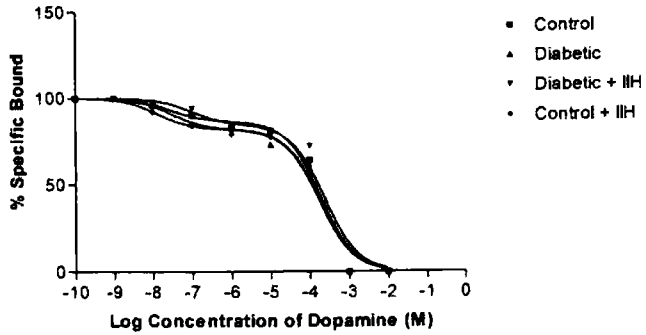
Animal status	Best-fit model	Log (EC_{50})	Ki	Hill slope
Control	One-site	-4.278	2.8070×10^{-5}	-0.9455
Diabetic	One-site	-4.411	3.5860×10^{-5}	-0.9227
Diabetic+IIH	One-site	-4.205	2.5610×10^{-5}	-0.9281
Control+IIH	One-site	-4.243	2.6490×10^{-5}	-0.9116

Values are mean of 4-6 separate experiments

IIH- Insulin Induced Hypoglycaemia

Figure – 23

Displacement analysis of [^{3}H]Dopamine against dopamine in corpus striatum of control and experimental rats



Data were fitted with 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 – 24

Scatchard analysis of DA D_1 receptor using [^{3}H]SCH-23390 against SCH-23390 in the corpus striatum of control and experimental rats

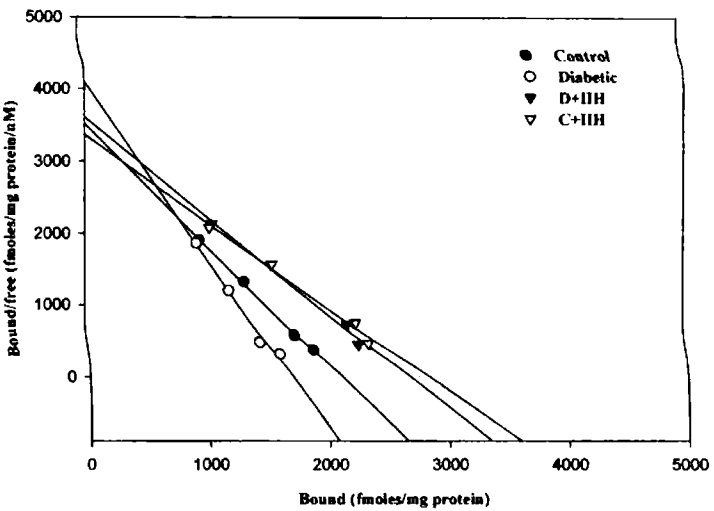


Table- 28

Binding parameters of [^{3}H]SCH-23390 against SCH-23390 in the corpus striatum of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	2600 ± 44	0.70 ± 0.02
Diabetic	2100 ± 52**	0.50 ± 0.06**
Diabetic+IIH	3400 ± 28***¶¶¶	0.94 ± 0.04**¶¶
Control+IIH	3600 ± 44***¶¶¶†	0.97 ± 0.01**¶¶

Values are Mean ± S.E.M. of 4-6 separate experiments
**p<0.01, ***p<0.001 when compared to control
¶¶p<0.01, ¶¶¶p<0.001 when compared to diabetic
†p<0.05 when compared to diabetic+IIH
IIH- Insulin Induced Hypoglycaemia

Table – 29

Binding parameters of [^{3}H]SCH 23390 against SCH 23390 in the corpus striatum of control and experimental rats

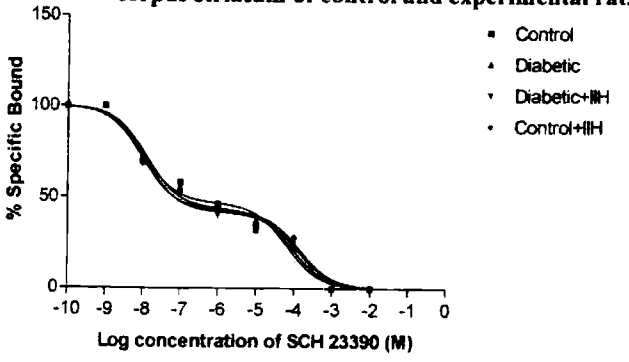
Animal status	Best fit model	Log(EC_{50})-1	Log(EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control	Two-site	-8.294	-4.413	2.8200×10^{-9}	1.7600×10^{-5}	-0.5349
Diabetic	Two-site	-8.258	-4.305	2.4340×10^{-9}	1.2950×10^{-5}	-0.5672
Diabetic+IIH	Two-site	-8.328	-4.533	3.0500×10^{-9}	2.3170×10^{-5}	-0.5860
Control+IIH	Two-site	-8.392	-4.666	3.3120×10^{-9}	2.9760×10^{-5}	-0.5754

Values are mean of 4-6 separate experiments

IIH- Insulin Induced Hypoglycaemia

Figure – 25

Displacement analysis of [^{3}H]SCH 23390 against SCH 23390 in corpus striatum of control and experimental rats



Data were fitted with iterative nonlinear regression software (Prism, GraphPad, San Diego, CA). Ki – The affinity of the receptor for the competing drug. The affinity for the first and second site of the competing drug are designated as $Ki_{(H)}$ (for high affinity) and $Ki_{(L)}$ (for low affinity). EC_{50} is the concentration of the competitor that competes for half the specific binding.

Figure – 26

Real-Time PCR amplification of Dopamine D_1 receptor mRNA from the corpus striatum of control and experimental rats

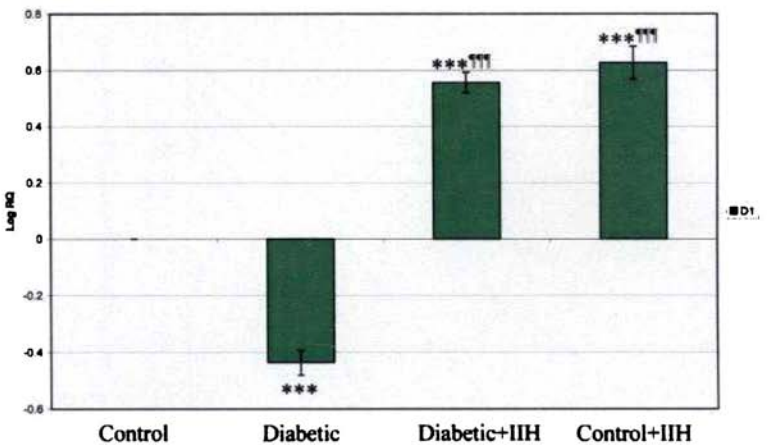


Table-30

Real-Time PCR amplification of Dopamine D_1 receptor mRNA from the corpus striatum of control and experimental rats

Animal status	Log RQ
Control	0
Diabetic	$-0.435 \pm 0.0428^{***}$
Diabetic+IIH	$0.556 \pm 0.036^{***¶¶¶}$
Control+IIH	$0.626 \pm 0.057^{***¶¶¶}$

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to control
¶¶¶p<0.001 when compared to diabetic
IIH- Insulin Induced Hypoglycaemia

Figure – 27

Scatchard analysis of DA D_2 receptor using [^{3}H]YM-09151-2 binding against sulpiride in the corpus striatum of control and experimental rats

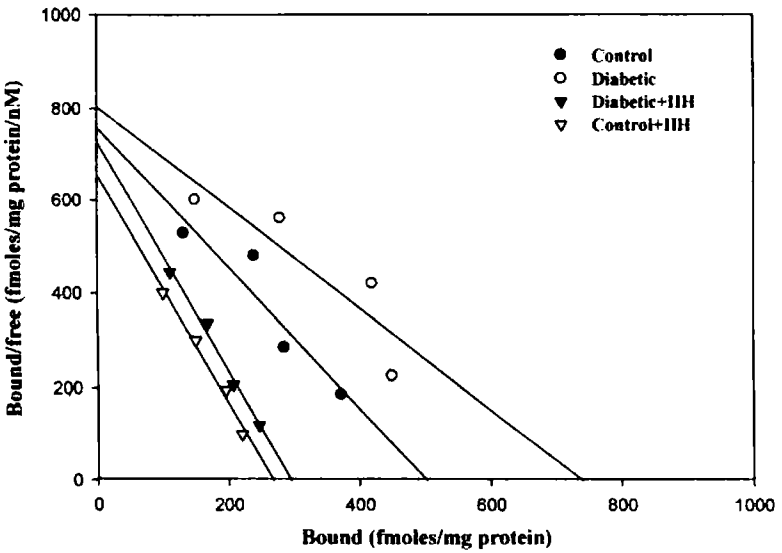


Table-31

Binding parameters of [^{3}H]YM-09151-2 against sulpiride in the corpus striatum of control and experimental rats

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Control	500.00 ± 12.09	0.64 ± 0.04
Diabetic	750.00 ± 15.19**	0.94 ± 0.06**
Diabetic+IIH	300.30 ± 10.87***¶¶¶	0.42 ± 0.01**¶¶¶
Control+IIH	280.63 ± 8.26***¶¶¶	0.45 ± 0.02**¶¶¶

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 diabetic
IIH- Insulin Induced Hypoglycaemia

Table 32

Binding parameters of [^{3}H]YM-09151-2 against sulpiride in the corpus striatum of control and experimental rats

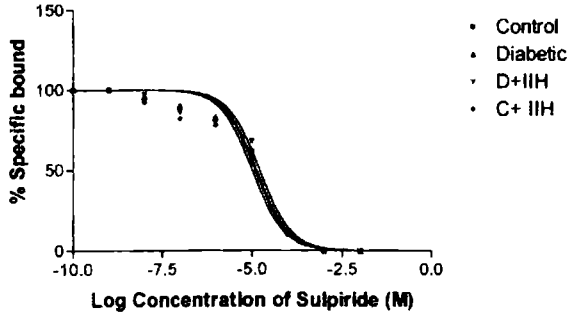
Animal status	Best-fit model	Log (EC_{50})	Ki	Hill slope
Control	One-site	-5.021	3.8070×10^{-5}	-0.9455
Diabetic	One-site	-6.976	4.1610×10^{-5}	-0.9227
Diabetic+IIH	One-site	-4.765	2.5860×10^{-5}	-0.9281
Control+IIH	One-site	-4. 905	2.6490×10^{-5}	-0.9116

Values are mean of 4-6 separate experiments

IIH- Insulin Induced Hypoglycaemia

Figure – 28

Displacement analysis of [^{3}H]YM-09151-2 against sulpiride in the corpus striatum of control and experimental rats



Data were fitted with 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 – 29

Real-Time PCR amplification of Dopamine D_2 receptor mRNA from the corpus striatum of control and experimental rats

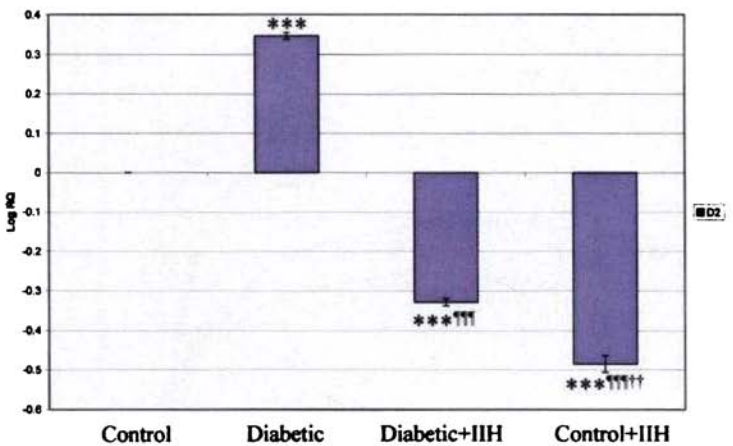


Table-33

Real-Time PCR amplification of Dopamine D_2 receptor mRNA from the corpus striatum of control and experimental rats

Animal status	Log RQ
Control	0
Diabetic	$0.346 \pm 0.008^{***}$
Diabetic+IIH	$-0.329 \pm 0.009^{***¶¶¶}$
Control+IIH	$-0.484 \pm 0.021^{***¶¶¶††}$

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to control
¶¶¶p<0.001 when compared to diabetic
††p<0.01 when compared to diabetic+IIH
IIH- Insulin Induced Hypoglycaemia

Figure – 30

Glutamate dehydrogenase activity in the brainstem of control and experimental rats

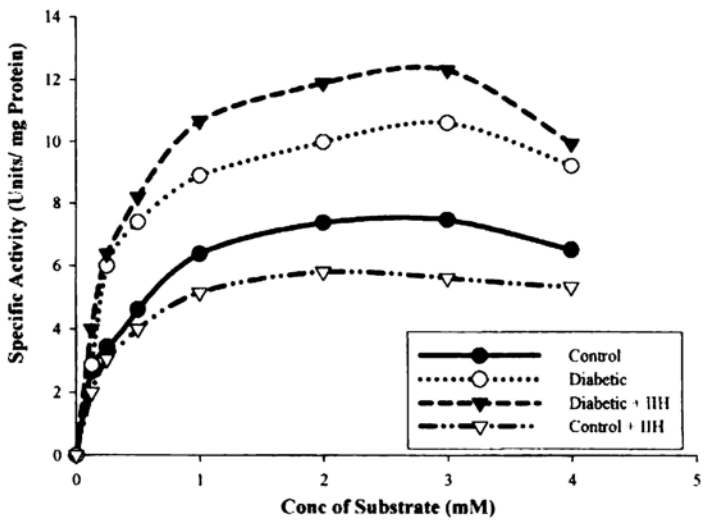


Table-34

Glutamate dehydrogenase activity in the brainstem of control and experimental rats

Animal status	V_{max} (Enyme Unit/mg protein)	K_m (mM)
Control	7.50 ± 0.72	0.35 ± 0.10
Diabetic	10.70 ± 0.17***	0.25 ± 0.06
Diabetic+IIH	12.50 ± 0.38***¶¶	0.25 ± 0.10
Control+IIH	5.90 ± 0.16***¶¶¶†††	0.25 ± 0.04

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to control
¶¶p<0.01, ¶¶¶p<0.001 when compared to diabetic
†††p<0.001 when compared to diabetic+IIH
IIH- Insulin Induced Hypoglycaemia

Figure – 31

Glutamate dehydrogenase activity in the cerebral cortex of control and experimental rats

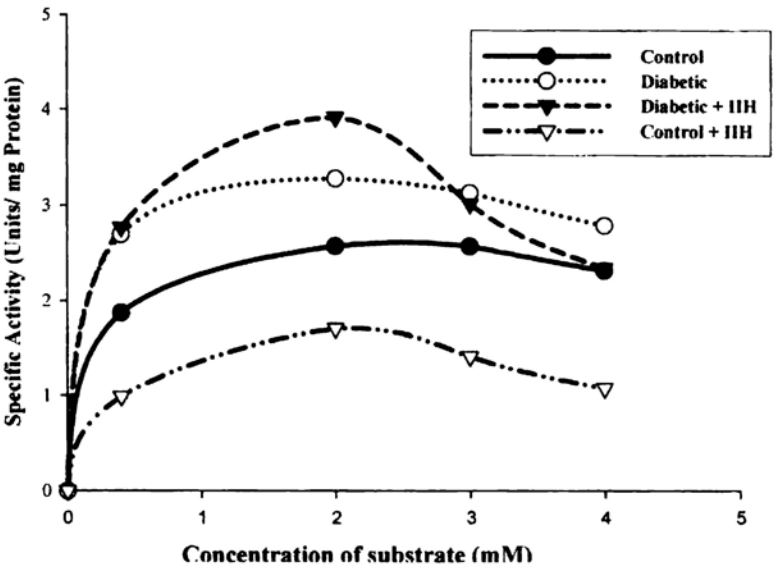


Table-35

Glutamate dehydrogenase activity in the cerebral cortex of control and experimental rats

Animal status	V_{max} (Enyme Unit/mg protein)	K_m (mM)
Control	2.6 ± 0.15	0.15 ± 0.10
Diabetic	3.3 ± 0.27***	0.15 ± 0.06
Diabetic+IIH	3.9 ± 0.33***¶	0.10 ± 0.10
Control+IIH	1.7 ± 0.11***¶¶††††	0.25 ± 0.04

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to control
¶p<0.05, ¶¶¶p<0.001 when compared to diabetic
†††p<0.001 when compared to diabetic+IIH
IIH- Insulin Induced Hypoglycaemia

Figure – 32

Malate dehydrogenase activity in the brainstem of control and experimental rats

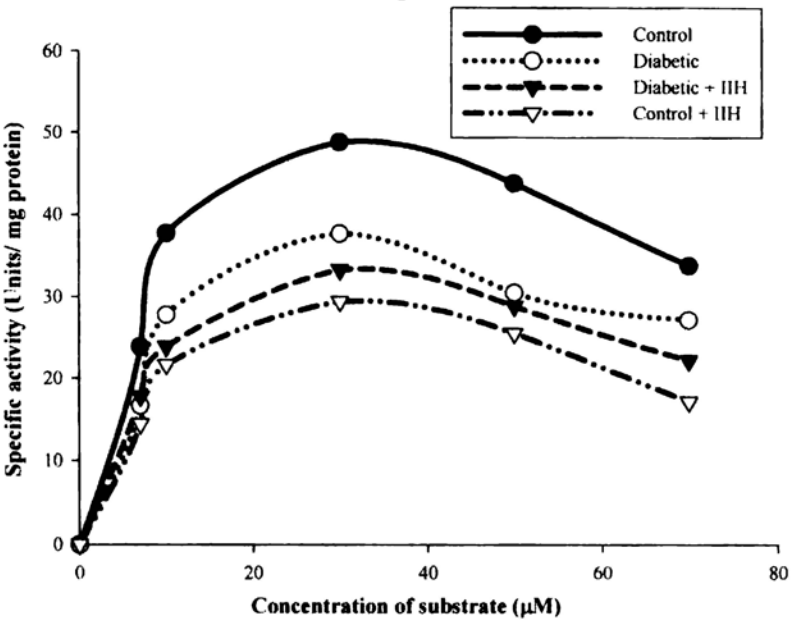


Table-36

Malate dehydrogenase activity in the brainstem of control and experimental rats

Animal status	V_{max} (Enyme Unit/mg protein)	K_m (μM)
Control	49.00 ± 2.67	7.25 ± 0.10
Diabetic	37.75 ± 3.33***	7.50 ± 0.82
Diabetic+IIH	33.50 ± 1.80***¶¶	7.00 ± 0.77
Control+IIH	30.00 ± 1.92***¶¶¶	7.20 ± 0.90

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to control
¶¶p<0.01, ¶¶¶p<0.001 when compared to diabetic
IIH- Insulin Induced Hypoglycaemia

Figure – 33

Malate dehydrogenase activity in the cerebral cortex of control and experimental rats

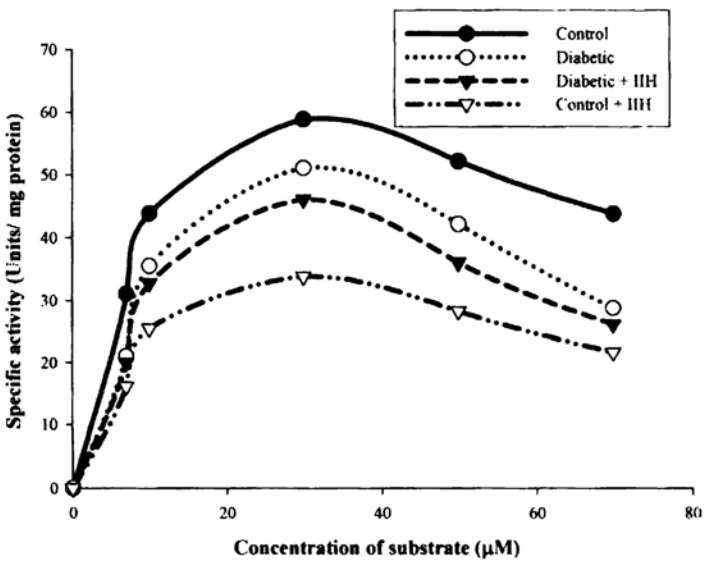


Table-37

Malate dehydrogenase activity in the cerebral cortex of control and experimental rats

Animal status	V_{max} (Enyme Unit/mg protein)	K_m (μM)
Control	59.5 ± 2.67	7.00 ± 0.10
Diabetic	51.5 ± 3.33 **	7.25 ± 0.82
Diabetic+IIH	47.0 ± 1.80 **¶	7.20 ± 0.77
Control+IIH	34.0 ± 1.92 ***¶¶††	7.25 ± 0.90

Values are Mean ± S.E.M. of 4-6 separate experiments
**p<0.01, ***p<0.001 when compared to control
¶p<0.05, ¶¶¶p<0.001 when compared to diabetic
†††p<0.001 when compared to diabetic+IIH
IIH- Insulin Induced Hypoglycaemia

Figure – 34

Real-Time PCR amplification of Glutamate receptor (NMDAR1) mRNA from the hippocampus of control and experimental rats

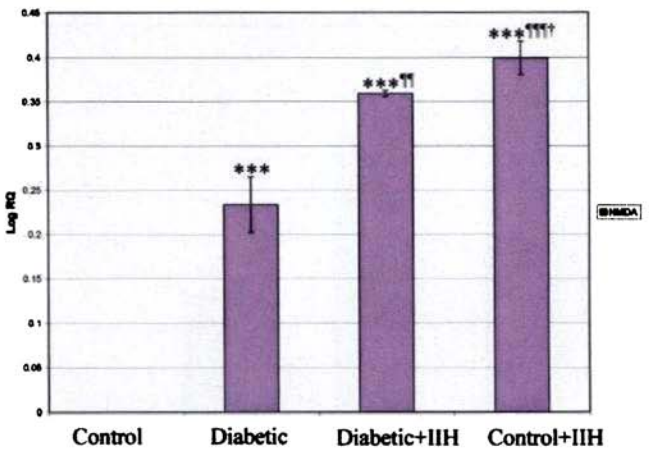


Table-38

Real-Time PCR amplification of Glutamate receptor (NMDAR1) mRNA from the hippocampus of control and experimental rats

Animal status	Log RQ
Control	0
Diabetic	$0.233 \pm 0.031^{***}$
Diabetic+IIH	$0.359 \pm 0.003^{***¶¶}$
Control+IIH	$0.399 \pm 0.019^{***¶¶¶†}$

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to control
¶¶p<0.01, ¶¶¶p<0.001 when compared to diabetic
†p<0.05 when compared to diabetic+IIH
IIH- Insulin Induced Hypoglycaemia

Figure – 35

Real-Time PCR amplification of Glutamate receptor (NMDAR1) mRNA from the brainstem of control and experimental rats

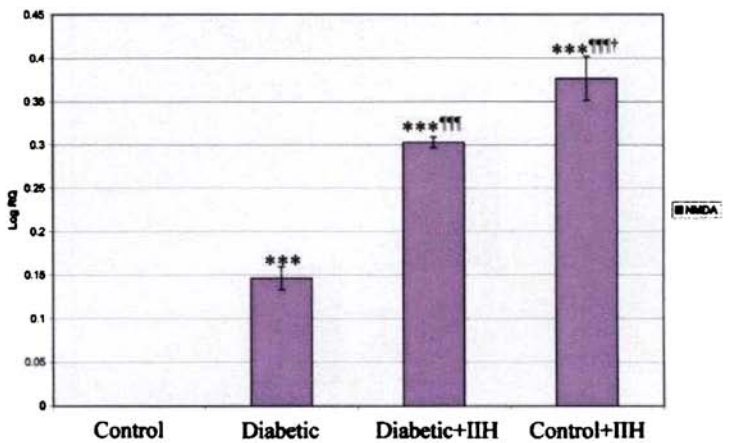


Table-39

Real-Time PCR amplification of Glutamate receptor (NMDAR1) mRNA from the brainstem of control and experimental rats

Animal status	Log RQ
Control	0
Diabetic	$0.146 \pm 0.013^{***}$
Diabetic+IIH	$0.302 \pm 0.006^{***¶¶¶}$
Control+IIH	$0.376 \pm 0.025^{***¶¶¶†}$

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to control
¶¶¶p<0.001 when compared to diabetic
†p<0.05 when compared to diabetic+IIH
IIH- Insulin Induced Hypoglycaemia

Figure – 36

Real-Time PCR amplification of Glutamate receptor (NMDAR1) mRNA from the cerebral cortex of control and experimental rats

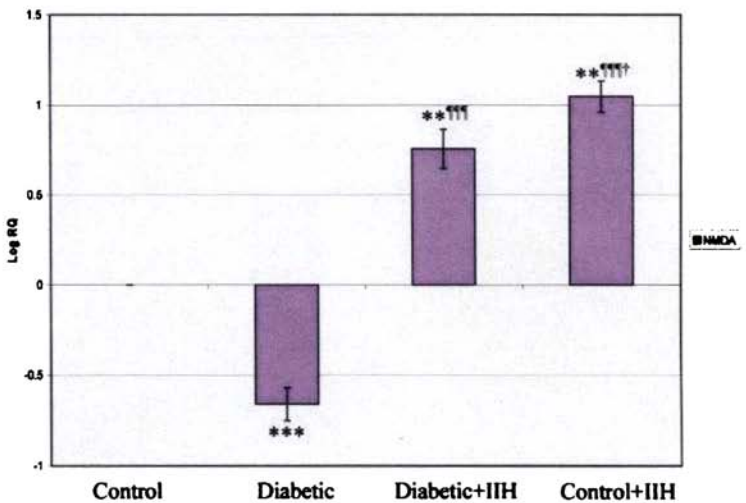


Table-40

Real-Time PCR amplification of Glutamate receptor (NMDAR1) mRNA from the cerebral cortex of control and experimental rats

Animal status	Log RQ
Control	0
Diabetic	-0.66 ± 0.091***
Diabetic+IIH	0.75 ± 0.108**¶¶¶
Control+IIH	1.04 ± 0.087**¶¶¶†

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 diabetic
†p<0.05 when compared to diabetic+IIH
IIH- Insulin Induced Hypoglycaemia

Figure – 37

Real-Time PCR amplification of Glutamate receptor (NMDAR1) mRNA from the corpus striatum of control and experimental rats

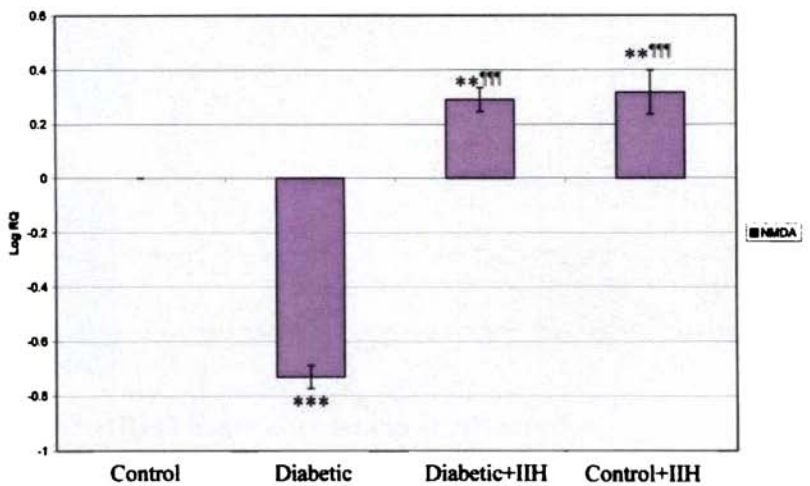


Table-41

Real-Time PCR amplification of Glutamate receptor (NMDAR1) mRNA from the corpus striatum of control and experimental rats

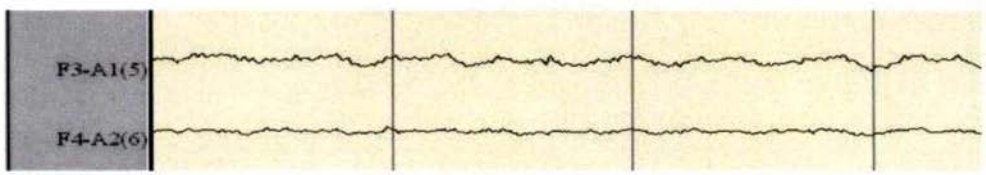
Animal status	Log RQ
Control	0
Diabetic	$-0.73 \pm 0.042^{***}$
Diabetic+IIH	$0.29 \pm 0.043^{**¶¶¶}$
Control+IIH	$0.31 \pm 0.08^{**¶¶¶}$

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 diabetic
IIH- Insulin Induced Hypoglycaemia

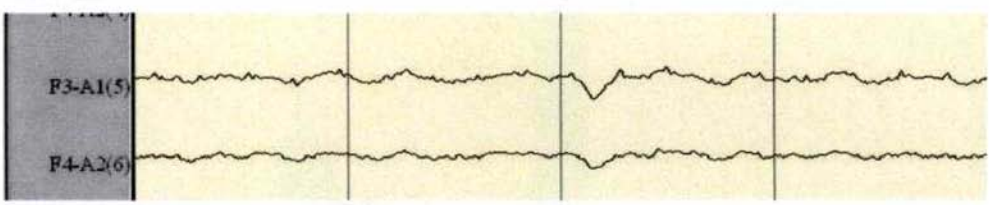
Figure – 38

EEG brain wave pattern in the frontal region on 10th day of experiment

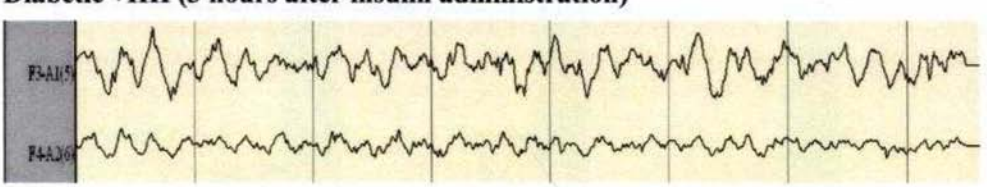
Control



Diabetic



Diabetic +IIH (3 hours after insulin administration)



Control + IIH (1 hour after insulin administration)

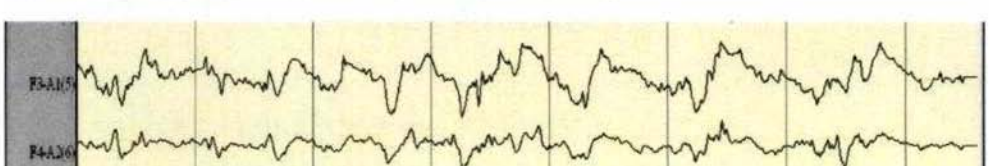
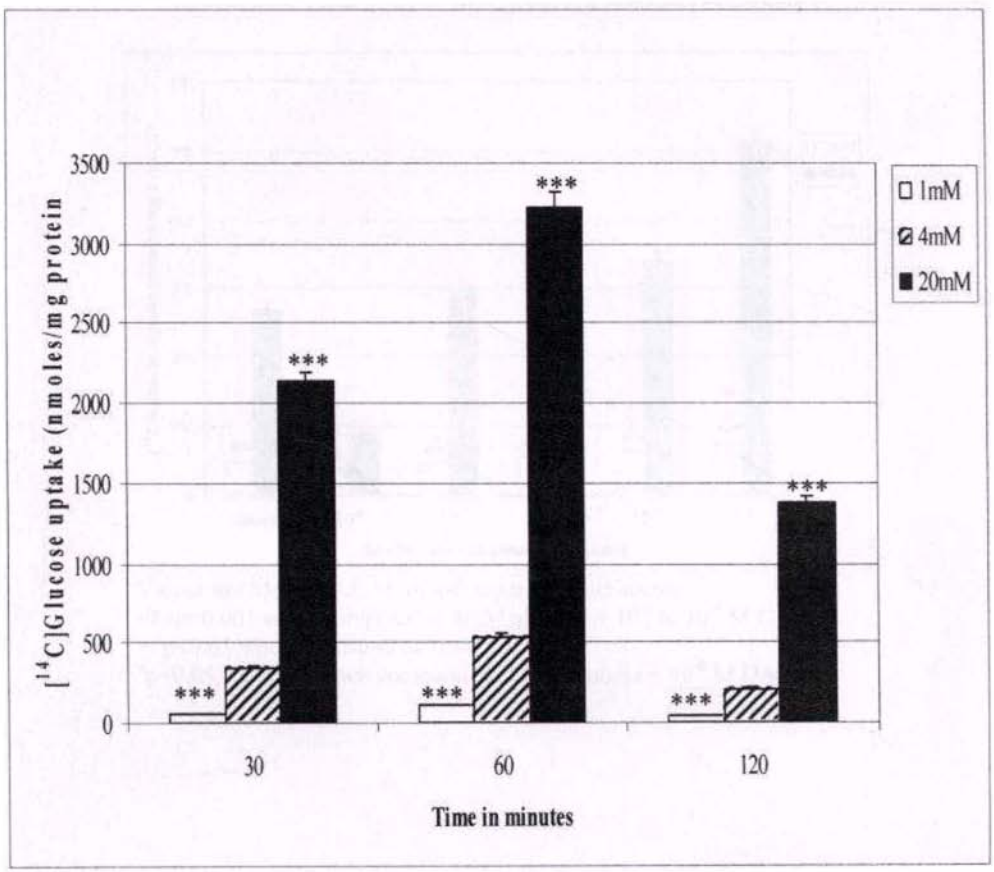


Figure – 39

Time dependant [^{14}C]Glucose uptake by pancreatic islets *in vitro*

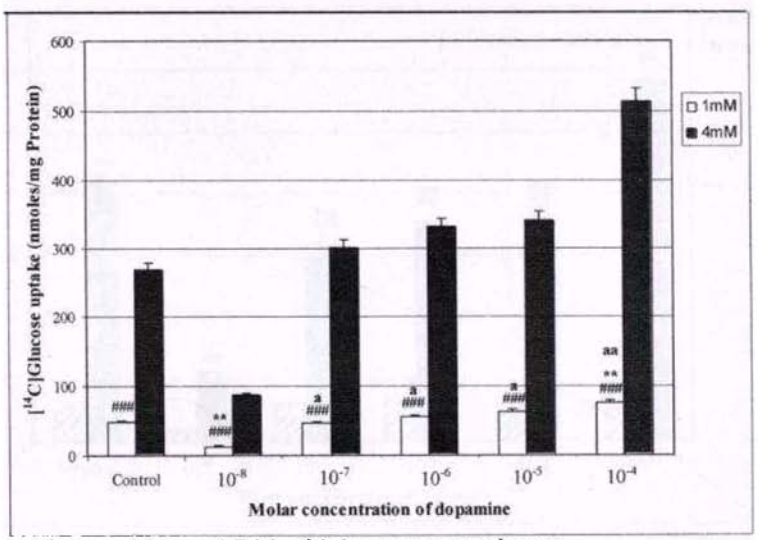


Values are Mean ± S.E.M. of 4-6 separate experiments

***p<0.001 when compared to 4mM glucose at respective time

Figure – 40

Effect of Dopamine (10^{-8} to 10^{-4} M) on [^{14}C]Glucose uptake by pancreatic islets *in vitro* in hypoglycaemic concentration



Values are Mean ± S.E.M. of 4-6 separate experiments

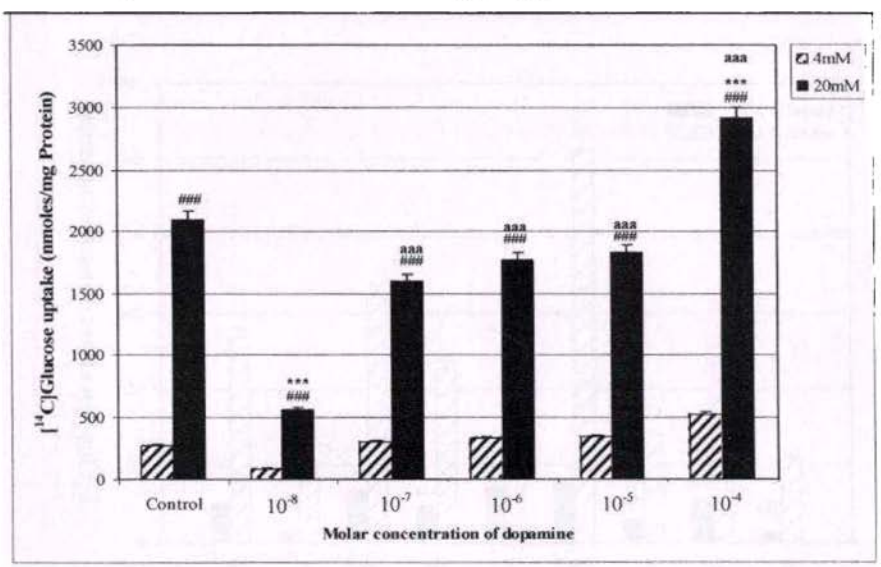
###p<0.001 when compared to 4mM glucose + 10^{-8} to 10^{-4} M DA

**p<0.01 when compared to 1mM control

^{a}p<0.05, ^{aa}p<0.01 when compared to 1mM glucose + 10^{-8} M DA

Figure – 41

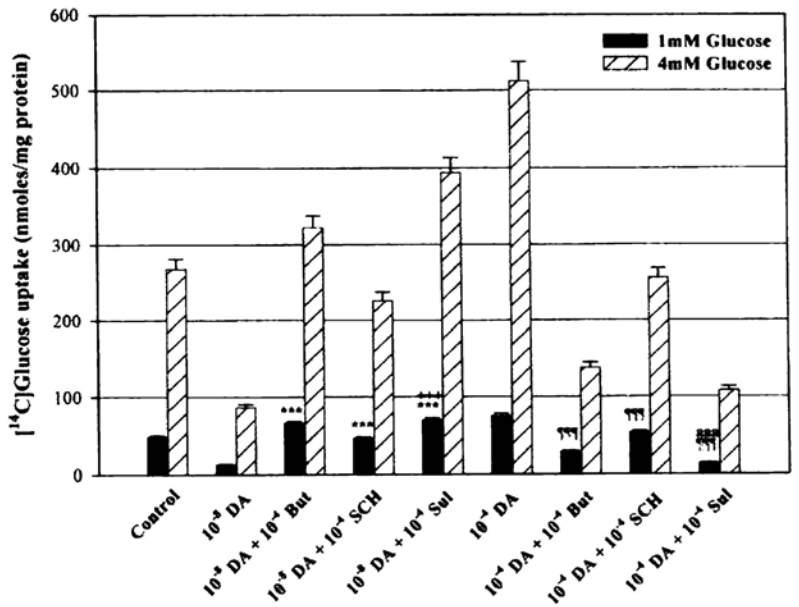
Effect of Dopamine (10^{-8} to 10^{-4} M) on [^{14}C]Glucose uptake by pancreatic islets *in vitro* in hyperglycaemic concentration



Values are Mean ± S.E.M. of 4-6 separate experiments
###p<0.001 when compared to 4mM glucose + 10^{-8} to 10^{-4} M DA
***p<0.001 when compared to 20mM control
^{aaa}p<0.001 when compared to 1mM glucose + 10^{-8} M DA

Figure – 42

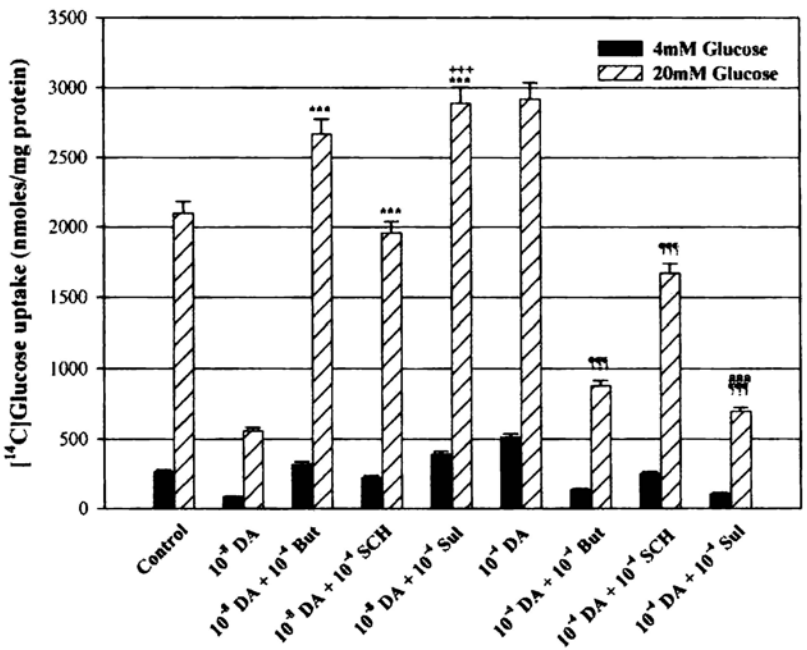
Effect of dopamine antagonists on [^{14}C]Glucose uptake by pancreatic islets *in vitro* in hypoglycaemic concentration



Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to 10^{-8} M DA
+++p<0.001 when compared to 10^{-8} M DA+10^{-4} M SCH 23390
¶¶¶p<0.001 when compared to 10^{-4} M DA
^{aaa} p<0.001 when compared to 10^{-4} M DA+10^{-4} M SCH 23390
But – Butaclamol, SCH – SCH 23390, Sul - Sulpiride

Figure – 43

Effect of dopamine antagonists on [^{14}C]Glucose uptake by pancreatic islets *in vitro* in hyperglycaemic concentration

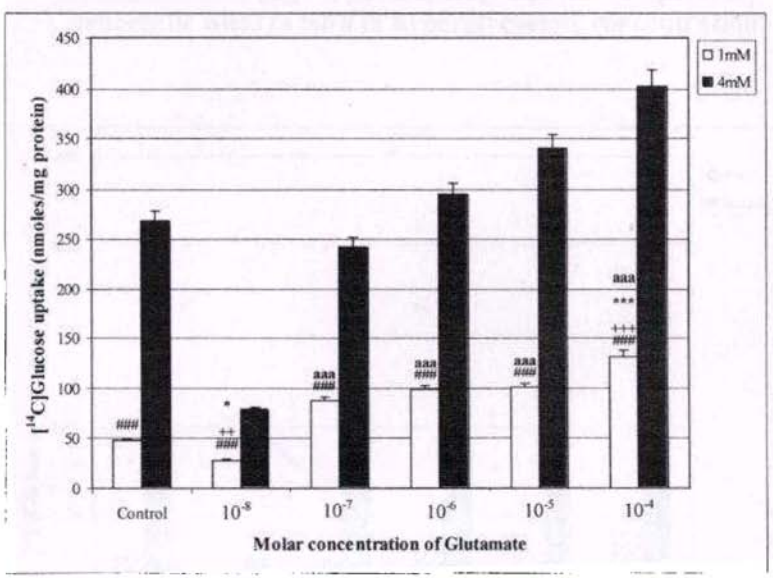


Molar concentration of neurotransmitters and ligands

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to 10^{-8} M DA
+++p<0.001 when compared to 10^{-8} M DA+10^{-4} M SCH 23390
¶¶¶p<0.001 when compared to 10^{-4} M DA
^{aaa} p<0.001 when compared to 10^{-4} M DA+10^{-4} M SCH 23390

Figure – 44

Effect of glutamate (10^{-8} to 10^{-4} M) on [^{14}C]Glucose uptake by pancreatic islets *in vitro* in hypoglycaemic concentration



Values are Mean ± S.E.M. of 4-6 separate experiments

#p<0.05, ###p<0.001 when compared to 4mM glucose + 10^{-8} to 10^{-4} M Glutamate

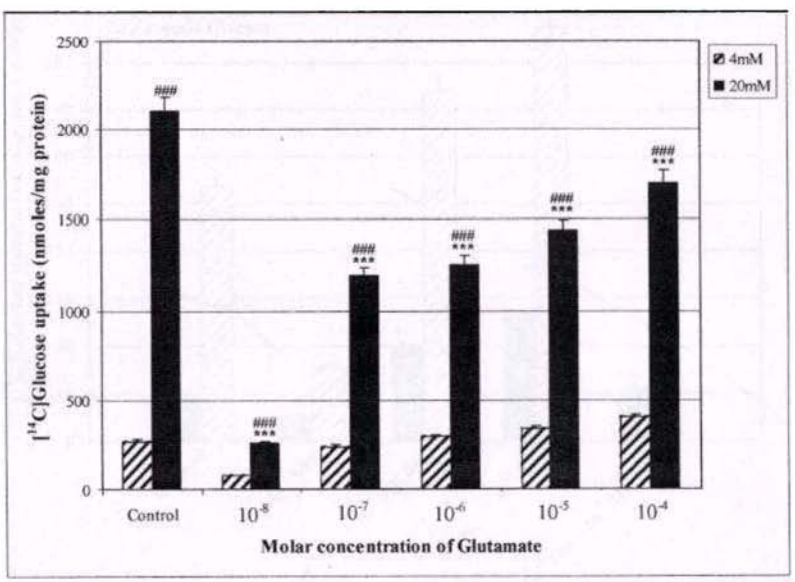
*p<0.05, ***p<0.001 when compared to 4mM control

++p<0.01, +++p<0.001 when compared to 1mM control

^{aaa}p<0.001 when compared to 1mM glucose + 10^{-8} M Glutamate

Figure – 45

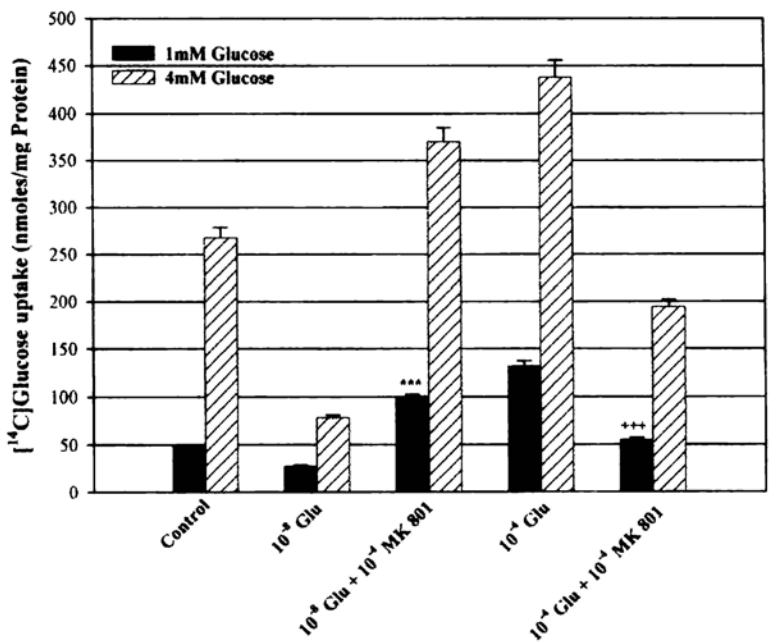
Effect of glutamate (10^{-8} to 10^{-4} M) on [^{14}C]Glucose uptake by pancreatic islets *in vitro* in hyperglycaemic concentration



Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to 20mM control
###p<0.001 when compared to 4mM glucose + 10^{-8} to 10^{-4} M Glutamate

Figure – 46

Effect of NMDA antagonist on [^{14}C]Glucose uptake by pancreatic islets *in vitro* in hypoglycaemic concentration

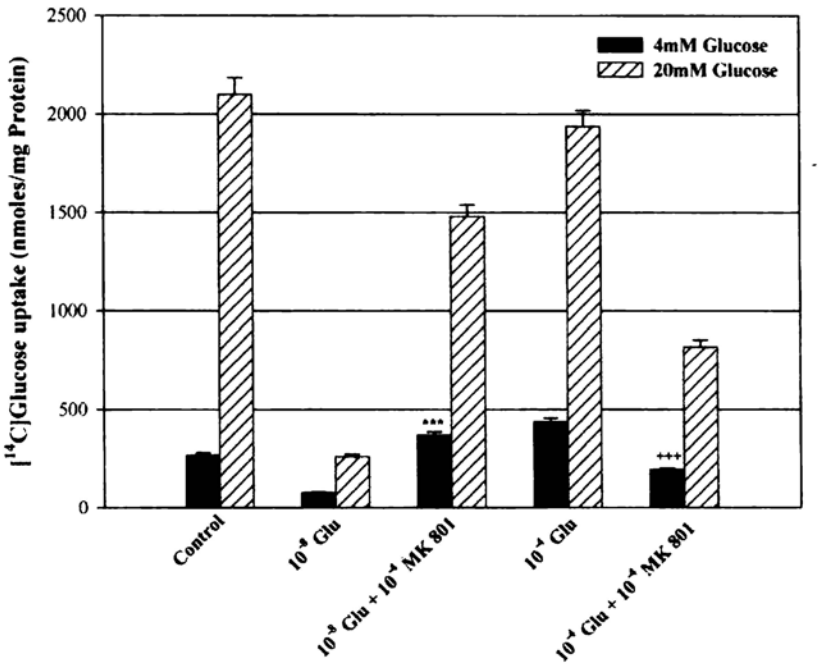


Molar concentration of neurotransmitter and ligands

Values are Mean ± S.E.M. of 4-6 separate experiments
***$p<0.001$ when compared to 10^{-8} M Glutamate
+++$p<0.001$ when compared to 10^{-4} M Glutamate

Figure – 47

Effect of NMDA antagonist on [^{14}C]Glucose uptake by pancreatic islets *in vitro* in hyperglycaemic concentration

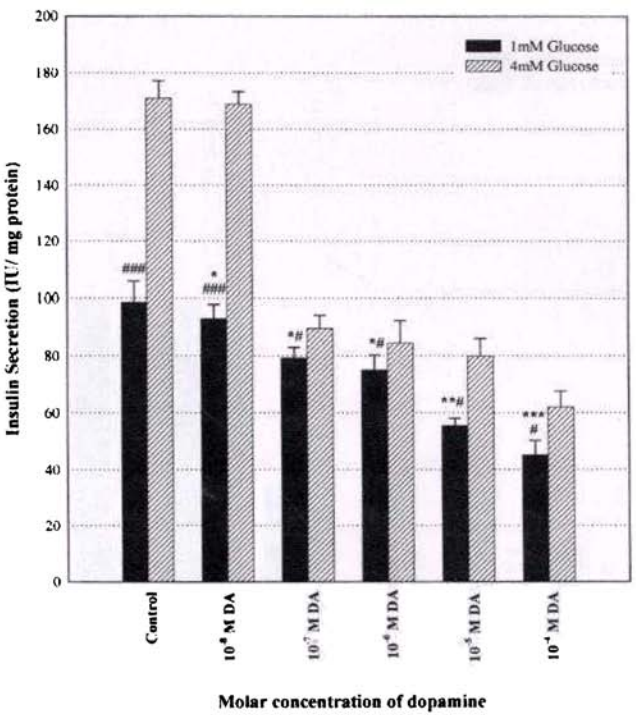


Molar concentration of neurotransmitter and ligands

Values are Mean ± S.E.M. of 4-6 separate experiments
***$p<0.001$ when compared to 10^{-8} M Glutamate
+++$p<0.001$ when compared to 10^{-4} M Glutamate

Figure – 48

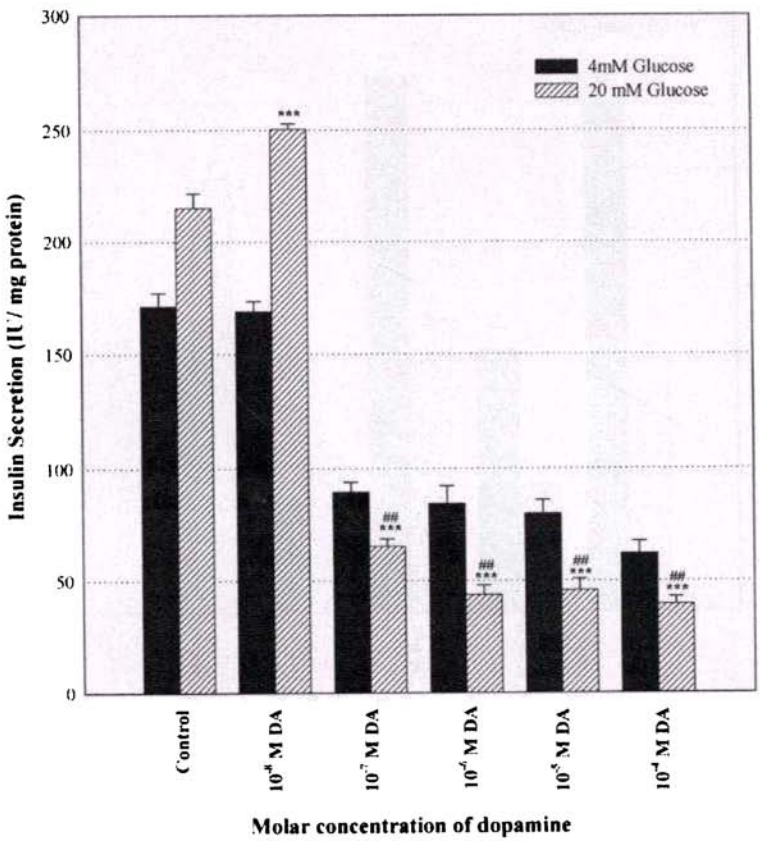
Effect of dopamine (10^{-8} to 10^{-4} M) on insulin secretion by pancreatic islets *in vitro* in hypoglycaemic concentration



Values are Mean ± S.E.M. of 4-6 separate experiments
*p<0.05, **p<0.01, ***p<0.001 when compared to 1mM control
#p<0.05, ###p<0.001 when compared to 4mM Glucose + 10^{-8} to10^{-4} M DA

Figure – 49

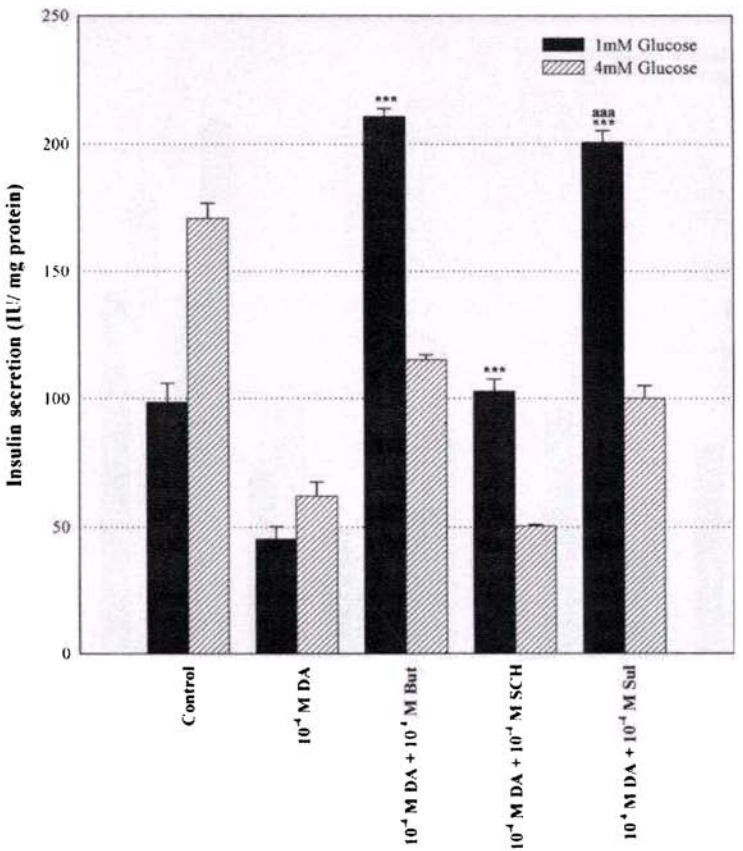
Effect of dopamine (10^{-8} to 10^{-4} M) on insulin secretion by pancreatic islets *in vitro* in hyperglycaemic concentration



Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to 20mM control
#p<0.05, ##p<0.01 when compared to 4mM Glucose + 10^{-7} to10^{-4} M DA

Figure – 50

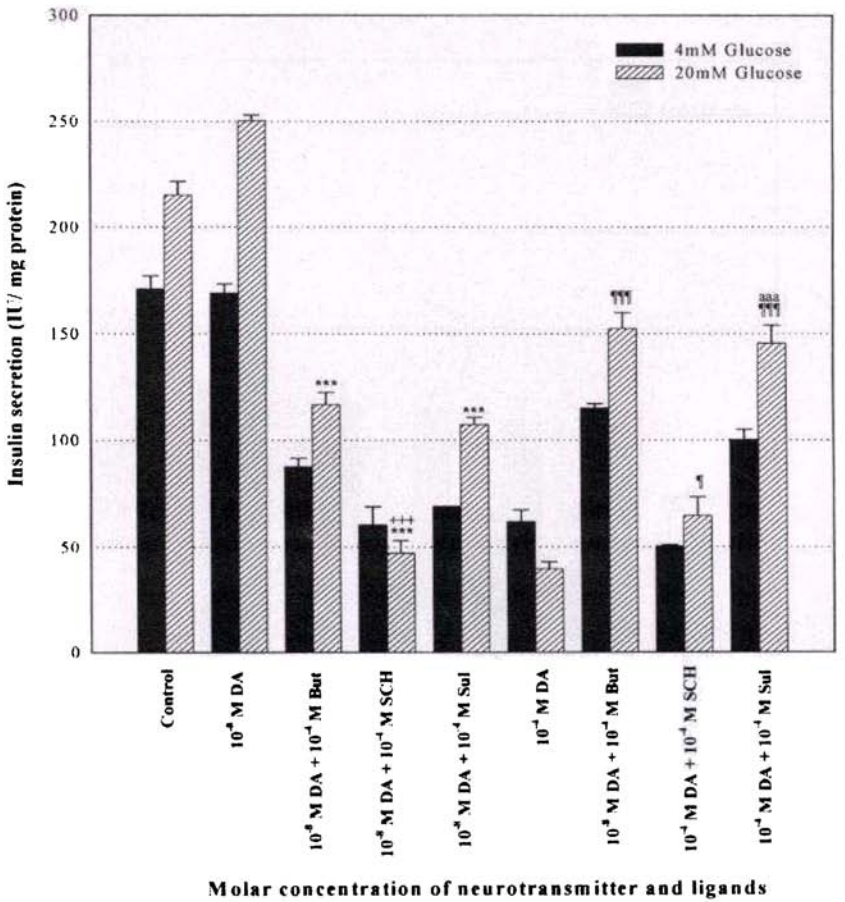
Effect of dopamine antagonists on insulin secretion by pancreatic islets *in vitro* in hypoglycaemic concentration



Values are Mean ± S.E.M. of 4-6 separate experiments
**p<0.01, ***p<0.001 when compared to 10^{-4} M DA
^{aaa} p<0.001 when compared to 10^{-4} M DA+10^{-4} M SCH 23390
But – Butaclamol, SCH – SCH 23390, Sul - Sulpiride

Figure – 51

Effect of dopamine antagonists on insulin secretion by pancreatic islets *in vitro* in hyperglycaemic concentration



Values are Mean ± S.E.M. of 4-6 separate experiments

***$p<0.001$ when compared to 10^{-8} M DA

+++$p<0.001$ when compared to 10^{-8} M DA+10^{-4} M Sulpiride

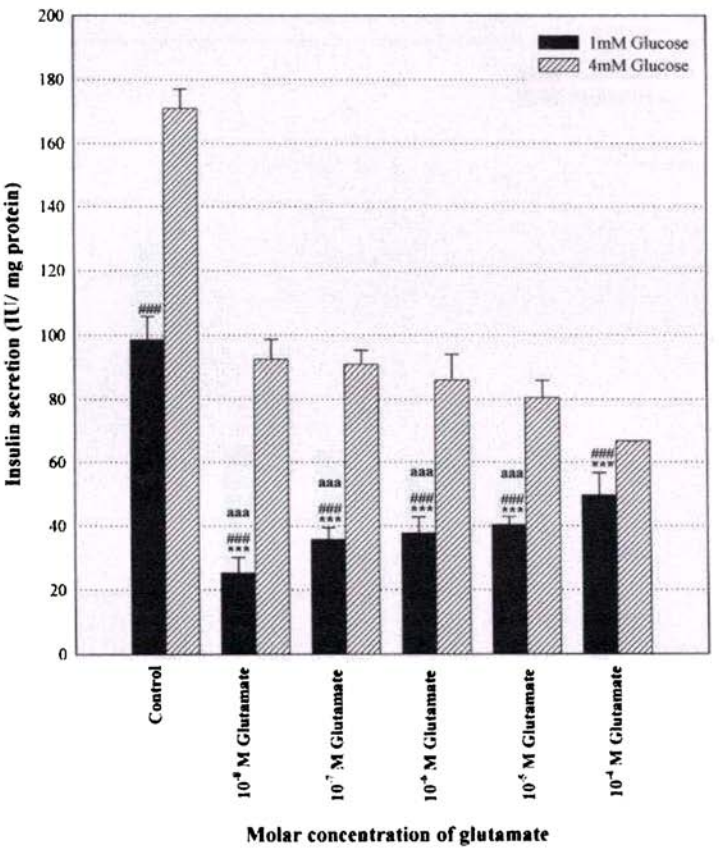
¶$p<0.05$, ¶¶¶$p<0.001$ when compared to 10^{-4} M DA

^{aaa} $p<0.001$ when compared to 10^{-4} M DA+10^{-4} M SCH 23390

But – Butaclamol, SCH – SCH 23390, Sul - Sulpiride

Figure – 52

Effect of glutamate (10^{-8} to 10^{-4} M) on insulin secretion by pancreatic islets *in vitro* hypoglycaemic concentration



Values are Mean ± S.E.M. of 4-6 separate experiments

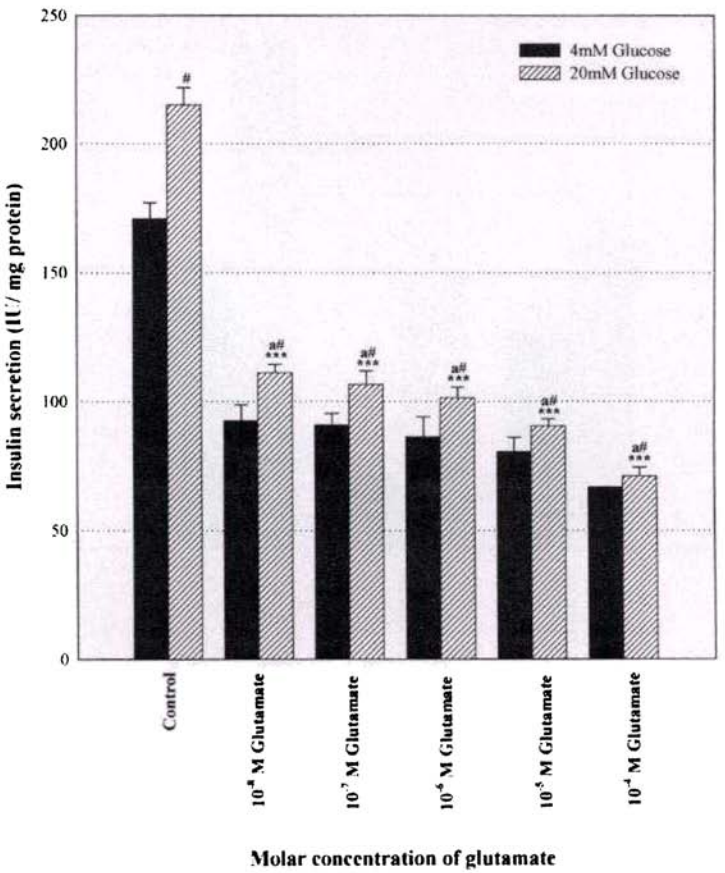
***p<0.001 when compared to 1mM control

###p<0.001 when compared to 4mM Glucose + 10^{-8} to 10^{-4} M Glutamate

^{aaa}p<0.001 when compared to 1mM Glucose + 10^{-4} M Glutamate

Figure – 53

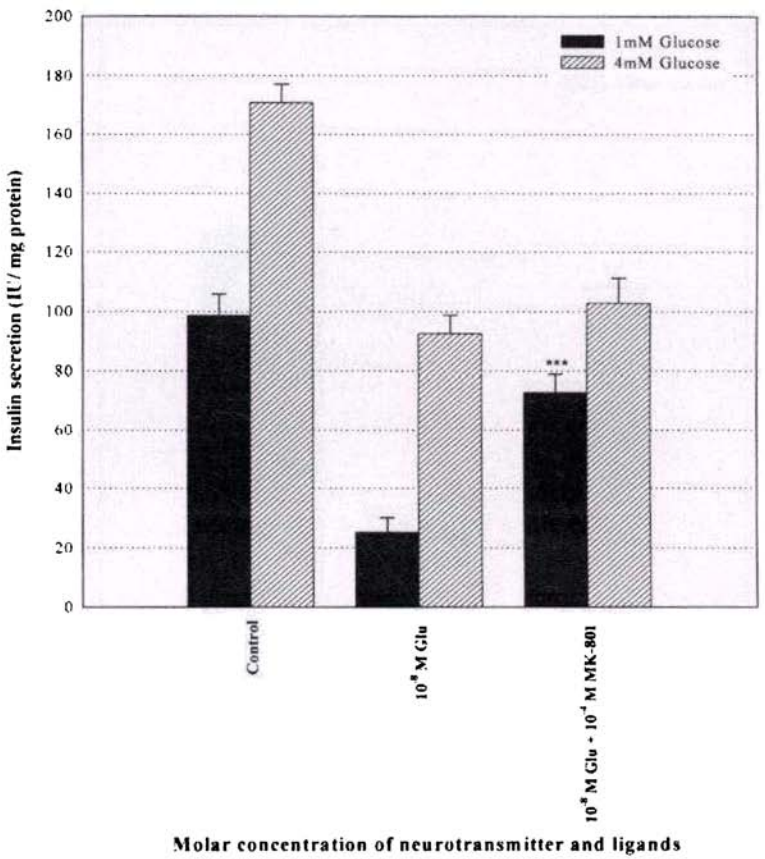
Effect of glutamate (10^{-8} to 10^{-4} M) on insulin secretion by pancreatic islets *in vitro* hyperglycaemic concentration



Values are Mean ± S.E.M. of 4-6 separate experiments
***$p<0.001$ when compared to 20mM control
#$p<0.05$ when compared to 4mM Glucose + 10^{-8} to10^{-4} M Glutamate
[a]$p<0.05$ when compared to 20mM Glucose + 10^{-8} M Glutamate

Figure – 54

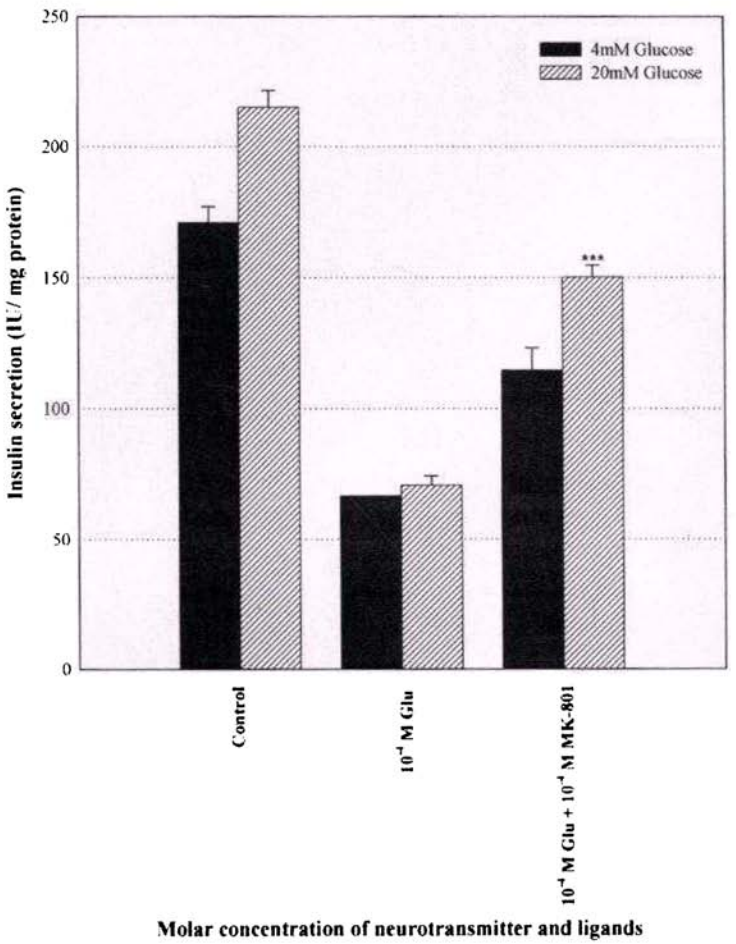
Effect of NMDA antagonist, MK-801 on insulin secretion by pancreatic islets *in vitro* at hypoglycaemic concentration



Values are Mean ± S.E.M. of 4-6 separate experiments
***$p<0.001$ when compared to 10^{-8} M Glutamate

Figure – 55

Effect of NMDA antagonist, MK-801 on insulin secretion by pancreatic islets *in vitro* at hyperglycaemic concentration



Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to 10^{-4} M Glutamate