DOPAMINE D_2 RECEPTOR FUNCTIONAL REGULATION: cAMP AND IP3 ROLE IN PANCREATIC REGENERATION

THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

UNDER THE FACULTY OF SCIENCE

OF

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

BY

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March 2006

CERTIFICATE

This is to certify that the thesis entitled **"DOPAMINE D_2 RECEPTOR FUNCTIONAL REGULATION: cAMP AND IP3 ROLE IN PANCREATIC REGENERATION"** is a bonafide record of the research work carried out by **Mr. SANTHOSH THOMAS K** under my guidance and supervision in the Department of Biotechnology, Cochin University of Science and Technology and that no part thereof has been presented for the award of any other degree.

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DECLARATION

I hereby declare that this thesis entitled **"DOPAMINE D_2 RECEPTOR FUNCTIONAL REGULATION: cAMP AND IP3 ROLE IN PANCREATIC REGENERATION"** 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, Head and Reader, 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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DEDICATED TO MY BELOVED PARENTS

ACKNOWLEDGEMENT

I wish to express my sincere heartfelt gratitude and indebtedness to my supervising guide, Dr. C.S. Paulose, Head Department of Biotechnology, Director Centre for Neuroscience for his sustained interest, motivation and exemplary guidance throughout the course of this work. His encouragement and timely advice will always be remembered with a deep sense of gratitude.

I would like to extend my sincere thanks to Dr. Oommen V. Oommen, Professor and Head, Department of Zoology, University of Kerala, and Dr. Babu Philip, Department of Marine Biology, Microbiology and Biochemistry, CUSAT for their valuable suggestions and encouragement. I thank Prof E. Vijayan for all his timely help and encouragement during the course of my Ph.D.

I wish to record my profound thanks to Prof. M. Chandrasekaran, Mr. Elyas K.K, Dr. Saritha G Bhat and Dr. Padma Nambisan for all their help, advice and encouragement. I also thank for their encouragement and friendly nature.

My special thanks to Dr.Mithradas M Panicker for allowing and helping me to work in his lab at NCBS, Bangalore. I can not forget the timely help that was provided by Basudha Basu, Aditi Bhattacharya, Parthasarathi, S.Suresh kannan, Imtiaz Zaffa and Geetha M.S.

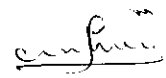
I sincerely acknowledge my seniors colleagues Dr. P.N Eswar Shankar, Dr.Renuka T.R, Dr. Mohanan V.V and Mr. Balarama Kaimal for their timely help and unfailing support.

My friends and colleagues Mr. Akash K George, Mr. Gireesh G, Mr.Reas Khan M.S and Rakesh S who had always been there with a helping hand through out the period of my work. I thank them for all their affection, friendship and help which I shall always cherish.

I express my sincere gratitude to the office staff of Department of Biotechnology, for their help and support. I also thank all other research scholars and all M.Sc. students of the department of Biotechnology for their friendship, help and co-operation. I thank Mr. Sabumon M .S and Mr. K.A. Maniram for their timely help and support.

I thank all the teachers of my school days, graduation and post-graduation for inspiring and encouraging me. I would like to extend my sincere thanks to Department of Science and Technology, Delhi for supporting this work with fellowships.

Words fail me when I speak about my beloved parents and only sister, Smitha Thomas K, for their constant encouragement, affection and support. There are so many others whom I may have inadvertently left out and I sincerely thank all of them for their help.



Santhosh Thomas K

My tribute... *To a number of animals who have paid a price with their lives and suffering in the name of human protection. I pay my tribute to their sacrifice and pray that it is not in vein. I look forward for a day when animal testing is no longer necessary and alternatives are found.*

ABBREVIATIONS USED IN THE TEXT

5-HIAA	5-Hydroxyindole acetic acid
5HT	5 Hydroxy tryptamine
5-HTP	5-hydroxy tryptophan
8-OH-DPAT	8-Hydroxy-2(di-n-propylamino)-tetralin
ACH	Acetylcholine
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
bHLH	basic Helix-loop-helix protein
B_{max}	Maximal binding
BS	Brain stem
C/EBP β	CCAAT /Enhancer binding protein β
cAMP	cyclic Adenosine mono phosphate
CC	Cerebral cortex
CNS	Central nervous system
CSF	Cerbro spinal fluid
Ct	crossing threshold
DA	Dopamine
DA D_2	Dopamine D_2
DEPC	Di ethyl pyro carbonate
DOI	1-(2,5-di-methoxy-4-iodophenyl)-2-aminopropane
DTT	Dithiothreitol
ECD	Electro chemical detector
EGF	Epidermal growth factor
EPI	Epinephrine
FCS	Fetal calf serum
GABA	Gamma aminobutyric acid

GABA	Gama Aminobutyric Acid
GH	growth hormone
GOD	Glucose oxidase
Gpp[NH]p	5'-Guanylyl-imidodiphosphate
GTP	Guanosine triphosphate
HPLC	High performance liquid chromatography
HVA	homovanillic acid
HYPO	Hypothalamus
i.p	Intraperitoneally
IAPP	Islet amyloid polypeptide
IBMX	3-isobutyl-1-methylxanthine
IP3	inositol 1,4,5-triphosphate
K_d	Dissociation constant
Ki	Dissociation Constant
MIF	Macrophage migration inhibiting factor
mRNA	messenger Ribonucleic acid
MUMLV	Murine moloney leukemia virus reverse transcriptase
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NE	Norepinephrine
NMN	Normetanephrine
NO	Nitric Oxide
p	Level of significance
p-CPA	para-Chlorophenylalanine
PCR	Polymerase Chain Reactor
PEG	Polyethylene glycol
PHV	parahypothalamic ventricular

Pi	Inorganic phosphate
PKC	Protein kinase C
PL	placental lactogen
POD	Peroxidase
PRL	prolactin
RIA	Radioimmuno assay
RT-PCR	Reverse-transcription-polymerase chain reaction
S.E.M	Standard error of mean
SDS-PAGE	Sodium dodecyl sulphate-poly acrilamide gel electrophoresis
T_3	Tri iodothyronine
T_4	Thyroxine
TRH	Thyrotropin releasing hormone
TSH	Thyroid stimulating hormone
VMH	Ventro medial hypothalamus
YM-09151-2	cis–N-(1-benzyl-2-methylpyrrolidine-3-yl)-5-chloro-2-methoxy-4-methylaminobenzamide

INTRODUCTION

Communication within and between the cell and organs are necessary for the co-ordination of an organism. In multi cellular organisms, neurotransmitters and the hormones are the major signaling molecules and its function through its receptor subtypes play an important role in governing the cellular activities such as metabolism, gene expression and cell division. However, these communication systems seem to be altered in response to changes within and out side of the living system. Living system has adopted two different but inter-related mode of communication; neurotransmitter and hormone mediated. Neurotransmitter mediated communication is faster than hormone mediated. The co-ordinated action of both hormonal and neurotransmitter communication help the body to regulate the vital activities of living system such as growth, movement and reproduction. Cellular functions such as metabolic activities, gene expression and cell division are also controlled by the central nervous system through the neurotransmitter and neurotransmitter-hormonal functional regulation.

Diabetes mellitus is a chronic condition associated with abnormally high levels of glucose in the blood. People with diabetes either do not produce enough insulin- a hormone that is needed for the metabolism of glucose-or cannot use the insulin that their body produces. As a result, glucose concentration increases in the bloodstream. Chronic hyperglycemia can induce multiple cellular changes leading to metabolic disorders. 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 receptors that trigger second messenger signaling pathways (Julius, *et al.*, 1989).

Dopamine (DA) is a major neurotransmitter in the central nervous system, and its receptors are associated with a number of neuro-pathological disorders such as Parkinson's disease and Schizophrenia. It also plays a major role in the regulation of appetite and growth hormone. Diabetes is reported to damage dopaminergic function as a result of hyperglycaemia. Diabetes reduces the activity of the nigrostriatal dopaminergic system. A reduction of norepinephrine (NE) and epinephrine (EP) synthesis, with increased storage probably due to a reduced release from terminal vesicles are also suggested (Gallego, *et al.*, 2003). Insulin pathway in the brain plays an important role in regulating dopamine transporter (DAT). The DAT regulates extra cellular DA levels and during diabetes there is a significant decrease in their number as a result of hypoinsulinemia which damages the dopaminergic activity (Figlewicz, *et al.*, 1996). It is reported that midbrain dopamine neurons, implicated to be critical in the mediation of motivational and reward aspects of stimuli, and are affected by alterations in insulin levels. Extensive co-expression of tyrosine hydroxylase (a marker for dopaminergic neurons) with insulin receptor was observed in the ventral tegmentum and substantia nigra. These findings suggest that midbrain dopamine neurons are direct targets of insulin and they participate in mediating the effects of these hormones on reward-seeking behavior (Figlewicz, *et al.*, 2003).

Dopamine 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. Dopamine receptors are classified into two major groups: Dopamine D_1 (DA D_1) like and Dopamine D_2 (DA D_2) like. Dopamine D_1 like receptors consists of DA D_1 and DA D_5 receptors. Dopamine D_2 like receptors consists of DA D_2, DA D_3 and DA D_4 receptors. These two receptors exert their biological actions by coupling to and activating different G protein complexes. The DA D_1 receptor interacts with the Gs complex to activate adenylyl cyclase, whereas the DA

D_2 interacts with Gi to inhibit cAMP production. The anatomical distributions of these two receptors overlap in the CNS, yet their quantitative ratios differ significantly in particular anatomical areas. With respect to mental disorders, it is noteworthy that both DA D_1 and DA D_2 receptors are present in the nigrostriatal and mesocorticolimbic pathways. Dopamine D_2 receptor activate the inositol phosphate second messenger system. Impairment of central dopamine neurotransmission causes muscle rigidity, hormonal regulation, thought disorder and cocaine addiction. Peripheral dopamine receptors mediate changes in blood flow, glomerular filtration rate, sodium excretion and catecholamine release. Dopamine itself has a regulatory effect on the synthesis of post-synaptic receptors. Dopamine D_2 receptors decreases when there is an increased dopaminergic transmission, while a decrease in the transmission has an opposite effect. Schizophrenia causes an increased dopamine DA D_2 receptor synthesis due to dopaminergic blockade by neuroleptics. In Parkinson's disease dopamine deficiency causes an increase in DA D_2 receptors.

The capacity of β-cells to proliferate plays a fundamental role in determining the onset and severity of carbohydrate intolerance in diabetes (Wang *et al.*, 1994). The ß-cell mass reduction is a critical event in the development of insulin dependent diabetes mellitus. The acute onset of the disease is preceded by a period of progressive destruction of the pancreatic islets (Swenne, 1992). The new concept is that β-cell mass is dynamic and increases and decreases both in function and mass to maintain the glycaemic level within a narrow physiological range. This is an important aspect to pursue in clinical research. The changes in mass can be in both number and individual volume of the β-cells. When the mass cannot increase adequately, diabetes ensues (Bonner-Weir., 2000).

The pancreatic islets are richly innervated by parasympathetic, sympathetic and sensory nerves. Several neurotransmitters- acetylcholine, norepinephrine, and dopamine are stored within the terminals. Stimulation of autonomic neurotransmitters and treatment with neurotransmitters affect insulin secretion. The facilitator action of vagal nerves and splanchnic inhibitory modulation of insulin release has been demonstrated. The cholinergic nerve fibres innervating the islets are of postganglionic origin and emanate from the intrapancreatic ganglia. These ganglia are controlled by the preganglionic fibres, originating primarily in the dorsal motor nucleus of the vagus. They enter the pancreas along the vessels and terminate at intrapancreatic ganglia, from which the postganglionic nerves pass to the islets. These nerves penetrate the islets to terminate close to the endocrine cells. The postganglionic nerve fibres innervating the islets release acetylcholine, which directly stimulates insulin secretion from the islet ß-cells through activation of muscarinic receptors (Ahren, 2000).

Dopamine exerts its inhibitory effect through the Dopamine D_1 receptors in the pancreatic exocrine secretion of conscious rats (Masuda, *et al.*, 1998). Pancreatic ß-cell secretory granules have the ability to store substantial amounts of calcium, dopamine and serotonin (5HT). Dopamine plays an important role in the modulation of the glucose-induced insulin secretion. L-3, 4-dihydroxyphenylalanine (L-DOPA) is rapidly converted to dopamine in islet ß-cells. Dopamine accumulation in pancreatic islets is accompanied by an increase in MAO activity which has an inhibitory effect on glucose-stimulated insulin response (Ahren & Lundquist, 1985). It is suggested that increased hydrogen peroxide production, following increased MAO activity augments the inhibitory effect of dopamine accumulation on insulin release.

Regeneration is a complex interplay of several factors - growth factors, hormones and neurotransmitters. Nutrients including glucose are reported to stimulate ß-cell

replication (Swenne, 1982; Hellerstrom *et al.*, 1985). The stimulatory effect of growth hormone on insulin production and ß-cell replication are well documented (Swenne *et al.*, 1987; Sjoholm *et al.*, 2000). *In vitro* and *in vivo* studies have established the role of insulin in ß-cell replication (Chick *et al.*, 1973). Insulin interacts with type1 IGF receptor and stimulates ß-cell proliferation.

Parasympathetic activity plays an important role in insulin secretion from pancreatic β-cells. Cholinergic agonist carbachol increases insulin secretion from isolated rat islets (Zawalich & Zawalich, 2002). The muscarinic receptor stimulation by acetylcholine (ACh) leads to activation of phospholipase C, which, in turn, hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP2) to produce IP_3 and diacylglycerol (Best & Malaisse, 1983; Zawalich *et al.*, 1989). In pancreatic β-cells, IP3 mobilizes Ca^{2+} from intracellular stores, resulting in an elevation of the intracellular concentration of Ca^{2+} and allowing activation of Ca^{2+}/calmodulin. Diacylglycerol on the other hand, activates PKC (Nishizuka, 1995; Renstrom *et al.*, 1996; Oliva *et al.*, 2005). PKC, like Ca^{2+}/calmodulin, accelerates exocytosis of insulin granules (Nakano *et al.*, 2002; Hashimoto *et al.*, 2005)

Scientific investigations so far has not given emphasis on the functional regulation of dopaminergic receptors and its down stream signaling through second messengers like cAMP, IP3 and intracellular calcium levels during pancreatic regeneration. In the present study a detailed investigation on the alterations of dopamine and dopamine D_2 receptors in the brain and regenerating pancreas were carried out. Our studies confirmed the functional role of dopamine through dopamine D_2 receptors and second messengers during pancreatic regeneration.

OBJECTIVES OF THE PRESENT STUDY

1. To study the changes in dopamine and homovanillic acid content in various rat brain regions – corpus striatum (CS) cerebral cortex (CC), brain stem (BS) and hypothalamus (Hypo) of Sham, 72 hours and 7 days partial pancreatectomised rats using High Performance Liquid Chromatography.

2. To study the changes in dopamine and homovanillic acid content in the pancreas, adrenals and plasma of sham, 72 hours and 7 days partial pancreatectomised rats using High Performance Liquid Chromatography.

3. To study the dopamine and dopamine D_2 receptor kinetic changes in corpus striatum (CS) cerebral cortex (CC), brain stem (BS) and hypothalamus (Hypo) of sham, 72 hours and 7 days partial pancreatectomised rats.

4. To study the dopamine and D_2 receptor changes in pancreatic islets of sham, 72 hours and 7 days partial pancreatectomised rats.

5. Displacement analysis was carried out to confirm the shift in affinity and the number of possible binding sites.

6. To study the gene expression of dopamine D_2 receptor using Real-Time PCR in the brain regions and pancreas of sham, 72 hours and 7 days pancreatectomised rats with specific primers

7. To study the effect of dopamine and its agonists and antagonists on glucose induced insulin secretion *in- vitro* by radio-immunoassay.

8. To study the effect of dopamine and its agonists and antagonists on pancreatic islets cell proliferation *in- vitro* by [^{3}H]thymidine incorporation.

9. To study the effect of dopamine and its agonists and antagonists on cAMP and IP3 level in pancreatic islets cell *in- vitro.*

10. To study the changes in cAMP and IP3 level in pancreatic islets during pancreatic regeneration.

11. To study the effect of dopamine and its agonists and antagonists on intracellular calcium release in pancreatic islets cell *in- vitro* by confocal microscopy.

REVIEW OF LITERATURE

Pancreas is a complex organ with endocrine and exocrine function. The mammalian endocrine pancreas is composed of four different types of functionally related cells. Each islet consists of cells responsible for the synthesis and release of glucagon (α-cells), insulin (β-cells), somatostatin (δ-cells) and pancreatic polypeptide (PP cells) (Munger, 1981; Smith & Davis 1983). The exocrine component includes acinar and ductal cells that secrete and transport digestive enzymes into the intestine. Exocrine cells make up the majority of the pancreas and are grouped together into acini and a highly branched ductal system. The endocrine cells account for approximately 4% of the volume of the pancreas and they are grouped together into islets of Langerhans. The islets are composed of a few hundred to several thousands of cells, of which 65–80% are insulin-secreting β-cells. These cells are mainly located in the center of the islet and are surrounded by a three other cell types, i.e., glucagon-secreting - alpha cells, somatostatin secreting - delta cells and pancreatic polypeptide secreting cells (PP-cells). Diabetes is characterized by a marked deficit in β-cell mass causing insufficient insulin secretion (Meier *et al.*, 2006)

Insufficient production of biologically active insulin is a common denominator in all forms of diabetes and the degree of the insulin deficiency determines both the severity of the disease and the choice of the therapy (Hellerstrom, 1984). Type 1 diabetes is a disease of β-cell destruction leading to insulin deficiency (Hokanson *et al.*, 2006). The total number of insulin-producing β-cells in the pancreas is a critical factor in the regulation of glucose homeostasis. Insulin dependent diabetes mellitus results when the number of β-cells is severely reduced due to autoimmune destruction (Bach, 1994; Tisch & McDevitt, 1996).

Age related changes in the capacity of β-cell for proliferation affect the insulin production and contribute to a decrease in glucose tolerance with advance in age (Hellerstrom, 1984). Cell cycle analysis of rat islets maintained in tissue culture indicates that proliferating β-cells proceed through the cell cycle at similar rates irrespective of the postnatal age (Swenne, 1983). The sensitivity to glucose in terms of DNA synthesis by the β-cells is also similar, but the proliferative capacity seems to be restricted by a decreasing number of cells capable of entering the cycle. The decrease in the capacity to proliferate with age may signify a gradual withdrawal of cells from the active cell cycle into an irreversible G_0 state. Therefore the capacity of β-cells to respond with proliferation to diabetogenic stimulus decreases with age (Hellerstrom, 1984).

Light and electron microscopic studies have demonstrated that there are three different types of nerve endings in the pancreas: sympathetic, parasympathetic and peptidergic nerves (Miller, 1981). The neurotransmitters found in the first two nerve terminals are catecholamines including DA and ACh. The peptidergic nerve terminals contain various peptides as neurotransmitters. The nerve fibres enter the pancreas in association with the vascular supply and they are dstributed to blood vessels, acinar tissue and islets. Adrenergic fibres innervate vessels, acini and islets. Cholinergic fibres are found mainly in islets. Peptidergic nerves are found in both exocrine and endocrine tissue (Ahren *et a.*, 1986).

The degree of regeneration of pancreas is variable, depending on the nature of the stimulus to regenerate (Logothetopaulos *et al.*, 1983). Exocrine cells exhibit significant regenerative potential after incomplete destruction, following the use of the selective pancreatic toxin ethionine or following cerulein induced pancreatitis (Kasai *et al.*, 1982). It appears that endocrine and exocrine regeneration are under different control mechanisms (Gepts *et al.*, 1990). There is much evidence to suggest that prolonged

stimulation of insulin secretion *in vivo* leads to a compensatory increase of the total volume of the pancreatic islets (Martin & Lacy 1963). Th insulin secretion from the β-cell is the result of a complex interaction between metabolic and neural (Campfield *et al.*, 1980) external inputs acting in concert with other controlling factors. Stat3-C a cell cycle regulator protein can induce cell proliferation of β-cells without loss of insulin secretion activity at the glucose challenge (Tsukiyama *et al.*, 2006).

INSULIN SECRETION REGULATING FACTORS

Glucose

Glucose is an important stimulator of insulin secretion. Glucose, over short intervals stimulates insulin biosynthesis at the level of translation (Permut *et al.*, 1972). Studies 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).

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). The glucose-phosphorylating enzyme glucokinase has structural, kinetic, and molecular genetic features that are ideal for its primary role as glucose sensor in a network of neuro / endocrine sentinel cells that maintain glucose homeostasis in many vertebrates including humans (Matschinsky *et al.*, 2006). The entry of glucose into β-cells is followed by an acceleration of metabolism that generates one or several signals that close ATP-sensitive K^+ channels in the plasma membrane. The resulting decrease in K^+ conductance leads to depolarisation of the membrane with subsequent opening of voltage dependent Ca^{2+} channels. The rise in the cytoplasmic free

Ca^{2+} eventually leads to the exocytosis of insulin containing granules (Dunne, 1991., Gembal *et al.*, 1992). Glucose induced insulin secretion is also partly dependent upon the activation of typical isoforms of protein kinase C (PKC) within the β-cell (Harris, 1996). It is suggested that PKC may be tonically active and effective in the maintenance of the phosphorylated state of the voltage-gated L-type Ca^{2+} channel, enabling an appropriate function of this channel in the insulin secretory process (Arkhammar, 1994).

Fatty acids

Short chain fatty acids and their derivatives are highly active stimulators of insulin release in sheep (Horino *et al.*, 1968). Exogenous saturated long chain fatty acids markedly potentiated glucose-induced insulin release and elevated long chain acyl-CoA esters in the clonal β-cell line, (Prentki *et.al.*, 1992). A novel ester of succinic acid 1,2,3-tri-(methyl-succinyl) glycerol ester displayed stimulation of insulin release and biosynthetic activity in pancreatic islets of Goto-Kakizaki rats (Laghmich *et al.*, 1997). A monomethyl ester of succinic acid along with D-glucose is required to maintain the β-cell response to D-glucose (Fernandez *et al.*, 1996). Chronic intake of high-fat and high-sucrose diets differentially affects glucose intolerance in mice (Sumiyoshi *et al.*, 2006).

Amino acids

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

Substrates derived from nutrients

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

Glucagon

Glucagon is the hormone secreted by pancreatic α-cells. It has been shown that glucagon has a striking stimulatory effect on insulin release in the absence of glucose (Sevi, 1966). The presence of specific glucagon receptors on isolated rat pancreatic β-cells as well as a subpopulation of α- and δ-cells shows the relevance of glucagon on regulation of insulin secretion. Intra-islet glucagon appears to be a paracrine regulator of cAMP *in vitro* (Schuit, 1996). Glucagon stimulates insulin release by elevating cAMP. cAMP through activation of protein kinase A, increases Ca^{2+} influx through voltage dependent L-type Ca^{2+} channels, thereby elevating Ca^{2+} and accelerating exocytosis (Carina, 1993). Protein phosphorylation by Ca^{2+}/Calmodulin and cAMP dependent protein kinase play a positive role in insulin granule movement which results in potentiation of insulin release from the pancreatic β-cell (Hisatomi, 1996). In pancreatic β-cells, cAMP potentiates Ca2+-dependent exocytosis and mediates the stimulation of insulin release exerted by the hormones glucagon and glucagon-like peptide-1 (Dyachok *et al*, 2006).

Somatostatin

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

Pancreastatin

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

Amylin

Amylin is a 37-amino acid peptide hormone co-secreted with insulin from pancreatic β-cells. Amylin appears to control plasma glucose *via* several mechanisms that reduce the rate of glucose appearance in the plasma. Amylin limits nutrient inflow into the gut and nutrient flux from the gut to blood. It is predicted to modulate the flux of glucose from liver to blood by its ability to suppress glucagon secretion. Amylin is absolutely or relatively deficient in type I - diabetes and in insulin requiring type II - diabetes (Young, 1997). It inhibits insulin secretion *via* an autocrine effect within pancreatic islets. Amylin fibril formation in the pancreas may cause islet cell dysfunction and cell death in type II - diabetes mellitus (Alfredo, 1994). Exogenously administered amylin agonists inhibit insulin secretion, at least partly via activation of an amylin-like receptor linked to Gi-mediated inhibition of cAMP in islets (Young, 2005).

Adrenomedullin

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

Galanin

Galanin is a 29 amino acid neuropeptide localised in the intrinsic nervous system of the entire gastrointestinal tract and the pancreas of man and several animal species. (Scheurink,1992). It inhibits insulin secretion in rat, mouse, and also in isolated human islets pig (Lindskog & Ahren, 1989). In isolated rat and mouse islets galanin inhibits insulin secretion by increasing the K^+ permeability and interfering with activation of adenylate cyclase and the activity of protein kinase C and cAMP (Lindskog and Ahren 1991). Among other functions, galanin inhibits insulin release (Ahren *et al.*, 1991), probably *via* activation of Gproteins (Renstrom, 1996) by the mediation of activated galanin receptors.

Macrophage migration inhibitory factor

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

insulin secretion in a positive manner and may play an important role in carbohydrate metabolism (Waeber, 1997).

Nerve growth factor

Nerve growth factor (NGF) is a neurotropic growth factor that promotes neurite outgrowth during development. This growth factor is capable of modulating β-cell plasticity because it promotes neurite-like outgrowth in fetal and adult pancreatic β-cells from primary cultures (Vidaltamayo *et al.*, 1996) and in RINm5F and insulinoma cells (Scharfmann *et al.*, 1993). In insulinoma cells NGF enhances glucose stimulated insulin secretion (Gonzalez *et al.*, 2001). In adult rat β-cells, *in vitro* NGF stimulates glucose induced insulin secretion. The presence of the high affinity receptor for NGF has been described in insulinoma cell lines as well as in foetal and adult β-cells (Rosenbaum *et al.*, 1993). The adult β-cells synthesise and secrete NGF in response to increasing extra cellular glucose concentration (Vidaltamayo *et al.*, 1996). The effect of NGF on insulin secretion is partly mediated by an increase in calcium current through calcium channels (Rosenbaum *et al.*, 2000). NGF has an important regulatory role in pancreatic β-cell function (Miao *et al*, 2006)

Neuropeptides

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

Gastrin releasing peptide

Gastrin releasing peptide (GRP) consists of a 27 amino acid residue. It is localised to pancreatic nerves, including islet nerve terminals of several species. GRP

released from the pancreas after vagal nerve activation and stimulates insulin secretion (Sundler& Bottcher, 1991; Knuhtsen *et al.*, 1987). In islets, activation by GRP receptors is coupled to PLC and phospholipase D (Gregersen & Ahren, 1996, Wahl *et al.*, 1992).

Vasoactive intestinal peptide

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

Pituitary adenylate cyclase activating polypeptide

Pituitary adenylate cyclase-activating polypeptide (PACAP) has been shown to play an important role in the regulation of islet function (Tsunekawa *et al.*, 2005). Pituitary adenylate cyclase activating polypeptide (PACAP) is localised to the parasympathetic nerves and released by the activation of the vagus nerve (Ahren, 2000). It exists in two forms consisting of 27 and 38 amino acids and shows 68% homology (Arimura & Shioda, 1995). PACAP stimulates insulin secretion in a glucose dependent manner accompanied by increased action of adenylate cyclase with increased formation of cAMP (Klinteberg *et al.*, 1996)..

NEURAL INNERVATIONS IN PANCREAS

The pancreas, like the gastrointestinal tract, embryologically develops from an outgrowth of the primitive foregut. Most of the nerve fibres enter the pancreas along the arteries (Woods and Porte, 1974; Miller 1981). Well differentiated synapses with islet cells have beenobserved (Orci, *et al.*, Watanabe & Yasuda., 1977). It is richly innervated

being composed of a variety of myelinated or unmyelinated nerve fibers, thick nerve bundles and aggregates of neural cell bodies known as intrapancreatic ganglia. These ganglionic structures are randomly scattered throughout the pancreatic parenchyma and represent the intrinsic neural component of the pancreatic nerve supply [Tiscornia, 1977; Holst, 1993]. The two main extrinsic components are anatomically identified in the vagus nerves (anterior and posterior branches) and the splanchnic nerve trunks. The vagus nerves reach the pancreas directly or, alternatively, they pass across the preaortic chain of the sympathetic ganglia. Post-ganglionic sympathetic fibers, whose neural cell bodies are located in the superior mesenteric and celiac ganglia, run with the splanchnic nerves. The afferent system, primarily involved in sensory/pain transmission to the central nervous system, is composed of thin unmyelinated fibers running with either the parasympathetic pathways (*vagi*) or the sympathetic inputs (*splanchnic nerves*). The cell bodies of these nerve processes can be located either in the dorsal root ganglia (the so-called *spinal afferents*) or in the nodose ganglia (*vagal afferents*) Furness & Bornstein,1991; Tiscornia, 1977; Holst, 1993].

Features of Pancreatic Peptidergic Innervation

Tissue Targets and Morpho- Functional Characteristics Using immunohistochemical methods, it was possible to thoroughly analyze the distribution of nerve fibers and neurons containing peptides in relation to a variety of innervation targets, including both exocrine (acini, ducts,blood vessels) and endocrine (islets of Langerhans) targets. Several studies from our group and other groups have clearly demonstrated that peptide-containing nerves are detectable in both the exocrine and endocrine pancreas (Su *et al.* 1987 and De *et al.* 1992) A detailed analysis of specific sites of innervation in mammalian species which was carried out by our group showed that nerve fibers containing CGRP, vasoactive intestinal polypeptide (VIP) and NPY, running in tiny bundles or in thick nerve trunks, were observed throughout the stroma and

around acini. Overall, the analysis of the density of nerve fibers showed a more abundant number of VIP, CGRP and NPY immunolabelled processes than those positive for the gastrin.

The parasympathetic innervations

The preganglionic fibres of the parasympathetic limb originate from the dorsal motor nucleus of the vagus (Luiten *et al.*, 1984 & Ahrén, 1986) and possibly also in the nucleus ambiguus (Luiten *et al.*, 1984), which are both under the control of the hypothalamus. They are organized in well separated branches traveling within the vagus nerves (cranial nerve X), and through the hepatic, gastric (Berthoud *et al.*, 1990), and possibly celiac branches of the vagus (Kinami *et al.*, 1997), they reach intrapancreatic ganglia that are dispersed in the exocrine tissue. These ganglia send unmyelinated postganglionic fibres toward the islets (Woods & Porte Jr., 1974). Preganglionic vagal fibres release ACh that binds to nicotinic receptors on intraganglionic neurons. Postganglionic vagal fibres release several neurotransmitters: ACh, vaso active intetsinal peptide (VIP), gastrin-releasing peptide (GRP), nitric oxide (NO), and pituitary adenylate cyclase-activating polypeptide (PACAP) (Ahrén, 2000). Cholinergic terminals are found in the neighborhood of all islet cell types at the periphery and within the islet (Love & Szebeni, 1999). Cholinergic synapses with endocrine cells have been observed in some species (Voss *et al.*, 1978). Vagal parasympathetic input to the islets of Langerhans is a regulator of islet hormone secretion, but factors promoting parasympathetic islet innervation are unknown (Rossi *et al.*, 2005)

The sympathetic innervation

The sympathetic innervation of the pancreas originates from the thoracic and upper lumbar segments of the spinal cord (Furuzawa *et al.*, 1996). The myelinated axons of these cells traverse the ventral roots to form the white communicating rami of the

thoracic and lumbar nerves that reach the paravertebral sympathetic chain (Chusid, 1979). Preganglionic fibres either communicate with a nest of ganglion cells within the paravertebral sympathetic chain or pass through the sympathetic chain, travel through the splanchnic nerves, and reach the celiac (Brunicardi *et al.*, 1995; Ahrén, 2000) and mesenteric ganglia (Furuzawa *et al.*, 1996). Ganglia within the paravertebral sympathetic chain, and the celiac and mesenteric ganglia, give off postganglionic fibres that eventually reach the pancreas. The existence of intrapancreatic sympathetic ganglia has also been reported (Liu *et al.*, 1998). The preganglionic fibres release ACh that acts on nicotinic receptors on intraganglionic neurons, whereas the postganglionic fibres release several neurotransmitters: NE, galanin, and NPY (Ahrén, 2000). A rich supply of adrenergic nerves in close proximity of the islet cells has been observed in several mammalian species (Esterhuizen *et al.*, 1968).

Sensory fibres

The sensory nerve fibres report pain information associated with diseases of the exocrine tissue, such as pancreatic cancer and pancreatitis (Rossi *et al.*, 1995; Di Sebastiano *et al.*, 2000), but there are no reports of sensations of pain associated with a destruction of the endocrine pancreas. However, it is possible that sensory fibres play a role in the control of insulin secretion. Thus, neonatal treatment of mice with capsaicin (to destroy these fibres) results in more glucose-stimulated insulin secretion than in nontreated mice, suggesting that sensory fibres exert a direct, tonic inhibition of insulin secretion (Karlsson *et al.*, 1994).

Other types of nerves

Immunocytochemistry has revealed the presence of neurotransmitters other than those described above in pancreatic nerves: cholecystokinin (Karlsson & Ahrén, 1992), 5-HT (Kirchgessner & Gershon, 1990), and methionine-enkephalin (Ahrén, 2000).

ROLE OF NEUROTRANSMITTERS IN INSULIN SECRETION

Gamma-Aminobutyric acid

Gamma aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system. GABA is reported to present in the endocrine pancreas at concentrations comparable with those found in central nervous system. The highest concentration of GABA within the pancreatic islet is confined to β-cells (Sorenson, 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, 1990). GABA through its receptors have been demonstrated to attenuate the glucagon and somatostatin secretion from pancreatic α-cells and δ-cells respectively (Gaskins, 1995). It is present in the cytoplasm and in synaptic-like microvesicles (Reetz, 1991) and is co-released with insulin from β-cells in response to glucose. The released GABA inhibits islet α-and δ-cell hormonal secretion in a paracrine manner. During diabetes the destruction of β-cells will lead to decrease in GABA release resulting in the enhancement of glucagon secretion from α-cells leading to hyperglycaemia. The brain GABAergic mechanisms also play an important role in glucose homeostasis. Inhibition of central $GABA_A$ receptors increases plasma glucose concentration (Lang, 1995). Thus, any impairment in the GABAergic mechanism in the central nervous system and/or in the pancreatic islets is important in the pathogenesis of diabetes. Gamma-aminobutyric acid up- and downregulates insulin secretion from β-cells in concert with changes in glucose concentration (Dong *et al.*, 2006)

5-Hydroxytryptamine

Brain serotonergic and adrenergic functional correlation with insulin secretion was established in diabetic rats (Vahabzadeh *et al.*, 1995). In mice 5-HT dose

dependently induced hyperglycemia and an increase in serum insulin level (Sugimoto, 1990). 5-HT content is increased in the brain regions and hypothalamic nuclei (Chen &Yang, 1991; Lackovic *et al.*, 1990). Chu *et al.*, (1986) reported lower 5-HT levels in both hypothalamus and brain stem but not in corpus striatum. Insulin treatment brought about an increase in the cerebral concentration of 5-hydroxyindole acetic acid (5-HIAA) and accelerated the cerebral 5-HT turnover (Juszkiewicz, 1985). The 5-HIAA concentration was reported to be approximately twice as high as the control regardless of duration of treatment. Brain tryptophan, the precursor of 5-HT, was also reduced in brain regions during diabetes (Jamnicky *et al.*, 1991). Studies from our lab have shown that 5-HT $_{2A}$ receptors upregulated in the cerebral cortex and brain stem of STZ induced diabetic rats (Jackson *et al.*, 1999). There are reports, glucose-induced insulin secretion is inhibited by 5-hydroxytryptamine (Zawalich *et al*, 2004).

Epinephrine and Norepinephrine

EP, the principal neurotransmitter of the sympathetic nervous system is inhibitory to insulin secretion. EP, when used in high doses *in vivo* or *in vitro*, reduces the insulin response to stimulators (Malaisse, 1972). EPI and NE have an antagonistic effect on insulin secretion and glucose uptake (Porte, 1966). Epinerphrine is, however, known to play a secondary role in the physiology of glucose counter-regulation. The inhibitory effect of EPI upon insulin secretion induced by glucose was reported by Coore and Randle (1964).

Adrenergic receptors are mainly classified into α and β-adrenergic receptors. (Lefkowitz, 1988). EPI and NE bind to these receptors in a concentration dependent manner. At low concentrations EPI and NE can bind and activate β-adrenergic receptors, which in turn stimulate the insulin secretion from pancreatic islets by activating adenylate cyclase through stimulatory G (Gs)-proteins. At high concentrations they can bind to α_{2A}

receptors and inhibit insulin secretion through inhibitory G_i proteins (Lacey, 1993). $Alpha_1$ receptors have also been observed to activate phospholipase A_2 and stimulate calcium influx through plasma membrane calcium channels. EPI and NE inhibit insulin secretion by α_2-adrenergic receptor activation. $Alpha_2$-adrenergic receptor activation leads to the inhibition of insulin release by a mechanism distal to those regulating β-cell cAMP production and $[Ca^{2+}]$ (Ullrich, 1985). $Alpha_2$-adrenergic receptor agonists are potent inhibitors of insulin release in the isolated islet preparation from rats (Morgan, 1985), as well as in mice *in vivo* (Skoglund, 1986) and in man (Porte, 1966).

EP and other adrenoceptor agonists are previously shown to induce a hyperglycaemic response following *in vivo* administration. Clonidine was used as a potent agonist for inducing hyperglycaemia by activating α_2-adrenoceptors (DiTullio, 1984). A peripherally active adrenoceptor agonist, (3,4-dihydroxyphenylimino)-2-imidazolidine (DPI), and a highly selective α_2-adrenoceptor agonist, UK 14.304, also could induce hyperglycaemia similar to clonidine (Angel, 1988).

Previous studies have shown that in diabetic condition, α_{2A}-adrenergic receptors are more activated which brought out the insulin inhibition and in turn hyperglycaemia (Lacey, 1993). Rat islet cell membrane is equipped with α_{2A}-adrenergic receptors (Filipponi, 1986) which are linked to adenylate cyclase and inhibits insulin secretion. Studies conducted in C57BL/KsJ mice revealed that all of the α_1- and α_2-adrenergic receptor population was elevated in the regional brain samples of diabetic compared with controls. However, β-adrenergic receptor populations were depressed in diabetes compared with age-matched controls (Garris, 1990). Studies from our lab have shown that α_1-adrenoceptors expressed altered affinity in hypothalamus and brain stem of diabetic rats (Pius, 1996).

Acetylcholine

Cholinergic system plays an important role in physiological and behavioural functions. ACh acts by binding to specific membrane receptors and can be divided into muscarinic and nicotinic receptors. Cholinergic stimulation of pancreatic β-cells increases insulin secretion (Kaneto *et al.*, 1967). This effect is mediated by muscarinic receptors (Grill & Ostenson, 1983; Henquin & Nenquin, 1988) and is dependent on extracellular glucose concentration (Henquin *et al.*, 1988). ACh stimulation-insulin secretion coupling is mediated by complex mechanisms of signal transduction. It has been proposed that ACh activates phospholipid turnover and thereby increases the intracellular calcium level. Normal β-cells' voltage-dependent sodium channels are important for membrane depolarisation. ACh increases sodium influx into the cells (Henquin *et al.*, 1988). ACh hyperpolarises the cell by increasing potassium permeability. Quist (1982) reported that carbachol causes Ca^{2+}-dependent stimulation of phosphate incorporation into phosphatidyl inositol phosphates in the canine heart. Cholinergic stimulation of phosphatidyl inositol phosphates synthesis is blocked by muscarinic antagonist atropine (Brown *et al.*, 1983). ACh is a recognized potentiator of glucose-stimulated insulin release in the normal β-cell (Dolz *et al.*, 2005)

Dopamine

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

DA is involved in the regulation of various physiological processes and motor functions, like feeding behaviour, and in the siphon-gill withdrawal reflex (Barbas *et al.*, 2006)

MOLECULAR BIOLOGY OF DOPAMINE RECEPTORS AND SUBTYPES

DA is present in most parts of the central nervous system (CNS) but in particular in the nigrostriatal pathway comprising the neurons of the substantia nigra (A9) and projecting to neurons of the neostriatum and the mesocorticolimbic pathway composed of neurons of the ventral tegmental area (A10) connecting with those of the limbic cortex and other limbic structures (Bjorklund, 1964).

The involvement of the dopaminergic nigrostriatal pathway in extrapyramidal dysfunctions was shown by the discovery that degeneration of this pathway occurs in the brains of patients afflicted with Parkinson's disease (Ehringer & Hornykiewicz, 1960; Felder *et al.*, 1989). The depletion of DA resulting from the degeneration of the nigrostriatal pathway led to the development of DA-replacement therapies which are successful in alleviating Parkinson's disease (Birkmayer & Hornykiewicz 1962, Hornykiewicz, 1966). The hypothesis that DA is involved in the pathogenesis of psychosis, in particular schizophrenia, rests on the finding that most antipsychotic drugs are DA receptor antagonists and that agents which cause excessive release of DA mimic schizophrenia-like states (Carlsson and Lindqvist, 1963, Creese *et al.*, 1976; Seeman *et al.*, 1976; Carlsson, 1988). The mesocorticolimbic pathway has been implicated as the principal dopaminergic pathway involved in the etiology of psychoses. These data explain the dilemma associated with DA-related drug therapies: the blockade of the dopaminergic system, desired for reducing psychoses, induces extrapyramidal dysfunctions and vice versa.

In 1979, Kebabian and Calne found that DA exerts its effects by binding to two receptors, known as the DA D_1 and DA D_2 receptors (Kebabian & Calne., 1979). These receptors could be differentiated pharmacologically, biologically, physiologically, and by their anatomical distribution (Creese., 1987). Pharmacologically, the hallmark of the DA D_1 receptor is to bind the benzazepine antagonist SCH 23390, while that of the DA D_2 receptor is to recognize with high affinity the butyrophenones: spiperone and haloperidol. These two receptors exert their biological actions by coupling to and activating different G protein complexes. The DA D_1 receptor interacts with the Gs complex to activate adenylyl cyclase, whereas the DA D_2 interacts with Gi to inhibit cAMP production. The anatomical distributions of these two receptors overlap in the CNS, yet their quantitative ratios differ significantly in particular anatomical areas. With respect to mental disorders, it is noteworthy that both DA D_1 and DA D_2 receptors are present in the nigrostriatal and mesocorticolimbic pathways. DA D_1 and DA D_5 receptors serve distinct functions in the mammalian nervous and endocrine systems (Jackson *et al.*, 2005).

This two-subtype classification has accounted for most of the activities attributed to the dopaminergic system. The existence of other DA receptors has been proposed but had been refuted when the "new" receptors were recognized to represent different affinity states of the canonical DA D_1 and DA D_2 receptor (Andersen, 1990; Levesque *et al.*, 1992). However, this classification was dramatically changed with the application of recombinant DNA technology to the molecular characterization of the DA receptors.

MOLECULAR CHARACTERIZATION OF THE DOPAMINE RECEPTORS

Cloning of the DA D2 Receptor

The cloning of the DA D_2 receptor resulted from the recognition that, on the basis of its inhibitory activity on adenylyl cyclase, it would belong to the supergene family of the G-protein-coupled receptors (Dohlman, 1987; Hall, 1987). Consequently, the use of a

cloning strategy based on the sequence homology known to exist among G-protein-coupled receptors could lead to the molecular characterization of the DA D_2 receptor. The DA D_2 receptor was cloned using the hamster a2-adrenergic receptor coding sequence as hybridization probe under conditions which would detect sequentially related DNA fragments (Bunzow *et al*, 1988). Via genomic and cDNA screenings, a rat brain cDNA was identified and shown to encode a protein featuring the characteristics expected for a G-protein-coupled receptor. The receptor encoded by this cDNA had the pharmacological profile and biological activity of the DA D_2 receptor found in the brain and pituitary, demonstrating that this cloned receptor is the same DA D_2 receptor as the one described in 1979 (Bunzow *et al*., 1988; Neve *et al*., 1989; Albert *et al* ., 1990). DA D_4 receptors has role in behavioral regulation (O'Sullivan *et al*., 2006)

Homology Screening Approach: Discovery of the Dopamine Receptor Heterogeneity

The success of the homology approach in the cloning of the D_2 receptor opened the door for the cloning of other DA receptors. Successful cloning of the D_1 receptor was reported by several groups (Dixon *et al*., 1988; Dearry *et al*, 1990; Zhou *et al*., 1990). The sequences derived from these clones share the characteristics expected of G-protein-coupled receptors in general and of the catecholamine receptors in particular (Zhou *et al*., 1990). These putative receptors were expressed by DNA transfection and were shown to bind DA D_1 receptor ligands and to stimulate adenylyl cyclase activity, the two hallmarks of the DA D_1 receptor. Molecular characterization of the DA D_1 receptor had been achieved.

The generality of the homology approach allowed for the search of other unexpected DA receptors. Using a DA D_2-receptor-specific DNA fragment as probe under low-stringency hybridization conditions, Sokoloff *et al*., 1990 identified another DA receptor, the DA D_3 receptor. When expressed in eukaryotic cells, this receptor was

shown to bind DA D2 but not DA D_1 ligands. Its structure and binding characteristics thus permitted its classification as a new DA receptor called the D_3 receptor. Noteworthy is its ability to affect second messenger systems, which has thus far not been demonstrated.

Furthermore, by analyzing the mRNAs of human neuroepithelioma SK-N-MC cells with DA D_2 receptor cDNA probes under conditions of low stringency, another DA D_2-related mRNA was detected (Van *et al.*, 1991). The corresponding cDNA and gene analyses led to the characterization of the DA D_4 receptor. The DA D_4 receptor, when expressed in COS-7 cells, binds DA D_2 antagonists with a pharmacological profile that is distinct but reminiscent of that of the DA D_2 receptor. The DA D_4 receptor was shown to couple to G proteins, although its potential at inducing second messenger systems is still being determined. In *Caenorhabditis elegans* D_4 stimulates cAMP accumulation in response to DA stimulation (Sugiura *et al.*, 2005)

Finally, the DA D_1 receptor clone was used as a hybridization probe to identify DA D_1-related genes. A human DA D_5 and a rat DA D_1b receptor have been characterized (Grandy *et al.*, 1991; Sokoloff *et al.*, 1990; Tiberi *et al.*, 1991). They display the same pharmacological profile, reminiscent of that of the DA D_1 receptor, and are able to stimulate adenylyl cyclase activity. On the basis of their sequences, the DA D_5 and DA D_1b receptors are human and rat equivalents of the same receptor, respectively.

Thus the application of homology screening techniques not only led to the deciphering of the molecular structures of the DA D_1 and DA D_2 receptors, but also led to the characterization of three new DA receptors: DA D_3, DA D_4, and DA D_5. These discoveries have, of course, medical implications. For example, most of what is known about DA agonists' and antagonists' action has to be reevaluated in view of the existence

of the different DA receptors. Our renewed knowledge of the dopaminergic system begins with the study of the dopaminergic receptor family.

COMMON FEATURES OF THE DOPAMINE RECEPTORS

Primary Sequences

In their putative transmembrane domains, the DA D_1 and DA D_5 receptors are 79% identical but are only 40–45% identical to the DA D_2, DA D_3, and DA D_4 receptors. Conversely, the DA D_2, DA D_3, and DA D_4 receptors are between 75% and 51% identical to each other, the first indication that the five receptors can be divided into the DA D_1-like and DA D_2-like receptor subfamilies. The topologies of the five DA receptors are predicted to be the same as all the other G-protein-coupled receptors. They should 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 C-tails (347 in DA D_1, the C-terminus in DA D_2-like receptors) (O'Dowd *et al.*, 1989). The DA receptors are probably glycosylated in their N-terminal domains; in addition, the DA D_1-like subtypes have potential glycosylation sites in their first extracytoplasmic loop.

Genomic Organization

The genomic organization of the DA receptors also supports the notion that they derive from the divergence of two gene subfamilies, the DA D_1-like and DA D_2-like receptor genes. The DA D_1 and DA D_5 receptor genes do not contain introns in their protein coding regions, whereas the DA D_2, DA D_3, and DA D_4 genes do. Furthermore, most of the introns in the DA D_2-like receptor genes are located in similar positions (Grandy *et al.*, 1989; Sokoloff *et al.*, 1990; Sunahara *et al.*, 1990; Van *et al.*, 1991; Zhou *et al.*, 1990).

Ligand Binding and Second Messenger Inductions

The cloned DA receptors, when expressed by transfection, exhibit binding profiles which can also differentiate them into the DA D_1-like and DA D_2-like subfamilies. The DA D_1-like receptors bind with high-affinity DA D_1 and not DA D_2 antagonists. A prototypic ligand for the DA D_1-like receptors is the benzazepine SCH23390 (*K*is < 1 nM); on the other hand, they bind the butyrophenone spiperone with low affinity (*K*is in the micromolar range). In contrast, the DA D_2-like receptors efficiently bind spiperone (*K*is < 1 nM) and not SCH23390 (*K*is for DA D_2 in the micromolar range); they also recognize most of the neuroleptics. Because there are 21 amino acid residues which differentiate DA D_1-like from DA D_2-like receptors in the transmembrane domains, these might participate in the selective recognition process. While there presently exists no ligand to differentiate the DA D_1 from the DA D_5 receptor, several DA D_2 antagonists can distinguish the different DA D_2-like receptors. The compound 7-OH-DPAT is selective for the DA D_3 receptor (Levesque *et al.*, 1992), whereas clozapine has the highest affinity for the DA D_4 receptor. It is noteworthy that DA binds to the DA D_3, D_A D_4, and DA D_5 receptors with nanomolar or submicromolar affinity constants, while its corresponding constants for the DA D_1 and DA D_2 receptors are in the micromolar ranges.

The predominant biological activities associated with DA D_1 and DA D_2 receptor stimulation are the activation and inhibition of adenylyl cyclase activity, respectively. Stimulation of the DA D_1 and DA D_5 receptors in transfected cells has been shown to result in activation of adenylyl cyclase, indicating similar pathways of second messenger induction for the DA D_1-like receptors. On the other hand, the D_3 and D_4 receptors have, thus far, not been shown to induce second messenger systems, thus preventing their subfamily classification based on biological activity. However, because receptors' interactions with G proteins involve the cytoplasmic loops (Dixon *et al.*, 1988; Lefkowitz

et al., 1988) and because DA D_2-like receptors have a large third cytoplasmic loop and a short C-terminal tail representative of the catecholamine receptors coupled to Gi proteins, the DA D_2-like relative homology suggests that they might couple to the same set of G proteins.

Thus, on the basis of their primary sequences, of their genomic organization, and of their pharmacological and, at least partly, biological activities, the different DA receptors can be classified into the DA D_1-like and DA D_2-like subtypes. This, and the fact that the DA D_3, DA D_4, and DA D_5 receptors are present in significantly lower amounts than are the DA D_1 and DA D_2 receptors, suggests that the existence of the former ones could not be found by pharmacological analyses.

PARTICULARITIES OF THE DIFFERENT DOPAMINE RECEPTORS

Pharmacological Profiles

As mentioned above, no selective ligand has been described which is able to differentiate the D1 from the D5 receptor. On the other hand, the pharmacological profiles of the D3 or D4 receptors show distinct striking differences when compared to that of the D2 receptor. Deletion of DA D1 and D3 receptors differentially affects spontaneous behaviour and cocaine-induced locomotor activity (Karasinska *et al.*, 2005).

Most neuroleptics were developed as DA D_2 receptor antagonists and thus are expected to bind to the D_2 receptor with higher affinity than to the DA D_3 and DA D_4 receptors. This is true for the majority of the neuroleptics, which implies that those neuroleptics are acting predominantly at DA D_2 receptors in the human brain. However, a few neuroleptics have been found to show selectivity for the DA D_3 or DA D_4 receptors; through these, some aspects of the functions of the D_3 and D_4 receptors may be revealed.

Two antagonists, UH232 and AJ76, bind to the DA D_3 receptor with a higher affinity than they do to the DA D_2 receptor (Sokoloff *et al.*, 1990). These compounds are classified as selective for presynaptic receptors or for autoreceptors. In addition, it was found that DA binds the DA D_3 receptor with a 20-fold higher affinity than the DA D_2 receptor, a characteristic expected for autoreceptors. Furthermore, the presence of DA D_3 receptor mRNA in the substantia nigra, a center of DA production, supports the hypothesis that the DA D_3 receptor may be a presynaptic receptor. Noteworthy is that the DA D_2 receptor mRNA is the predominant DA receptor mRNA in the substantia nigra (Meador-Woodruff *et al.*, 1991) and that, as for the DA D_3 receptor, 6-OHDA lesions show its presence in the DA-secreting neurons (Chen *et al.*, 1991; Gerfen *et al.*, 1990; Mansour *et al.*, 1990; Sokoloff *et al.*, 1990). Therefore both the DA D_2 and the DA D_3 receptors are autoreceptors. Interestingly, the recent involvement of the DA D_3 receptor in modulating cocaine self-administration has also been associated with its autoreceptor properties.

Clozapine, an "atypical" neuroleptic (i.e., a neuroleptic whose actions are not accompanied by adverse motor control side effects), shows a higher selectivity for the DA D_4 receptor than for any other DA D_2-like receptors. In schizophrenia therapy, clozapine is administered at a concentration 10-fold lower than its affinity constant for the DA D_2 receptor, indicating that clozapine may not be primarily acting at the DA D_2 receptor. The DA D_4 receptor binds clozapine with a 10-fold higher affinity than does the DA D2 receptor (Van *et al.*, 1991). Therefore the DA D_4 receptor may be the specific target of clozapine. A corollary of this is that antagonism of DA binding to the DA D_4 receptor could be an important step in prevention of psychoses, a hypothesis reinforced by the low abundance of DA D_4 mRNA in the striatum (Van *et al.*, 1991). Thus the lack of extrapyramidal side effects observed with clozapine treatment may be a reflection of

DA D_4 receptor localization in the CNS. These observations point to the DA D_4 receptor as an important molecule in the etiology of psychoses.

TISSUE DISTRIBUTION

Because there are no current antibodies against all the different DA receptors, our knowledge of their tissue distribution comes primarily from in situ hybridization experiments. In the CNS, the five DA receptors exhibit overlapping but also distinct localizations. In the periphery, the different receptors are mostly expressed in a tissue-specific fashion.

The tissue distribution of the DA D_1 and DA D_2 mRNAs in the CNS supports their participation in the different aspects of dopaminergic neurotransmission which have been described on the basis of ligand binding and receptor autoradiography experiments. The DA D_1 and DA D_2 receptor mRNAs are present in all dopaminoceptive regions of the rat brain (Mansour *et al.*, 1990; Meador-Woodruff *et al.*, 1989; Fremeau *et al.*, 1991, Meador-Woodruff *et al.*, 1991, Mengod *et al.*, 1989, Najlerahim *et al.*, 1989, Weiner and Brann, 1989). High levels of DA D_1 and DA D_2 mRNAs are present in the caudate-putamen, nucleus accumbens, and olfactory tubercule, and lower levels are present in the septum, hypothalamus, and cortex. Regions where DA D_2 but no DA D_1 mRNAs were detected are the substantia nigra and ventral tegmental area, where the DA D_2 mRNA is expressed at a high level, and the hippocampus. Conversely, the amygdala contains DA D_1 mRNA but little, if any, DA D_2 mRNA.

The DA D_3, DA D_4, and DA D_5 receptor mRNAs are mostly present in tissues where the DA D_1 and/or the DA D_2 mRNAs are also expressed. However, their relative abundances are one to two orders of magnitude lower than that of the DA D_1 or DA D_2 mRNAs (Sokoloff *et al.*, 1990, Van *et al.*, 1991). It has been shown that, relative to the

DA D_1 or DA D_2 receptors, the DA D_3 and DA D_4 receptors are more selectively associated with the "limbic" brain, a region which receives its DA input from the ventral tegmental area and is known to be associated with cognitive, emotional, and endocrine functions. The location of the DA D_3 receptor mRNA, on the other hand, is highly specific. The DA D_5 mRNA is found only in the hippocampus, the hypothalamus, and the parafascicular nucleus of the thalamus and thus might be involved in affective, neuroendocrine, or pain-related aspects of dopaminergic functions (Meador-Woodruff *et al.*, 1992). Finally, using in situ hybridization experiments, it has also been possible to demonstrate that DA D_1 and DA D_2 mRNA are colocalized in 26–40% of all caudate-putamen cells and in about 50% of all DA receptor mRNA-positive cells (Meador-Woodruff *et al.*, 1991). DA modulates ACh levels in the brain by binding to DA receptors located directly on cholinergic cells. The DA D5 receptor, a D1-class receptor subtype, potentiates ACh release (Berlanga *et al.*, 2005).

DA receptor reactivities have also been described in several peripheral organs. mRNA detection by Northern blot analyses have shown that neither DA D_1 nor DA D_3 receptor mRNA are detectable outside the CNS (Sokoloff *et al.*, 1990, Zhou *et al.*, 1990). On the other hand, the DA D_2 receptor mRNA is expressed at high levels in the pituitary (Bunzow *et al.*, 1988) and in the adrenal gland and also in the retina. Of particular interest are the kidney and the heart in which both DA D_1- and DA D_2-like activities have been described (Andersen *et al.*, 1990, Felder *et al.*. 1989). The DA D_5 receptor mRNA is expressed, albeit at low levels, in the kidney (Meador-Woodruff & Grandy, 1992). Whether it is the expected DA D_1-like receptor has yet to be demonstrated. None of the cloned DA D_2-like receptor mRNAs is present in the kidney. On the other hand, the DA D_4 mRNA is expressed in the heart (O'Malley *et al.*, 1992) and might account for the expected DA D_2-like reactivity reported for this tissue. None of the DA D1-like receptor mRNAs exists in significant amount in the heart. These data open the possibility that the

DA D_4 and DA D_5 receptors carry the DA receptor reactivities detected in the kidney and the heart.

In conclusion, one can foresee that an advantage for the organism of having heterogeneous population of receptors is that it permits tissue-specific expression. mRNA detection experiments show that the different DA receptors exhibit specificity in their tissue distribution in the periphery, while in the CNS they often share tissue locations and, possibly, individual neurons as in the case of the DA D_1 and DA D_2 receptors. Although selectivity in cellular distributions has also been found in the CNS, it does not seem to be the rule for the different receptor subtypes. Another factor to consider in our understanding of the importance of the receptor diversity is the comparison of the relative abundance of the subtypes. Variable levels of distinct receptors, added to the fact that interactions between different DA receptor subtypes exist (Bertorello *et al.*, 1990, Seeman *et al.*, 1989; Waddington, 1989; Weiner and Brann, 1989), generate a high degree of diversity in responses that reflect the broad spectrum of the physiological activities known to be regulated by DA.

ALTERNATIVE SPLICING AND GENE POLYMORPHISM

Although the human genome contains five DA receptor genes, the number of DA receptor mRNA species that it encodes is higher. This results from the fact that polymorphism and alternative splicing events play a role in DA receptor gene expression and leads to the existence of more than five different receptor binding sites. DA D1 / DA D2 receptor-selective binding are located either in that loop or in the transmembrane helices (Lan *et al.*, 2006).

The existence of two forms of DA D_2 receptors (Chio *et al.*, 1990, Dal Toso *et al.*, 1989, Giros *et al.*, 1989; Grandy *et al.*, 1989, Miller *et al.*, 1990, Monsma *et al.*,

1989, O'Malley *et al.*, 1990, Selbie *et al.*, 1989). These two forms differ in 29 amino acid residues located in the putative third cytoplasmic loop of the receptor. They are generated by an alternative splicing event which occurs during the maturation of the D2 receptor pre-mRNA (Dal Toso *et al.*, 1989, Grandy *et al.*, 1989, O'Malley *et al.*, 1990). The two D2 receptor forms are neither species- nor tissue-specific; they coexist in all tissues analyzed but at a highly variable ratio. Because of its location in the third cytoplasmic loop, the 29-residue addition was expected to affect G protein coupling and consequently second messenger systems. It has been shown that both forms can inhibit cAMP accumulation (Dal Toso *et al.*, 1989) and that their efficiencies are somewhat variable (Hayes *et al.*, 1992, Montmayeur and Borrelli, 1991). Alternative splicing events have also been shown to occur during the maturation of the DA D3 receptor pre-mRNA (Giros *et al.*, 1991, Snyder *et al.*, 1991).

The existence of different variants of the human D_4 receptor has also been demonstrated, although their generation is not by alternative splicing. These variants differ in the number of 48 base-pair repeats contained in their putative third cytoplasmic loop (Van *et al.*, 1992) and they have been detected in the genomes of different individuals, showing that a genetic polymorphism is responsible for the generation of the DA D_4 receptor variants. These repeats are not present in the rat gene, making the polymorphism specific to humans. When expressed by DNA transfection, the variants containing 2, 4, and 7 repeats bind clozapine with equal affinities in the presence of sodium chloride. In the absence of sodium ions, however, the variants containing 2 and 4 repeats had a six- to eightfold lower dissociation constant for clozapine, while the affinity of the variant containing seven repeats was practically unaffected (Van *et al.*, 1992). Although it is not understood what effects the sodium ions have on receptors, these data indicate that the variants can behave differently with respect to the mechanism of ligand

recognition. Inherited variants of D_4 may explain some of the interindividual variation seen in patient response to drugs like clozapine (Zhao *et al.*, 2005).

Finally, the DA D_5 receptor gene is peculiar among the G-protein-coupled receptors because it is associated with two pseudogenes in the human genome (Grandy *et al.*, 1991). The three DA D_5-related genes are found on different chromosomes (Grandy *et al.*, 1992). Only one gene (DRD5, chromosome 4 q15.1-q15.3) codes for the active receptor; the two others contain an 8-base-pair insertion which leads to a frame shift and are genuine pseudogenes. Interestingly, these pseudogenes appear to be specific to humans, suggesting that the evolution of the DA D_5 pseudogenes is a recent event which may be restricted to primates. Stimulation of the DA D_5 receptor inhibits NADPH oxidase activity, and thus the production of reactive oxygen species (Yang *et al.*, 2006).

CENTRAL NERVOUS SYSTEM REGULATION OF INSULIN SECRETION

Studies conducted have demonstrated that insulin secretion in response to glucose from β-cells of the endocrine pancreas can be modified by the activity of both the sympathetic and parasympathetic branches of the autonomic nervous system (Burr *et al.*, 1976; Campfield *et al.*, 1980). Electrical stimulation of the sympathetic nerves to the pancreas or exposure of the pancreas to exogenous NE decreased glucose-induced insulin secretion. Sympathetic inhibition was observed at glucose concentrations greater than 5mM (Campfield *et al.*, 1976; Campfield *et al.*, 1980). Brain glucagon-like peptide-1 increases insulin secretion (Knauf *et al.*, 2005).

It is demonstration that, central nervous system cell groups projecting into the pancreatic vagal motor neurons received inputs from adrenergic, noradrenergic and serotonergic neurons from the lower brain stem and a dopaminergic input from paraventricular nucleus of hypothalamus (Lowey *et al.*, 1994). It is well established that

the autonomic fibres supplying the pancreas travel via the vagus and splanchnic nerves (Helman *et al.*, 1982). These nerves are clearly related to the ventral hypothalamus. The hypothalamus plays a central role in the integration of neurohormonal function (Oommura & Yoshimatsu, 1984). The ventro-medial hypothalamic nucleus is considered as the sympathetic centre and the stimulation of this area decreases insulin secretion (Helman *et al.*, 1982). Lesions in the ventro-medial hypothalamus, resulted in behavior alterations and morphological changes in pancreatic islets (Scalfani, 1981). Ventro-lateral hypothalamus is the parasympathetic centre, stimulation of which increases the circulating level of insulin (Helman *et al.*, 1982). Lesions in ventro-lateral hypothalamus results in decreased body weight; food intake, plasma insulin levels and decrease in islet size (Powley & Opsahl, 1976). Hyper activation of the HPA axis in diabetes is associated with increased expression of hypothalamic corticotrophin-releasing hormone (CRH) mRNA and hippocampal mineral corticoid receptor (MR) mRNA (Chan *et al.*, 2003)

MECHANISMS OF β- CELL FORMATION

The developing pancreas appears as a protrusion for the dorsal surface of the embryonic gut. At this time the endocrine α-cells differentiate within the epithelial cell matrix. Later pancreatic duct is formed from the pancreatic primordia and the first lobulations containing the differentiating exocrine tissue appear in the body of the gland. During development, β-cells arise from progenitor cells localised in the pancreatic duct to populate new islets. This suggests that pancreatic duct is a source of endocrine stem cells throughout embryogenesis without the need to postulate a neuroendocrine origin. This is supported by the finding that the pancreatic duct is able to regenerate a new pancreas containing exocrine and endocrine cells (Teitelman *et al.*, 1987; Dudck & Lawrence, 1988). Rosenberg and co workers have shown that pancreatic ductal cells are capable of differentiating upon stimulation into adult endocrine cells secreting insulin in a fully

regulated manner (Rosenberg *et al.*, 1987). The different islet cell types appear sequentially during the development *in vivo*. In mouse α-cells appear on embryonic day 14, β-cells at 17, δ-cells at 19 and PP cells at birth.

DIFFERENTIATION OF THE PANCREATIC β-CELL

The new concept is that β-cell mass is dynamic, as it increases and decreases both in function and mass to maintain the normoglycaemic level within a narrow physiological range. The changes in mass can be in both number and individual volume of the β-cells. When the mass cannot increase adequately, diabetes ensues. If β-cell could be induced to replicate a higher rate, this may prove beneficial in maintaining normoglycaemia (Bonner-Weir, 2000).

The two mechanisms of β-cell formation from the embryo, neogenesis, or differentiation from ductal precursor cells and replication of a differentiated β-cell are maintained postnatally even in the adult. Experimentally increased proliferation of differentiated β-cells is seen in a number of models including partial pancreatectomy (Bonner-Weir *et al.*, 1989).

The likely source of precursor cells would be the pancreatic ducts because the adult duct epithelium retains the ability to give rise to all the differentiated cells of the pancreas. It was suggested that (Bonner-Weir *et al.*, 1997) true stem cells are very few in the normal rat pancreas and are not involved in normal pancreatic growth nor in the massive regeneration after partial pancreatectomy. The adult pancreatic duct cells have the capacity to expand and differentiate during the pancreatic regeneration in rats. After replication of the duct cells the transcription factor PDX1/IDX1 was transiently expressed. This protein is expressed in the embryonic pancreatic ducts but is repressed in the ducts shortly before birth (Sharma *et al.*, 1999). The large Maf family of basic

leucine-zipper-containing transcription factors are known regulators of key developmental and functional processes in various cell types, including pancreatic islets (Artner, 2006)

PANCREATIC β-CELL GROWTH REGULATING FACTORS

Glucose

Glucose is one of the best stimuli for β-cell replication *in vivo* and *in vitro*. The chronic glucose infused rat showed that the β-cell mass could increase 50% with a 4-5 fold increase in β-cell replication and by hypertrophy (Bonner-Weir *et al.*,1989; Bonner-Weir & Smith, 1997).

Glucose has been reported to stimulate β-cell proliferation both *in vivo* and *in vitro*. It was suggested that glucose stimulates the β-cell proliferation by regulating the number rather than the rate at which the β-cells enter the cycle (Hellerstrom, 1977; Swenne, 1982;). High glucose increases extracellular matrix production in pancreatic stellate cells by activating the renin-angiotensin system (Ko *et al.*, 2006).

Insulin

Recent observations indicate that insulin can stimulate pancreatic islet β-cell growth *in vivo*. McEvoy and Herge (1978) reported that administration of insulin to diabetic rats implanted with foetal pancreas resulted in a three-fold increase in β-cell mass in some of the pancreatic recipients. Rabinovitch *et al.*. (1982) have demonstrated that insulin can stimulate islet β-cell replication directly, possibly through a receptor for multiplication stimulating activity or insulin like growth factor .Insulin favored regeneration of β-cell by activating the neogenesis of the β-cells from precursor cells (Movassat *et al.*, 1997). It is reported that mannoheptulose, an agent believed to inhibit

insulin release, inhibits β-cell replication *in vitro* (King *et al.*, 1978). It has been reported that foetal rat pancreas explanted *in vitro* in the presence of added insulin had greater β-cell volume and a greater insulin content than those grown without insulin (McEvoy, 1981).

Role of growth factors

There are several reports on effects of growth factors in the normal β-cell growth. The growth hormone (GH), prolactin (PRL) and placental lactogen (PL) were found to stimulate proliferation of normal rat β-cells (Nielsen, 1986). Among the large number of protein hormones GH and lactogenic peptides, PRL and PL have an important role in β-cell proliferation. GH has been reported to stimulate the *in vitro* replication of foetal (Dudek *et al.*, 1984), neonatal (Brelje *et al.*, 1989) and adult rat β-cells (Swenne & Hill, 1989). In most of the studies there was also a stimulatory effect of GH on the insulin content or secretion, and the majority of effects were mimicked by PRL and PL. Growth hormone appears to elicit its biological activities by inducing local production of insulin-like growth factors (IGF) in target cells (Milner & Hill, 1984). It is reported that in both foetal and adult islets, growth factors, but not glucose stimulated release of IGF-I partially counteracted the mitogenicity of GH (Swenne *et al.*, 1987; Swenne & Hill, 1989). Culture of islets with platelet derived growth factor (PDGF) and IGF-I caused an increase in the islet content of polyamines resembling the effect of GH. These two growth factors elicited a stimulation of DNA synthesis in islets (Sjoholm *et al.*, 1990). Epidermal growth factor (EGF) is known to stimulate DNA replication in many systems like hepatocytes and pituitary cells. Chatterjee *et al.*, (1986) have shown that EGF stimulates proinsulin biosynthesis as well as [^{3}H]thymidine uptake into pancreatic islets. They suggested that EGF behaves like glucose in stimulating both insulin biosynthesis and β-cell replication. Insulin-like growth factor 1 (IGF-1) is a potent mitogenic and secretory factor (Mergler *et al.*, 2005).

Amino acids and polyamines

The amino acid enrichment in the organ culture appears to favour the growth of pancreatic rudiments suggesting that metabolites other than glucose might influence the development of pancreatic β-cells. DeGasparo *et al.*, (1978) have shown that enrichment of amino acids in the culture medium is a factor which induce the growth of β-cells in organ culture. Amino acids are also able to stimulate β-cell replication, and it appears in the early foetal life as they are more important than glucose in this respect. Amino acids, and human amniotic fluid were recently also identified as potent stimulators of cell proliferation in adult mouse islets (Dunger *et al.*, 1990). It is shown that glucose regulates polyamine content *in vitro*. Polyamines like, putrescine and spermidine are necessary for the maintenance of normal insulin and protein biosynthesis, whereas spermine may exert a role in some other cellular processes such as DNA replication, RNA transcription and glucose stimulated insulin release (Welsh & Sjoholm, 1988).

Regulatory proteins

A pancreatic gene celled *reg*, encoding a 165-amino acid protein was isolated from regenerating rat islets after partial pancreatectomy. The *reg* gene is expressed in experimentally induced regenerating or hyperplastic islets. The ectopic expression of the *reg* gene occurs in some human colonic and rectal tumors, suggesting that enhanced *reg* expression may be related to the proliferative state of tumor cells. At present, any direct relationship between Reg protein and β-cell replication remains to be established. However, since the Reg protein is a secretory protein and it can be expressed at an early stage of pancreatic cell differentiation, the Reg protein may act on the stem cells of β-cells in an autocrine or paracrine manner. In normal mature exocrine cells, the *reg* gene is expressed and the gene product may be necessary to maintain adequate exocrine pancreatic function (Unno *et al.*, 1992). The Reg protein is synthesised and secreted from regenerating β-cells, and that the expression of Reg was closely associated with β-

cell regeneration. Recently, Reg protein was shown to stimulate pancreatic β-cell growth, further strengthening the notion that it is involved in pancreatic islet growth and regeneration (Watanabe *et al.*, 1994).

Prolactin and placental lactogen are reported to exert insulin antagonistic effects and are supposed to play a role in the increased insulin demand during pregnancy (Freinkel, 1980). It is reported that hGH, PRL and hPL stimulated both insulin production and DNA synthesis in isolated islets from rats and mice maintained *in vitro* (Nielsen 1982; Nielsen *et.al.*, 1992). Growth hormone and prolactin are important growth factors for pancreatic β-cells. The effects exerted by these hormones on proliferation and on insulin synthesis and secretion in β-cells are largely mediated through the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway (Lindberg *et al.*, 2005).

The insulin like growth factors (IGF I and IGF II) are mitogenic peptides that are structurally related to insulin. The biological effects of IGF are mediated by cell surface receptors (LeRoit *et al.*, 1992). DNA synthesis in β-cells was also stimulated by over expression of growth factor receptors such as the platelet-derived growth factor receptor (PDGF-R) and fibroblast growth factor receptor (FGF-R). Growth factor mediated signal transduction pathways lead to changes in expression of cell cycle proteins, eventually, resulting in the increased proliferation effects. Identification of cell cycle modulators of β-cell proliferation will provide insights into the replication potential of fetal, young and adult islet cells.

Other factors

Inhibitors of β-cell proliferation include transforming growth factor β (TGF-β), the cytokine interleukin 1-β (IL1-β), pancreastatin and the diazepam binding inhibitor, all of which inhibit fetal rodent β-cell proliferation. TGF-β inhibits glucose stimulated β-cell replication (Sjoholm & Hellerstrom, 1991(b)). IL1-β suppresses islet cell proliferation in adult mice and rats (Southern *et al.*, 1990). However, the role of IL1-β in fetal islet cell proliferation is slightly complex with the first 24 hrs of stimulation leading to a suppression of β-cell proliferation followed by a potent mitogenic stimulus after 3 days of cytokine exposure. Sjoholm *et al.*, (1991 (c)) identified pancreastatin and diazepam-binding inhibitor (acyl-CoA binding protein) as inhibitors of β-cell replication. Both pancreastatin and diazepam-binding inhibitor are produced by islet cells (Chen *et al.*, 1988) and inhibit insulin secretion and may function as inhibitors of β-cell replication *in vivo*.

Neurotransmitters as growth signals

In the adult nervous system, neurotransmitters act as chemical mediators of intercellular communication by the activation of specific receptors and second messengers in postsynaptic cells. This view derives from the finding that a number of 'classical' neurotransmitters are present in primitive organisms and early embryos in the absence of a nervous system, and pharmacological evidence that these substances regulate morphogenetic activities such as proliferation, differentiation, cell motility and metamorphosis. These phylogenetically old functions may be reiterated in the developing nervous system and in the humoral functions of neurotransmitters outside the nervous system (Lauder, 1993)

NE induces the production of EGF and HGF at distal sites and also enhances the response to HGF at target tissues (Broten *et al.*, 1999). NE rises rapidly in the plasma

within one hour after PH (Knopp *et al.*, 1999). Prazosin, a specific antagonist of α_1 adrenergic receptor, as well as sympathetic denervation greatly decreases DNA synthesis at 24 hrs after PH (Cruise *et al.*, 1989). Addition of NE to hepatocytes stimulates Ca^{++} mobilisation or PI turnover and either or both of these processes were proposed to be involved in the mitogenicity of NE (Exton, 1981 & 1988). Hepatic neoplasm are characterised by an increase in α_2-and β-adrenergic receptors and a concomitent decline in α_1-receptors (Sanae, 1989).

ACh analogue carbachol stimulated DNA synthesis in primary astrocytes derived from perinatal rat brain (Ashkenazi *et al.*, 1989). ACh is reported to induce proliferation of rat astrocytes and human astrocytoma cells (Guzzetti *et al.*, 1996).

GABA inhibits the growth of murine squamous cell carcinoma and HeLa cell lines (Boggust & Al-Nakib, 1986). Gliomas with high proliferation rate lack the expression of functional GABA binding sites (Labrakakis *et al.*, 1988). GABA also plays an important role in terminating the growth of rapidly developing tissues *in utero* (Gilon *et al.*, 1987). Studies from our lab have shown that hypothalamic GABAergic system plays an important role in the neoplastic transformation of rat liver. $GABA_A$ receptor agonist muscimol, dose dependently inhibited EGF induced DNA synthesis and enhanced the TGFβ1 mediated suppressed DNA synthesis in rat primary hepatocyte culture (Biju *et al.*, 2001). Increased $GABA_A$ receptor activity inhibits proliferation of HepG2, human hepatocyte carcinoma cell line. The inhibition is prolonged in the cell line co-transfected with $GABA_A$ receptor β_2 and γ_2 subunit genes (Zhang *et al.*, 2000).

Effect of dopamine and dopamine receptors on cell proliferation.

In the developing mammalian brain, DA contributes to morphogenesis by governing the activity of neural stem cells (Cameron *et al.*., 1998). DA appears early

during ontogenesis, as do its receptors (Lindow and Rakic, 1995,Diaz, *et al.*, 1997), and affects cell proliferation in the embryonic germinal zone (Ohtani, *et al.*, 2003). DA specifically inhibits forebrain neural stem cell proliferation (Kippin *et al.*, 2005). DA D2-like receptors are expressed in pancreatic β-cells and mediate inhibition of insulin secretion (Rubi *et al.*, 2005). Studies have demonstrated that dopaminergic agonists ameliorated hyperglycaemia and hyperlipidaemia in obese and diabetic rodents (Liang *et al.*, 1998)

Second messengers and cell proliferation

Some information is available concerning second messengers activated by these neurotransmitters in developing cells, little is known about subsequent steps involving signal transduction cascades leading to their final outcomes. This review attempts to provide testable hypotheses regarding possible cellular and molecular mechanisms downstream of second messengers activated by neurotransmitters, based on recent insights into signal transduction cascades activated by classical growth-regulatory signals. In many cases, there are clear points of convergence between these pathways, raising the interesting possibility that neurotransmitters and other growth-regulatory signals may cooperate to regulate developmental functions of cells and tissues (Weiss et al., 1998)

MATERIALS AND METHODS

BIOCHEMICALS AND THEIR SOURCES

Biochemicals used in the present study were purchased from Sigma Chemical Co., USA. All other reagents were of analytical grade purchased locally. HPLC solvents were of HPLC grade obtained from SRL and MERCK, India.

Biochemicals:

(±)Norepinephrine, (±)epinephrine, 5-hydroxytryptamine, dopamine, homovanillic acid, sodium octyl sulfonic acid, ethylene glycol bis (β-aminoethyl ether)-EGTA, ethylene diamine tetra acetic acid-EDTA, HEPES - [n′ (2-hydroxy ethyl)] piperazine-n′-[2-ethanesulfonic acid], ascorbic acid, pargyline, D-glucose, calcium chloride, butaclamol, (±) 7-hydroxy-2-(di-n-propylamino) tetralin hydrogen bromide, (-) sulpiride, collagenase type XI and bovine serum albumin fraction V were purchased from Sigma Chemical Co., St. Louis, MI, USA.

YM-09151-2: *cis–N-(1-benzyl-2-methylpyrrolidine-3-yl)-5-chloro-2-methoxy-4-methylaminobenzamide* was a gift from Yamanouchi Pharmaceuticals Ltd, Tokyo, Japan, Bromocriptine was a gift from and Dr. Jacqueline Trouillas *Laboraitoire d'Histologie-Embryologie (J.T.,P.C., C.G.), Alexis Carrel, France.*

Radiochemicals

[^{3}H] Dopamine (Sp. activity- 45.1Ci/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.. Radioimmunoassay kit for insulin assay was purchased from Bhabha

Atomic Research Centre, Mumbai, India. cAMP and IP3 assay kits were purchased from Amersham Bioscience. USA

Molecular biology chemicals

Reverse transcriptase enzyme MuMLV was purchased from Amersham Biosciences, USA, random hexameres, human RNAse inhibitor, DNA molecular weight markers and dNTPs were purchased from Bangalore Genei Pvt. Ltd. India. Tri-reagent kit was purchased from Sigma Chemical Co., USA. Dopamine D_2 receptor primers for PCR and ß-actin primers for PCR were synthesised by Sigma Chemical Co., USA.

Animals

Wistar weanling rats of 80-100g body weight were purchased from Amritha Institute of Medical Science, Cochin 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.*

Partial pancreatectomy

Male Wistar weanling rats, 45 weeks old, were anaesthetised under aseptic conditions, the body wall was cut opened and 60-70% of the total pancreas, near to the spleen and duodenum, was removed (Pearson, 1977). The removal of most of the pancreas was done by gentle abrasion with cotton applications, leaving the major blood vessels supplying other organs intact [Zangen, 1997]. The sham was done in an identical procedure except that the pancreatic tissue was only lightly rubbed between fingertips using cotton for a minute instead of being removed. Bodyweight and blood glucose levels were checked routinely. The rats were maintained for different time intervals (72 hours and 7 days) and sacrificed.

Tissue preparation

Rats were sacrificed by decapitation and the brain regions - cerebral cortex, brain stem, corpus striatum and hypothalamus were dissected out quickly over ice according to the procedure of [Glowinski & Iversen 1966]. The tissues were stored at -70^{0} C until assay. Pancreas was also dissected out and stored.

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 $KHCO_3$, 10mM HEPES. Autoclaved triple distilled water was used for the preparation of the buffer.

The pancreas was aseptically dissected out into a sterile Petri-dish containing ice cold HBSS and excess fat and blood vessels were removed. The pancreas was cut into small pieces and transferred to a sterile glass vial containing 2ml collagenase type XI solution (1.5 mg/ml in HBSS), pH 7.4. The collagenase digestion was carried out for 15 minutes at 37°C in an environmental shaker with vigorous shaking (300rpm/min). The tissue digest was filtered through 500 µm nylon screen and the filtrate was washed with three successive centrifugations and resuspensions in cold HBSS. Islets visible as yellowish white spheres were handpicked carefully by finely drawn pasture pipettes and aseptically transferred to HBSS. The islets prepared by this method were used for all other experiments.

Quantification of monoamines and their metabolites

The monoamines were assayed according to Jackson *et al.*, (1997). The tissues from brain regions were homogenised in 0.4N perchloric acid. The homogenate was centrifuged at 5000 x g for 10 minutes at 4°C and the clear supernatant was filtered through 0.45µm HPLC grade filters and used for quantification of catecholamines. Norepinephrine, epinephrine, dopamine and homovanillic acid were determined in high performance liquid chromatography (HPLC) with electrochemical detector (HPLC-ECD) (Shimadzu, Japan) fitted with CLC-ODS reverse phase column of 5µm particle size. The mobile phase consisted of 75 mM sodium dihydrogen orthophosphate, 1mM sodium octyl sulfonate, 50mM EDTA and 7% acetonitrile. The pH was adjusted to 3.25 with orthophosphoric acid, filtered through 0.22µm filter (Millipore) and degassed. A Shimadzu (model 10 AS) pump was used to deliver the solvent at a rate of 1 ml/min. The catecholamines were identified by an amperometric detection using an electrochemical detector (Model 6A, Shimadzu, Japan) with a reduction potential of + 0.8 V, with the range set at 16 and a time constant of 1.5 seconds. Twenty microlitre aliquots filtered through 0.22µm filter were injected into the system. The peaks were identified by relative retention times compared with external standards and quantitatively estimated using an integrator (Shimadzu, C-R6A -Chromatopac) interfaced with the detector. Data from different brain regions of the experimental and control rats were statistically analysed and tabulated.

In the case of pancreas and adrenals, the tissues were homogenised in 0.1N perchloric acid. The homogenate was centrifuged at 5000xg for 10 minutes at 4°C (Kubota refrigerated centrifuge) and the clear supernatant was filtered through 0.22µm HPLC grade filters and used for HPLC analysis. Data from pancreas and adrenals of the experimental and control rats were statistically analysed and tabulated.

Determination of plasma monoamines and their metabolites

Plasma monoamines were assayed as per Jackson *et al.*, (1997). 1.0 ml of plasma was diluted with 1.0 ml of distilled water. To this was added 50µl of 5mM sodium bisulfite was added and mixed, followed by 25µl of 1M Tris buffer pH 8.6. Acid alumina (20mg) was then added and the contents were mixed well using a shaker. The supernatant was aspired out by means of a pasture pipette. The alumina was washed twice with 2.0 ml of 5mM sodium bisulfite. To the final pellet of alumina 0.2ml of 0.1 N perchloric acid was added and mixed in a shaker for 15 minutes. The supernatant was filtered using a syringe top filter (0.22 µm) and used in the determination of monamines and its metabolites. Data from the plasma of the experimental and control rats were statistically analysed and tabulated

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.

DOPAMINE RECEPTOR BINDING STUDIES USING [^{3}H] RADIOLIGANDS IN THE BRAIN REGIONS

Dopamine 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 10 volumes of cold 50mM Tris-HCl buffer, along with 1mM EDTA, 0.01% ascorbic acid, 4mM $MgCl_2$, 1.5 mM $CaCl_2$, pH.7.4 and centrifuged at 38,000 x g for 30min. at 4°C. The pellet was washed twice by rehomogenisation 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 1mM EDTA, 0.01%, ascorbic acid, 1mM $MgCl_2$, mM $CaCl_2$ 2, 120mM NaCl, 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 butaclamol. Competition studies were carried out with 0.25nM [^{3}H] DA in each tube with unlabelled ligand concentrations varying from 10^{-9}M - 10^{-3}M of unlabelled DA.

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.

Dopamine D_2 receptor binding studies using [^{3}H] YM-09151-2 in the brain regions of rats.

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 corpus striatum, hypothalamus, cerebral cortex and brain stem were weighed and homogenized in 10 volumes of ice cold 50mM Tris-HCl buffer, along with 1mM EDTA, 5mM $MgCl_2$, 1.5 mM $CaCl_2$, 120mM NaCl, 5mM KCl pH.7.4. The homogenate was centrifuged at 48,000xg for 30 min. The pellet was washed and centrifuged with 50 volumes of the buffer at 48,000xg 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., 0.25nM-2.0nM of [^{3}H] YM-09151-2 in 50mM Tris-HCl buffer, along with 1mM EDTA, 5mM $MgCl_2$, 1.5 mM $CaCl_2$, 120mM NaCl, 5mM KCl with 10μM pargyline and 0.1% ascorbic acid in a total incubation volume of 250μl containing 200-300μg of protein. Specific binding was determined using 5.0 μM unlabelled sulpiride. Competition studies were carried out with 0.25nM [^{3}H] YM-09151-2 in each tube with unlabelled ligand concentrations varying from 10^{-12} - 10^{-3}M of unlabelled YM-09151-2.

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.

DOPAMINE RECEPTOR BINDING STUDIES USING [^{3}H] RADIOLIGANDS IN THE PANCREATIC ISLETS OF RATS

Dopamine D_2 receptor binding studies using [^{3}H] dopamine in the islets experimental rats

Pancreatic islets of control, diabetic and insulin treated diabetic rats were isolated by standard collagenase digestion procedure 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 $KHCO_3$, 10mM HEPES with 0.2% (w/v) BSA (Fraction V), equilibrated with 5% CO_2 and pH 7.3 at room temperature. Autoclaved triple distilled water was used in the preparation of the buffer.

Pancreatic islets isolated as per the above mentioned procedure was homogenised in a polytron homogeniser with 10 volumes of cold 50mM Tris-HCl buffer, along with 1mM EDTA, 0.01 ascorbic acid, 4mM $MgCl_2$, 1.5mM $CaCl_2$, pH.7.4 and centrifuged at 38,000xg for 30min. at 4°C. The pellet was washed twice by rehomogenization and centrifuged twice at 38,000xg 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., 0.1nM-2.0 nM of [^{3}H] DA in 50mM Tris-HCl buffer, along with lmM EDTA, 0.01% ascorbic acid, 1mM $MgCl_2$, 2mM $CaCl_2$, 120mM NaCl, 5mM KCl, 10μM pargyline pH.7.4 in a total incubation volume of 250μl containing 150-200μg of proteins. Specific binding was determined using 100μM unlabelled butaclamol. Competition studies were carried out with 0.5nM [^{3}H] DA in each tube with unlabelled ligand concentrations varying from 10^{-12}M- 10^{-3}M of DA.

Tubes were incubated at 25°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 in the pancreatic islets of rats.

The assay was done in a similar way as in brain regions. After isolation the islets were homogenized in 10 volumes of ice cold 50mM Tris-HCl buffer, along with 1mM EDTA, 5mM $MgCl_2$, 1.5 mM $CaCl_2$, 120mM NaCl, 5mM KCl pH.7.4. The homogenate was centrifuged at 48,000xg for 30 min. The pellet was washed and recentrifuged with 50

volumes of the buffer at 48,000xg 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., 0.1nM-2.0nM of [^{3}H] YM-09151-2 in 50mM Tris-HCl buffer of pH 7.4, along with 1mM EDTA, 5mM $MgCl_2$, 1.5 mM $CaCl_2$, 120mM NaCl, 5mM KCl with 10μM pargyline and 0.1% ascorbic acid in a total incubation volume of 250μl containing 150-250 μg 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^{-12} - 10^{-3}M unlabelled YM-09151-2.

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).

IN VITRO DNA SYNTHESIS STUDIES IN THE PANCREAS

Pancreatic islets were prepared by the collagenase digestion method as mentioned earlier. The isolated islets were then suspended in RPMI 1640 medium containing 10% FCS, and incubated for 16hrs at 37^0C and 5% CO_2 to remove the fibroblasts. After fibroblast removal the cells were re-cultured for three days to remove all other non-endocrine tissue. After the incubation the medium will be rich in β-cells. At the end of culture period, groups of 100 islets were transferred to 1ml fresh medium containing 5% FCS, antibiotics, different concentrations of glucose (4 and 20mM), DA (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4}) and dopamine antagonists- butaclamol and sulpiride, and added and cultured free floating for an additional 24 hrs in the presence of 1μCi of [^{3}H] thymidine [Sjoholm, 1991]. The radioactivity incorporated was determined by counting in a scintillation counter.

INSULIN SECRETION STUDY – 24 HOURS

The islets were harvested after removing the fibroblasts and cultured for 24hrs in RPMI-1640 medium. Cells were incubated with dopamine (10^{-12} M - 10^{-4} M), antagonist- butaclamol, sulpiride and agonist - bromocriptine in the presence of glucose (4mM and 20mM) to study the effect of dopamine on insulin synthesis and release [MacDonald, 1990]. The cells were then harvested and washed with fresh KRB and then incubated for another 24 hr in the presence (4mM and 20mM) glucose concentrations. At the end of incubation period, the medium was collected and insulin content was measured by using radio-immuno assay kit from BARC, Mumbai.

cAMP AND IP3 ASSAY

Pancreatic islets were isolated by standard collagenase digestion method. Cells were incubated for one hour in the presence of various concentrations of dopamine, DA antagonists-butaclamol and dopamine D_2 receptor specific antagonist sulpiride in the presence of both 4mM and 20 mM glucose. Assay of cAMP and IP3 was done according to the procedure of Amersham Biosciences cAMP assay kit.

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 were separated by the second antibody-polyethylene glycol (PEG) aided separation method. The radioactivity associated with bound fraction of sample and standards were used for quantitating insulin concentration in samples

Assay Protocol

Standards, ranging from 0 to 200μU/ml, insulin free serum and insulin antiserum (50μl each) were added together and the volume was made up to 250μl with assay buffer. Samples of appropriate concentration from the experiments from the secretion studies were used for the assay. They were incubated overnight at 2°C. Then [^{125}I] insulin (50μl) was added and incubated at room temperature for 3hours. The second antibody was added (50μl) along with 500μl 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 $\%B/B_o$ on the Y-axis and insulin concentration/ml on the X-axis of a log-logit graph. $\%B/B_o$ 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).

EXPRESSION STUDIES OF DOPAMINE D2 RECEPTOR IN DIFFERENT BRAIN REGIONS AND PANCREAS OF RATS USING REAL-TIME PCR

Preparation of RNA

RNA was isolated from the different brain regions of control and experimental 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 12,000 x *g* for 10 minutes at 4°C. 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 12,000 x *g* for 15 minutes at 4°C. 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 12,000 x *g* for 10 min at 4°C.

RNA precipitate forms a pellet on the sides and bottom of the tube. The supernatant was removed and the RNA pellet was washed with 500µl of 75% ethanol, vortexed and centrifuged at 12,000 x *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 absorbances were measured at 260nm and 280nm. Pure RNA preparation has the ratio of absorbance at 260/280 as ≥ 1.7. The concentration of RNA was calculated as one A_{260} = 42µg.

RT -PCR Primers

The following primers were used for dopamine D_2 receptors and β-actin mRNA expression studies.

5'- GCC AAA CCA GAG AAG AAT GG -3' 5'- GAT GTG CGT ATG AAG GAA GG -3' PRODUCT SIZE: 500bp	DA D_2
5'- CAA CTT TAC CTT GGC CAC TAC C -3' 5'- TAC GAC TGC AAA CAC TCT ACA CC -3' PRODUCT SIZE: 150bp	β-ACTIN

RT-PCR of dopamine D_2 receptors and β-actin

RT-PCR was carried out in a total reaction volume of 20µl reaction mixture in 0.2ml tubes. RT-PCR was performed on an Eppendorf Personal thermocycler. cDNA synthesis of 2ng RNA was performed in a reaction mixture containing MuMLV reverse transcriptase (40U/reaction), 2mM dithiothreitol, 4 units of human placental RNAse inhibitor, 0.5µg of random hexamer and 0.25mM (dNTPS dATP, dCTP, dGTP and

dTTP). The tubes were then incubated at 42°C for one hour. Then reverse transcriptase, MuMLV, was inactivated by heating at a temperature of 95°C.

Thermocycling profile for Realtime PCR

For obtaining higher stringency conditions RT-PCR profile was adopted. PCR was carried out in a 25µl volume reaction mixture in the specially designed Real-Time PCR tubes provided by Takara Japan., containing 2ml of cDNA, 12.5µl of reaction mixture and 1.0ml of primer and 9.5 ml of DEPC treated water. The reaction mixture

95°C..... 60 Sec Initial denaturation

95°C ..... 10 sec Denaturation

65°C ..... 30 sec Annealing

72°C ..... 30 sec extension

Analysis of Realtime PCR products

The crossing threshold (Ct) represents the comparative expression of the mRNA of the gene of interest from the sample used. The Ct values are taken from the graph directly in the software of the RealTime PCR provided along with the (Cephaied Smartcycler software v2.0) and selected for each experimental group separate. The Ct value is inversely proportional to the gene expression.

CALCIUM IMAGING USING CONFOCAL MICROSCOPY

Pancreatic islets cell were cultured over night. Media was removed and washed thrice with calcium free balanced solution. DA at 10^{-8}M concentration was added and the final volume was adjusted to 1.5ml in a Petri dish. Cells were loaded with Rohd-2 dye. A continuous scanning was done with 1 second interval. Pictures at various time intervals were taken and analysed for the intensity of emission which is directly proportional to the intracellular calcium level.

STATISTICS AND SOFTWARES USED FOR ANALYSIS

Statistical evaluations were done by ANOVA using InStat (Ver.2.04a) computer programme. Linear regression analysis for displacement assay was done using GraphPad Prism (Ver.3.02) Scatchard plot analysis was done by SigmaPlot (Ver. 8.01 SSP). Confocal image was captured by Confocal assistant (Ver.4.01). Confocal image analysis was done using ImageJ (Ver. 1.34S).

DA AND HVA CONTENT IN THE CEREBRAL CORTEX OF EXPERIMENTAL RATS

DA content decreased significantly ($p<0.01$) in the cerebral cortex of 72 hours pancreatectomy and reversed to near sham value after 7 days of partial pancreatectomy. HVA content did not show significant change in 72 hours pancreatectomy and increased significantly after 7 days of partial pancreatectomy ($p<0.01$). HVA/DA ratio increased significantly in 72 hours pancreatectomy ($p<0.05$) (Table-1).

Table 1: Dopamine and homovanillic acid content (nmoles / g wet weight tissue)

Animal status	DA	HVA	HVA/DA RATIO
Sham	1.425 ± 0.122	0.485 ± 0.074	0.341 ± 0.020
72 hours pancreatectomy	0.703 ± 0.055 **	0.427 ± 0.020	0.185 ± 0.006 *
7 days pancreatectomy	1.304 ± 0.130	0.924 ± 0.079 **	0.531 ± 0.063

Values are Mean ± S.E.M. of 4-6 separate experiments
**$p<0.01$ when compared to sham; *$p<0.05$ when compared to sham

DA AND HVA CONTENT IN THE CORPUS STRIATUM OF EXPERIMENTAL RATS

DA content decreased significantly ($p<0.001$) in the corpus striatum of 72 hours pancreatectomy and reversed to near sham value after 7 days of partial pancreatectomy. HVA content increased significantly ($p<0.001$) in 72 hours pancreatectomy and restored to near sham value after 7 days of partial pancreatectomy. There was a significant increase in the HVA/DA ratio in 72 hours pancreatectomy ($p<0.001$) (Table-2).

Table 2: Dopamine and homovanillic acid content (nmoles / g wet weight tissue)

Animal status	DA	HVA	HVA/DA RATIO
Sham	19.75 ± 0.58	0.070 ± 0.004	0.078 ± 0.004
72 hours pancreatectomy	8.28 ± 0.67 ***	0.290 ± 0.070 ***	0.203 ± 0.016 ***
7 days pancreatectomy	22.31 ± 1.48	0.072 ± 0.001	0.069 ± 0.003

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to sham

DA AND HVA CONTENT IN THE BRAIN STEM OF EXPERIMENTAL RATS

DA content decreased significantly (p<0.001) in the brain stem of 72 hours pancreatectomy and was not restored to sham value after 7 days of partial pancreatectomy. There was a significant (p< 0.01) increase in the DA content after 7 days of pancreatectomy compared to 72 hours after pancreatectomy. HVA content decreased significantly (p<0.001) in 72 hours pancreatectomy and restored to near sham value after 7 days of partial pancreatectomy. There was a significant increase in the HVA/DA ratio in 72 hours pancreatectomy (p<0.001) (Table-3).

Table 3: Dopamine and homovanillic acid content (nmoles / g wet weight tissue)

Animal status	DA	HVA	HVA/DA RATIO
Sham	2.141 ± 0.113	1.242 ± 0.059	0.497 ± 0.047
72 hours pancreatectomy	0.931 ± 0.016 ***	0.886 ± 0.022 ***	0.942 ± 0.028 ***
7 days pancreatectomy	1.447 ± 0.167 **	1.301 ± 0.037	0.575 ± 0.047

Values are Mean ± S.E.M. of 4-6 separate experiments
**p<0.01 when compared to sham; ***p<0.001 when compared to sham

DA AND HVA CONTENT IN THE HYPOTHALAMUS OF EXPERIMENTAL RATS

DA content decreased significantly (p<0.001) in the hypothalamus of 72 hours pancreatectomy and was not restored to sham value after 7 days pancreatectomy. HVA content decreased significantly (p<0.001) in 72 hours pancreatectomy and restored to

near sham value after 7 days of partial pancreatectomy. There was a significant decrease in the HVA/DA ratio in 72 hours pancreatectomy ($p<0.001$) (Table-4).

Table 4: Dopamine and homovanillic acid content (nmoles / g wet weight tissue)

Animal status	DA	HVA	HVA/DA RATIO
Sham	1.241 ± 0.09	0.765 ± 0.06	0.616 ± 0.05
72 hours pancreatectomy	0.731 ± 0.03 ***	0.224 ± 0.06 ***	0.306 ± 0.05 ***
7 days pancreatectomy	0.926 ± 0.02	0.645 ± 0.05	0.695 ± 0.06

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to sham

DA AND HVA CONTENT IN THE PANCREAS OF EXPERIMENTAL RATS

DA content decreased significantly ($p<0.001$) in the pancreas of 72 hours pancreatectomy and was not restored to sham value after 7 days of partial pancreatectomy. But there was a significant ($p<0.05$) increase in the DA content 7 days after pancreatectomy compare to 72 hours after pancreatectomy. HVA content decreased significantly ($p<0.01$) in 72 hours pancreatectomy and restored to near sham value after 7 days of partial pancreatectomy. HVA/DA ratio remained unchanged in 72 hours pancreatectomy (Table-5).

Table 5: Dopamine and homovanillic acid content (nmoles / g wet weight tissue)

Animal status	DA	HVA	HVA/DA RATIO
Sham	1.569 ± 0.164	1.682 ± 0.050	1.072 ± 0.048
72 hours pancreatectomy	0.801± 0.040 ***	0.919 ± 0.015 **	1.147 ± 0.051
7 days pancreatectomy	1.139 ± 0.131 *	1.115 ± 0.070	0.978 ± 0.036

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to sham; **p<0.01 when compared to sham; *p<0.05 when compared to sham

DA AND HVA CONTENT IN THE ADRENALS OF EXPERIMENTAL RATS

DA content decreased significantly ($p<0.001$) in the adrenals of 72 hours pancreatectomy. A further significant ($p<0.001$) decrease was observed after 7 days of partial pancreatectomy. HVA content increased significantly ($p<0.01$) during pancreatic regeneration. A further significant ($p<0.001$) increase in HAV level was observed after 7 days of partial pancreatectomy. HVA/DA ratio increased significantly ($p<0.01$) after 72 hours of pancreatectomy and a further significant increase ($p<0.001$) was observed in 7 days after pancreatectomy (Table-6).

Table 6: Dopamine and homovanillic acid content (nm oles /g wet weight tissue)

Animal status	DA	HVA	HVA/DA RATIO
Sham	28.056 ± 1.988	0.621 ± 0.109	0.022 ± 0.001
72 hours pancreatectomy	11.308 ± 0.269 ***	1.044 ± 0.125	0.183 ± 0.020**
7 days pancreatectomy	7.434 ± 0.287 ***	11.128 ± 0.358 ***	1.554 ± 0.110 ***

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 when compared to sham

NE AND EPI CONTENT IN THE ADRENALS OF EXPERIMENTAL RATS

NE and EPI content in adrenals were decreased significantly ($p<0.001$) during pancreatic regeneration and it was found to be reversed to near sham value in 7 days after pancreatectomy (Table-7).

Table 7: Norepinephrine and epinephrine content (nm oles/ wet weight tissue)

Animal status	Epinephrine	Norepinephrine
Sham	3.28 ± 0.11	2.89 ± 0.12
72 hours pancreatectomy	1.81 ± 0.16 ***	1.24 ± 0.02***
7 days pancreatectomy	3.17 ± 0.18	2.71 ± 0.04

Values are Mean ± S.E.M. of 4-6 separate experiments; ***p<0.001 when compared to sham.

DA AND HVA CONTENT IN THE PLASMA OF EXPERIMENTAL RATS

DA content increased significantly ($p<0.001$) in the plasma of 72 hours pancreatectomy and restored to near sham value after 7 days of partial pancreatectomy. HVA content remained unchanged in 72 hours pancreatectomy. But there was a significant decrease in HVA content after 7 days of pancreatectomy compare to 72 hours and sham ($p<0.01$, $p<0.001$). HVA/DA ratio decreased significantly ($p<0.01$) and there was no change in 7 days pancreatectomy (Table-8).

Table 8: Dopamine and homovanillic acid level (nmoles / ml)

Animal status	DA	HVA	HVA/DA RATIO
Sham	0.013 ± 0.001	0.041 ± 0.001	1.983 ± 0.006
72 hours pancreatectomy	0.029 ± 0.001 ***	0.038 ± 0.003	1.451 ± 0.044 **
7 days pancreatectomy	0.013 + 0.001	0.022 + 0.001**	1.477 + 0.117 **

Values are Mean ± S.E.M. of 4-6 separate experiments
**p<0.01 when compared to sham; ***p<0.001 when compared to sham

DOPAMINE D_2 RECEPTOR ANALYSIS IN BRAIN REGIONS AND PANCREAS.

SCATCHARD ANALYSIS USING [^{3}H] YM-09151-2 IN CEREBRAL CORTEX

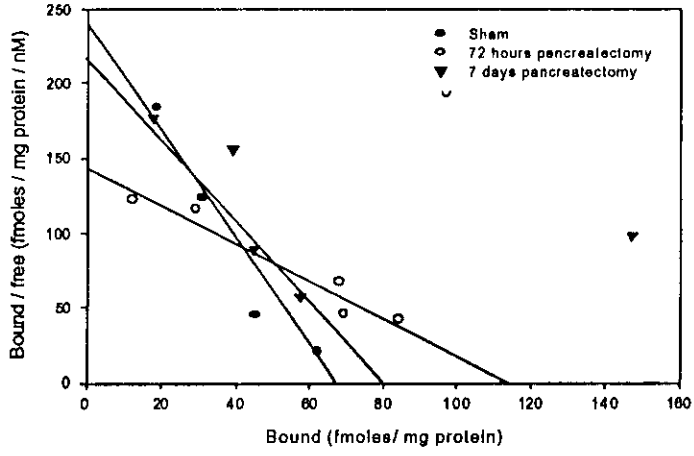
Scatchard analysis of [^{3}H]YM-09151-2 in 72 hours pancreatectomised rats showed a significant increase in B_{max} ($p<0.001$) and K_d ($p<0.001$). Both B_{max} and K_d ($p<0.01$) reversed to sham value after 7 days of pancreatectomy (Table-9; Fig.-1).

Table 9: Scatchard analysis of [^{3}H]YM-09151-2 binding against sulpiride in the cerebral cortex of partially pancreatectomised rat

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	065.63 ± 3.75	0.268 ± 0.005
72 hours pancreatectomy	122.81 ± 4.27 ***	0.778 ± 0.055 **
7 days pancreatectomy	088.14 ± 7.01*	0.464 ± 0.101

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 compared to sham; **p<0. compared to sham; *p<0.05 compared to sham
B_{max} – Maximal binding; K_d – Dissociation constant

Figure-1: Scatchard analysis of [^{3}H]YM-09151-2 binding against sulpiride in cerebral cortex of pancretectomised rat



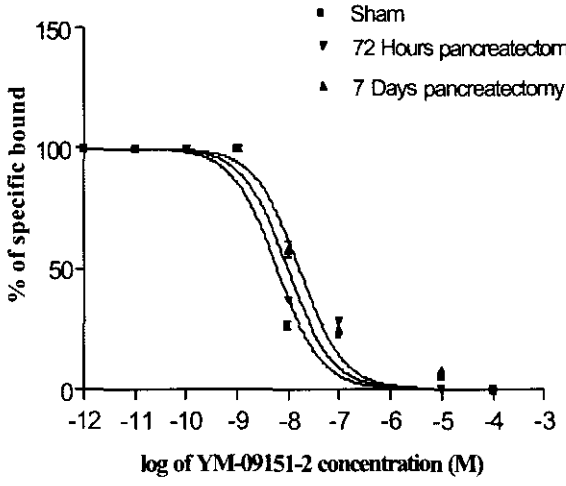
DISPLACEMENT ANALYSIS OF [^{3}H]YM-09151-2 AGAINST YM-09151-2

Competition binding assay was carried out in the cerebral cortex using 10^{-12}-10^{-3}M concentrations of unlabelled YM-09151-2 against [^{3}H]YM-09151-2. All the experimental groups fitted best to a one-site model with the hill slope value within unity. The log (EC-50) value increased during 72 hours pancreatectomy. Both log (EC-50) and Ki value reversed to near sham value after 7days of pancreatectomy (Table-10; Fig.-2).

Table 10: Displacement of [^{3}H]YM-09151-2 with YM-09151-2 in cerebral cortex

Animal status	Best-fit model	Log (EC_{50})	Ki	Hill slopes
Sham	One site	-8.218	2.05×10^{-9}	0.94
72 hours pancreatectomy	One site	-7.784	5.58×10^{-9}	0.98
7 days pancreatectomy	One site	-8.009	3.33×10^{-9}	9.96

Figure-2: Displacement of [^{3}H]YM-09151-2 with YM-09151-2 in cerebral cortex



Data were fitted with an interactive 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 specific binding.

RT-PCR ANALYSIS OF DOPAMINE D_2 RECEPTORS IN CEREBRAL CORTEX

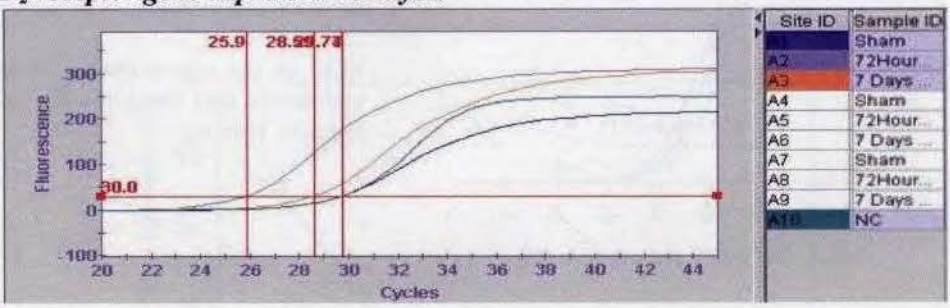
RT-PCR analysis was done to check the changes in receptor number at gene expression level. The Crossing threshold (Ct) value was taken as a measure to analysis the gene expression in mRNA level. Ct value is inversely proportional to gene expression. RT-PCR analysis in the cerebral cortex showed a decrease in Ct value representing an increased expression of DA D_2 receptor mRNA during 72 hours pancreatectomy. The Ct value reversed to near sham value after 7 days of pancreatectomy. A house keeping gene - ß-actin was also analyzed. There was no change in ß-actin gene expression. (Table 11; Fig.-3).

Table-11: Real-Time PCR analysis of dopamine D_2 receptor gene expression in cerebral cortex of partially pancreatectomised rats

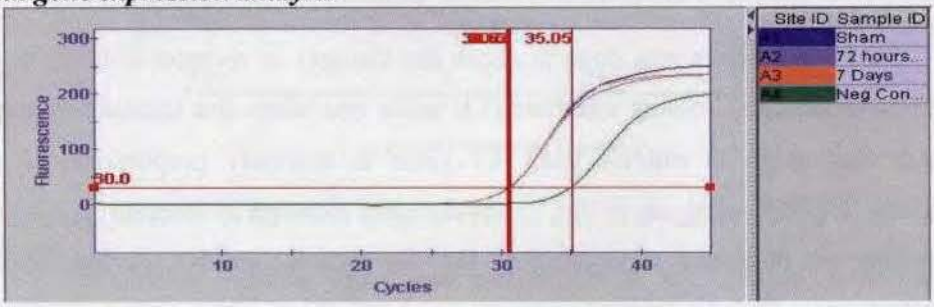
Animal status	Ct-Value
Sham	29.77
72 hours pancreatectomy	25.90
7 days pancreatectomy	28.59

Figure-3: Real-Time PCR analysis of dopamine D_2 receptor gene expression in cerebral cortex of partially pancreatectomised rats

DA D_2 receptor gene expression analysis



β-actin gene expression analysis



SCATCHARD ANALYSIS USING [^{3}H]YM-09151-2 IN CORPUS STRIATUM

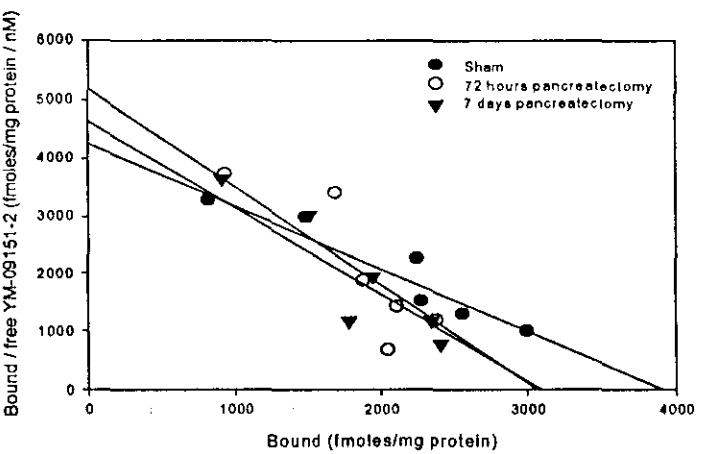
YM-09151-2 is a potent antagonist for DA D_2 receptor. Both B_{max} ($p<0.001$) and K_d ($p<0.05$) decreased significantly (($p<0.001$)) in 72 hours pancreatectomised rats when compared to sham operated. The B_{max} and K_d did not reversed to sham value after 7 days pancreatectomy (Table 12; Fig.-4).

Table-12: Scatchard analysis of [^{3}H]YM-09151-2 binding against sulpiride in the corpus striatum of partially pancreatectomised rat

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	3770.8 ± 37.55	0.856 ± 0.088
72 hours pancreatectomy	2958.3 ± 57.56 ***	0.525 ± 0.005*
7 days pancreatectomy	2963.1 ± 58.88	0.607 ± 0.022*

Values are Mean ± S.E.M. of 4-6 separate experiments
*$p<0.05$ compared to sham; ***$p<0.001$ compared to sham
B_{max} – Maximal binding; K_d – Dissociation constant

Figure-4: Scatchard analysis of [^{3}H]YM 09151-2 binding against sulpiride in corpus striatum of pancretectomised rat



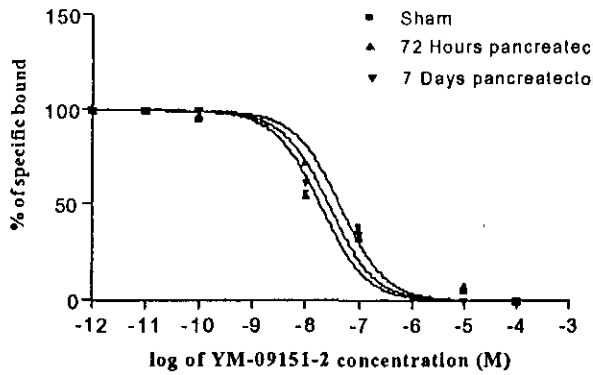
DISPLACEMENT ANALYSIS OF [^{3}H]YM-09151-2 AGAINST YM-09151-2

Competition binding assay was carried out in the corpus striatum using 10^{-12}-10^{-3} M concentrations of unlabelled YM-09151-2 against [^{3}H] YM-09151-2. All the experimental groups fitted best to a one-site model with the hill slope value within unity. The log (EC-50) value decreased with an increase in affinity during 72 hours pancreatectomy. The decreased log (EC-50) and Ki value partially reversed to near sham value after 7 days of pancreatectomy (Table-13; Fig.-5).

Table 13: Displacement of [^{3}H]YM-09151-2 with YM-09151-2 in corpus striatum

Animal status	Best-fit model	Log (EC_{50})	Ki	Hill slopes
Sham	One site	-7.566	1.46×10^{-8}	0.97
72 hours pancreatectomy	One site	-7.730	6.32×10^{-9}	0.94
7 days pancreatectomy	One site	-7.573	9.07×10^{-9}	0.96

Figure-5: Displacement of [^{3}H]YM-09151-2 with YM-09151-2 in corpus striatum



Data were fitted with an interactive 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 specific binding.

RT-PCR ANALYSIS OF DOPAMINE D_2 RECEPTORS IN CORPUS STRIATUM

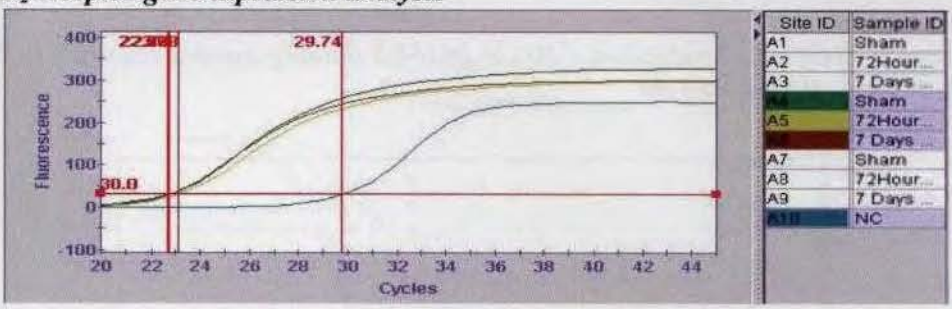
RT-PCR analysis was done in corpus striatum to study the DA D_2 receptor mRNA level during pancreatic regeneration. DA D_2 receptor mRNA expression did not alter in corpus striatum during 72 hours pancreatectomy. There was no change in ß-actin gene expression (Table-14; Fig.-6).

Table-14: Real-Time PCR analysis of dopamine D_2 receptor gene expression in corpus striatum of partially pancreatectomised rats

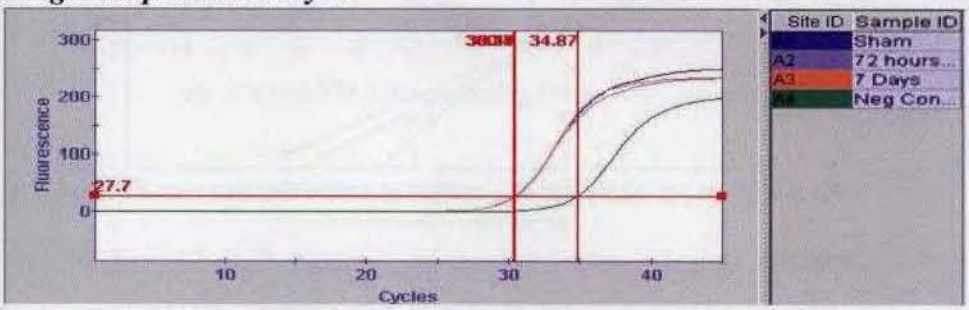
Animal status	Ct-Value
Sham	22.73
72 hours pancreatectomy	23.08
7 days pancreatectomy	22.67

Figure-6: Real-Time PCR analysis of dopamine D_2 receptor gene expression in corpus striatum of partially pancreatectomised rats

DA D_2 receptor gene expression analysis



β-actin gene expression analysis



SCATCHARD ANALYSIS USING [^{3}H]YM-09151-2 BRAIN STEM

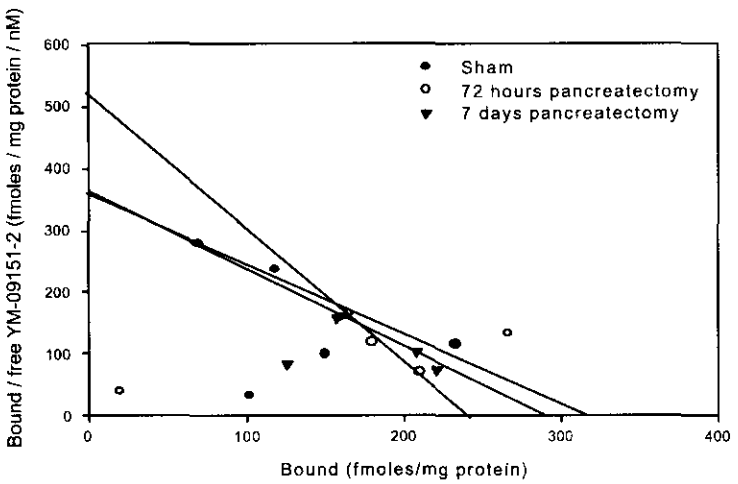
Scatchard analysis of [^{3}H]YM-09151-2 in brain stem of 72 hours pancreatectomised rats showed a significant decrease in B_{max} ($p<0.01$) and K_d ($p<0.01$)

compared to sham. The B_{max} reversed to sham value after 7 days of pancreatectomy. A further decrease in K_d was observed after 7 days pancreatectomy (Table 15-; Fig.-7).

Table-15: Scatchard analysis of [H]YM-09151-2 binding against sulpiride in the brain stem of partially pancreatectomised rat

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	331.3 ± 06.324	0.932 ± 0.092
72 hours pancreatectomy	223.1 ± 18.997 **	0.576 ± 0.074**
7 days pancreatectomy	262.4 ± 12.508*	0.508 ± 0.030

Figure-7: Scatchard analysis of [^{3}H]YM 09151-2 binding against sulpiride in brain stem of pancretectomised rat



DISPLACEMENT ANALYSIS OF [^{3}H]YM-09151-2 AGAINST YM-09151-2

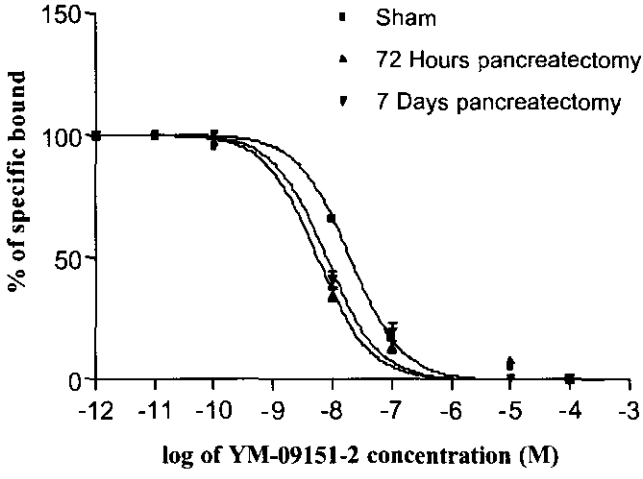
Competition binding assay was carried out in the brain stem using 10^{-12}-10^{-3} M concentrations of unlabelled YM-09151-2 against [^{3}H]YM-09151-2. All the experimental groups fitted best to a one-site model with the hill slope value within unity. The log (EC-50) decreased during 72hours after pancreatectomy. There was a further decrease in log

(EC-50) and Ki was observed in 72 hours after pancreatectomy indicating a further increase in affinity in 7 days after pancreatectomy (Table 16; Fig.-8).

Table 16: Displacement of [^{3}H]YM-09151-2 with YM-09151-2 in brain stem

Animal status	Best-fit model	Log (EC_{50})	Ki	Hill slopes
Sham	One site	-7.702	6.74×10^{-9}	0.99
72 hours pancreatectomy	One site	-8.048	2.71×10^{-9}	0.98
7 days pancreatectomy	One site	-8.297	1.91×10^{-9}	0.98

Figure-8: Displacement of [^{3}H]YM-09151-2 with YM-09151-2 in brain stem



Data were fitted with an interactive 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 specific binding.

RT-PCR ANALYSIS OF DOPAMINE D_2 RECEPTORS IN BRAIN STEM

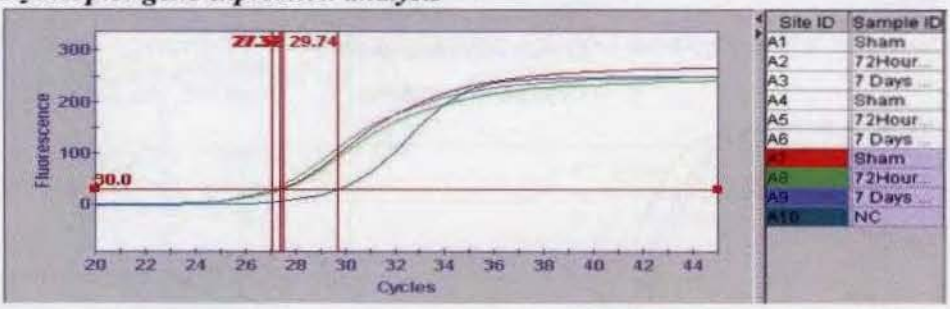
The RT-PCR analysis in the brain stem showed an increase in Ct value representing a decreased expression of DA D_2 receptor mRNA during 72 hours pancreatectomy. The Ct value reversed to near sham value after 7 days of pancreatectomy. There was no change in ß-actin gene expression. (Fig-17; Table-9).

Table-17: Real-Time PCR analysis of dopamine D_2 receptor gene expression in brain stem of partially pancreatectomised rats

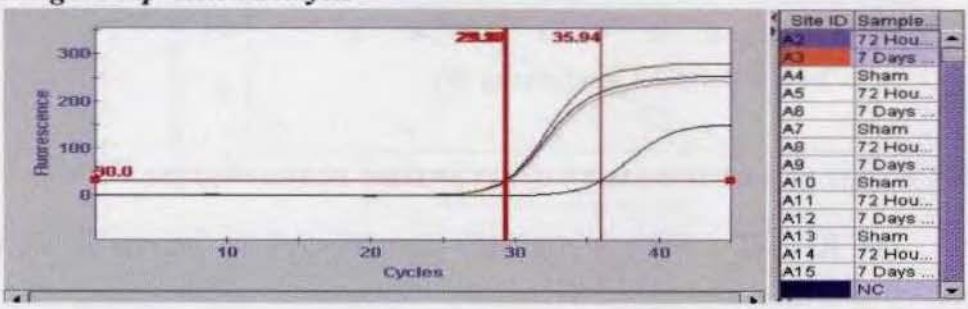
Animal status	Ct-Value
Sham	27.37
72 hours pancreatectomy	27.52
7 days pancreatectomy	27.10

Figure-9: Real-Time PCR analysis of dopamine D_2 receptor gene expression in corpus striatum of partially pancreatectomised rats

DA D_2 receptor gene expression analysis



β-actin gene expression analysis



SCATCHARD ANALYSIS USING [^{3}H]YM-09151-2IN HYPOTHALAMUS

Scatchard analysis of [^{3}H]YM-09151-2 in hypothalamus of 72 hours pancreatectomised rats showed a significant increase in B_{max} ($p<0.001$) when compared to sham. The K_d in 72 hours pancreatectomised rats decreased significantly ($p<0.01$)

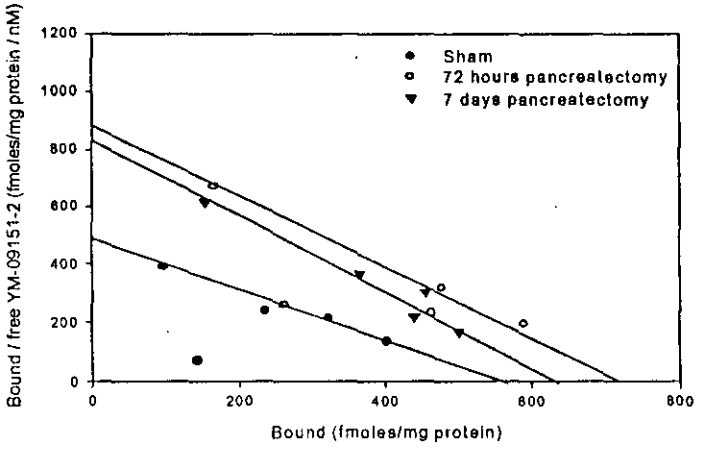
when compared to sham. The B_{max} partially reversed to sham value after 7 days of pancreatectomy. A further decrease in K_d was observed after 7 days of pancreatectomy (Table-18; Fig.-10)

Table-18: Scatchard analysis of [H]YM-09151-2 binding against sulpiride in the hypothalamus of partially pancreatectomised rat

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	541.18 ± 2.81	1.04 ± 0.009
72 hours pancreatectomy	746.28 ± 1.21 ***	0.91 ± 0.004**
7 days pancreatectomy	610.28 ± 1.91	0.99 ± 0.003

Values are Mean ± S.E.M. of 4-6 separate experiments
***p<0.001 compared to sham; **p<0.01 compared to sham;
B_{max} – Maximal binding; K_d – Dissociation constant

Figure-10: Scatchard analysis of [^{3}H]YM-09151-2 binding against sulpiride in hypothalamus of pancretectomised rat



DISPLACEMENT ANALYSIS OF [^{3}H]YM-09151-2 AGAINST YM-09151-2

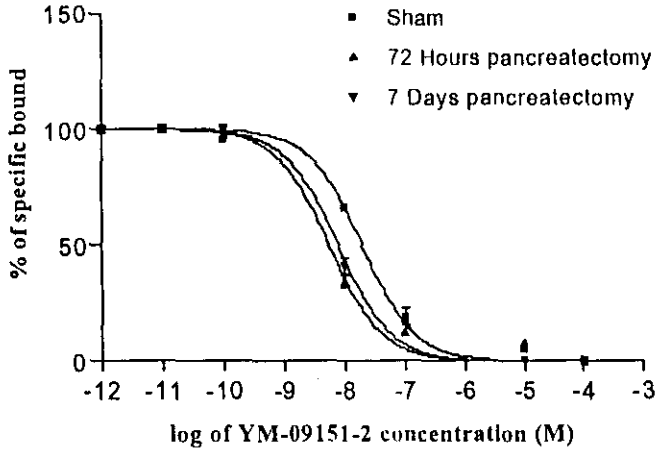
Competition binding assay was carried out in the hypothalamus using 10^{-12}-10^{-3}M concentrations of unlabelled YM-09151-2 against [^{3}H]YM-09151-2. All the

experimental groups fitted best to a one-site model with the hill slope value within unity. The Log (EC_{50}) value decreased in partially pancreatectomised rats. A further decrease in Log (EC_{50}) and Ki was observed in 7 days after pancreatectomy. (Table-19; Fig.-11)

Table 19: Displacement of [^{3}H]YM-09151-2 with YM-09151-2 hypothalamus

Animal status	Best-fit model	Log (EC_{50})	Ki	Hill slopes
Sham	One site	-7.599	5.70×10^{-9}	0.95
72 hours pancreatectomy	One site	-8.011	3.32×10^{-9}	0.99
7 days pancreatectomy	One site	-8.122	2.22×10^{-9}	0.96

Figure-11: Displacement of [3H]YM-09151-2 with YM-09151-2 hypothalamus



Data were fitted with an interactive 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 specific binding.

RT-PCR ANALYSIS OF DOPAMINE D_2 RECEPTORS IN HYPOTHALAMUS

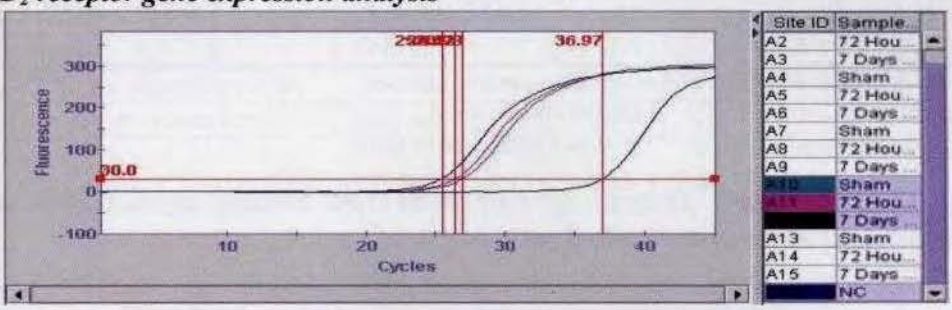
RT-PCR analysis in the hypothalamus showed a decrease in Ct value representing an increased expression of DA D_2 receptor mRNA during 72 hours pancreatectomy. The Ct value reversed to near sham value after 7 days of pancreatectomy. There was no change in ß-actin gene expression (Table 20; Fig.-12).

Table-20: Real-Time PCR analysis of dopamine D_2 receptor gene expression in hypothalamus of partially pancreatectomised rats

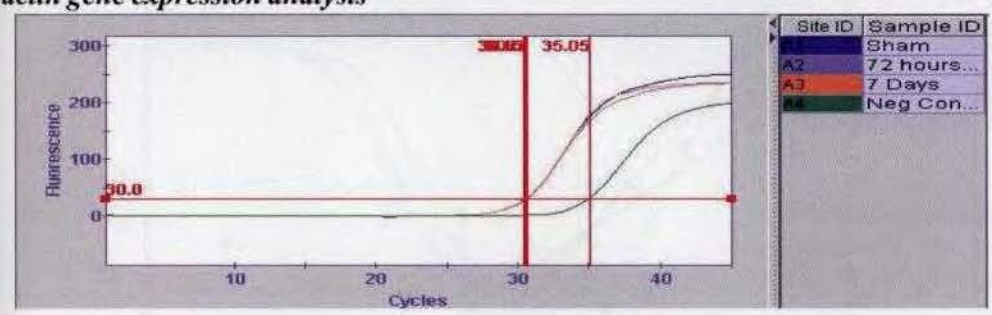
Animal status	Ct-Value
Sham	26.98
72 hours pancreatectomy	26.42
7 days pancreatectomy	25.46

Figure-12: Real-Time PCR analysis of dopamine D_2 receptor gene expression in hypothalamus of partially pancreatectomised rats

DA D_2 receptor gene expression analysis



β-actin gene expression analysis



SCATCHARD ANALYSIS USING [^{3}H]YM-09151-2 IN PANCREATIC ISLETS CELLS

Scatchard analysis of [^{3}H]YM-09151-2 in pancreatic islets of 72 hours pancreatectomised rats showed a significant increase in B_{max} ($p<0.001$) and K_d ($p<0.001$)

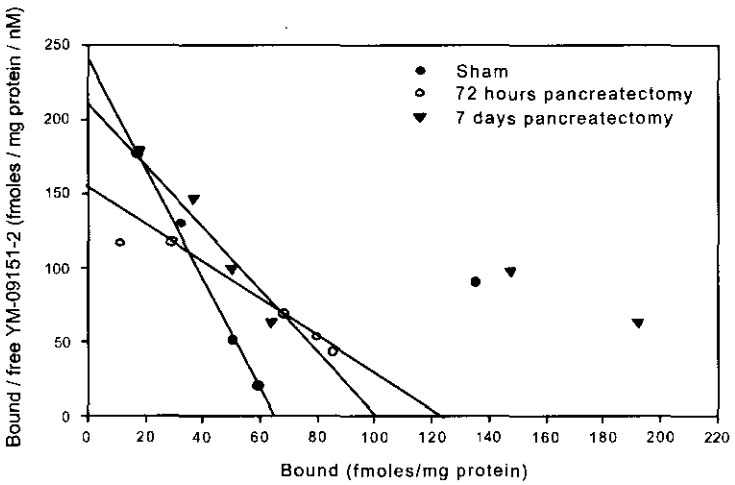
compared to sham. Both B_{max} and K_d trends to reversed to near sham value after 7 days of pancreatectomy. (Table 21; Fig.-13).

Table-21: Scatchard analysis of [^{3}H]YM-09151-2 binding against sulpiride in the pancreatic islets of partially pancreatectomised rat

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	065.54 ± 0.76	0.274 ± 0.014
72 hours pancreatectomy	119.13 ± 0.87 ***	0.701 ± 0.010 ***
7 days pancreatectomy	080.89 ± 3.96 *	0.330 ± 0.005

Values are Mean ± S.E.M. of 4-6 separate experiments
B_{max} – Maximal binding; K_d – Dissociation constant
*p<0.05 compared to sham; ***p<0.001 compared to sham

Figure-13: Scatchard analysis of [^{3}H]YM-09151-2 binding against sulpiride in hypothalamus of pancretectomised rat



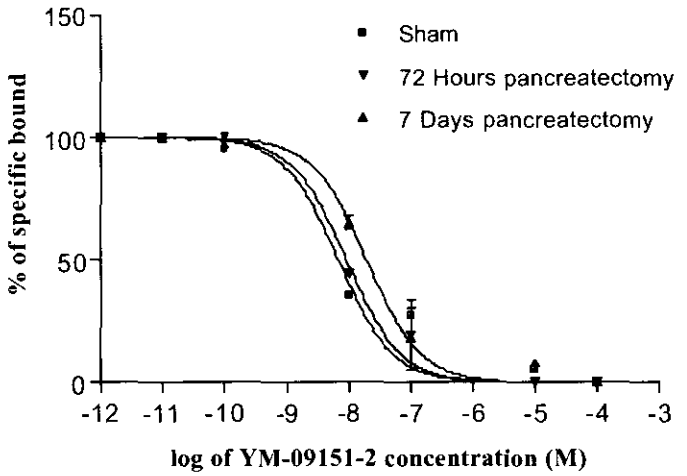
DISPLACEMENT ANALYSIS OF [^{3}H]YM-09151-2 AGAINST YM-09151-2

Competition binding assay was carried out in the pancreatic islets using 10^{-12}-10^{-3}M concentrations of unlabelled YM-09151-2 against [^{3}H] YM-09151-2. All the experimental groups fitted best to a one-site model with the hill slope value within unity. The log (EC-50) increased during 72hours after pancreatectomy. The log (EC-50) and Ki reversed to near sham value in 7 days after pancreatectomy (Table -22; Fig.-14).

Table-22: Displacement of [^{3}H]YM-09151-2 with YM-09151-in pancreas

Animal status	Best-fit model	Log (EC_{50})	Ki	Hill slopes
Sham	One site	-8.066	2.31 x 10^{-9}	0.94
72 hours pancreatectomy	One site	-7.711	6.60 x 10^{-9}	1.03
7 days pancreatectomy	One site	-7.935	3.13 x 10^{-9}	1.09

Figure-14: Displacement of [3H]YM-09151-2 with YM-09151-in pancreas



Data were fitted with an interactive 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 specific binding.

RT-PCR ANALYSIS OF DOPAMINE D_2 RECEPTORS IN PANCREATIC ISLETS

The RT-PCR analysis in the pancreatic islets showed a decrease in Ct value representing an increased expression of DA D_2 receptor mRNA during 72 hours

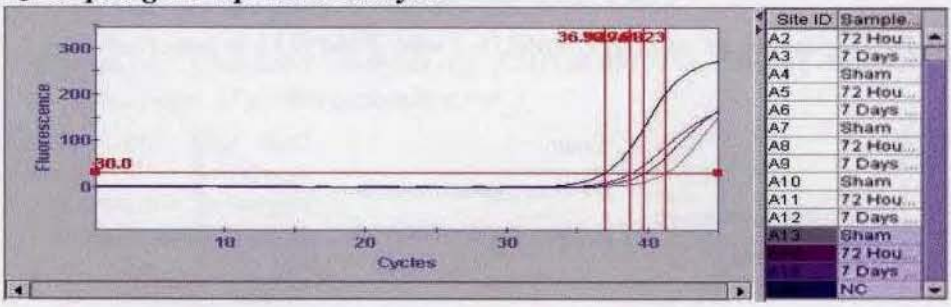
pancreatectomy. The Ct value was reversed to near sham value after 7 days of pancreatectomy. There was no change in ß-actin gene expression (Fig-23; Table-15).

Table-23: Real-Time PCR analysis of dopamine D_2 receptor gene expression in pancreatic islets of partially pancreatectomised rats

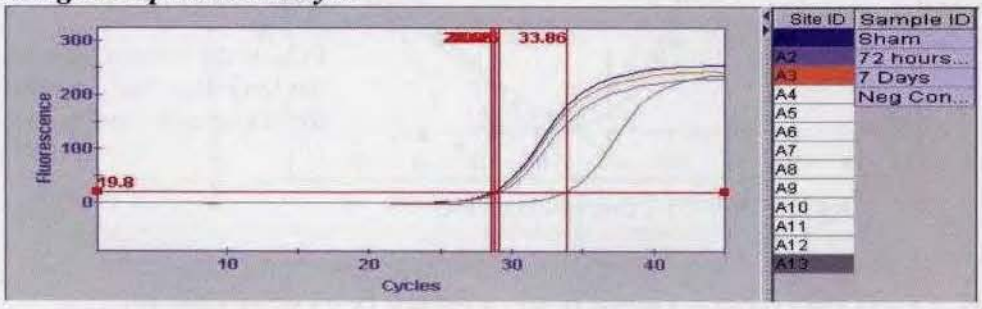
Animal status	Ct-Value
Sham	41.23
72 hours pancreatectomy	38.76
7 days pancreatectomy	39.68

Figure-15: Real-Time PCR analysis of dopamine D_2 receptor gene expression in pancreatic islets of partially pancreatectomised rats

DA D_2 receptor gene expression analysis



β-actin gene expression analysis



DOPAMINE GENERAL RECEPTOR ANALYSIS IN BRAIN REGIONS AND PANCREAS.

SCATCHARD ANALYSIS USING [^{3}H]DOPAMINE IN CEREBRAL CORTEX

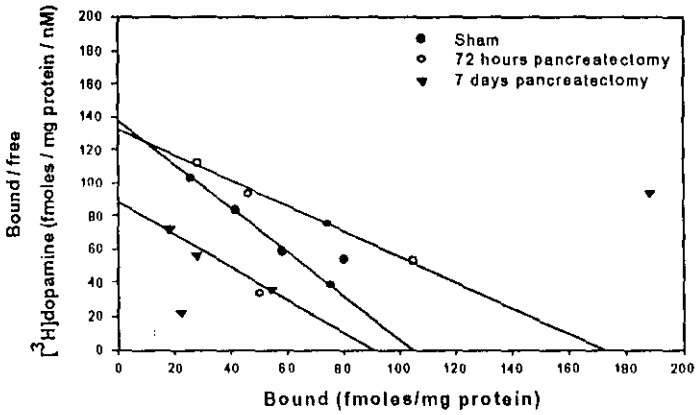
Scatchard analysis of [^{3}H]DA in 72 hours pancreatectomised rats showed a significant increase in B_{max} ($p<0.001$) and K_d ($p<0.001$). Both B_{max} and K_d reversed to near sham value after 7days of pancreatectomy (Table-24-; Fig.-16).

Table-24: Scatchard analysis of [^{3}H]dopamine binding against butaclamol in cerebral cortex of pancretectomised rat

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	097.19 ± 3.84	0.70 ± 0.03
72 hours pancreatectomy	169.23 ± 8.49 ***	1.21 ± 0.04**
7 days pancreatectomy	083.84 ± 4.09	0.80 ± 0.11

Values are Mean ± S.E.M. of 4-6 separate experiments
**p<0.01 when compared to sham; ***p<0.001 when compared to sham
B_{max} – Maximal b inding; K_d – Dissociation constant

Figure-16: Scatchard analysis of [^{3}H]dopamine binding against butaclamol in cerebral cortex of pancretectomised rat



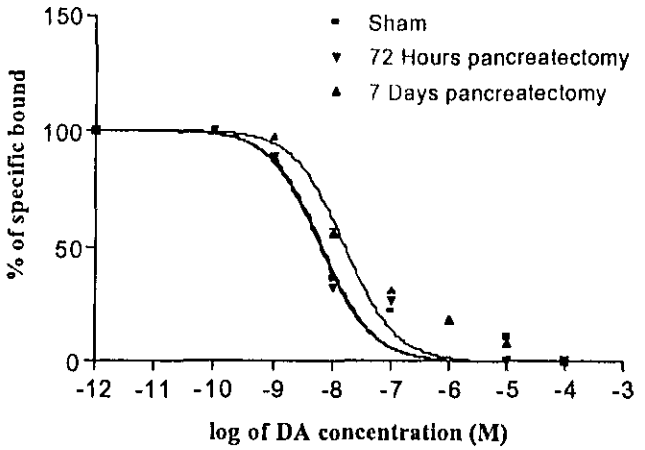
DISPLACEMENT ANALYSIS OF [^{3}H]DOPAMINE AGAINST DOPAMINE

Competition binding assay was carried out in the cerebral cortex using 10^{-12}-10^{-3}M concentrations of unlabelled DA against [^{3}H] DA. All the experimental groups fitted best to a one-site model with the hill slope value within unity. The log (EC-50) value increased during 72 hours pancreatectomy. Both log (EC-50) and Ki value partially reversed to near sham value after 72days of pancreatectomy (Table-25; Fig.-17).

Table-25: Displacement of [^{3}H]dopamine with dopamine in cerebral cortex

Animal status	Best-fit model	Log (EC_{50})	Ki	Hill slopes
Sham	One site	-8.180	3.15×10^{-9}	0.99
72 hours pancreatectomy	One site	-7.726	6.28×10^{-9}	0.99
7 days pancreatectomy	One site	-8.099	4.12×10^{-9}	0.97

Figure-17: Displacement of [3H]dopamine with dopamine in cerebral cortex



Data were fitted with an interactive 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 specific binding.

SCATCHARD ANALYSIS USING [^{3}H]DOPAMINE IN CORPUS STRIATUM

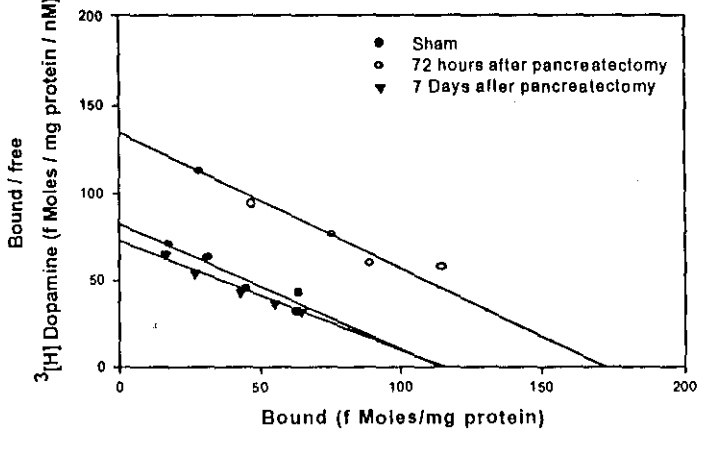
Scatchard analysis of [^{3}H] DA in corpus striatum of 72 hours pancreatectomised rats showed a significant increase in B_{max} ($p<0.001$). There was a significant ($p<0.05$) decrease in K_d during 72 hours pancreatectomised rats compared to sham. The B_{max} reversed to sham value after 7 days of pancreatectomy. The K_d was reversed to sham after 7 days pancreatectomy (Table 26; Fig.-18).

Table-26: Scatchard analysis of [^{3}H]dopamine binding against butaclamol in corpus striatum of pancretectomised rat

Aanimal status	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	111.03 ±1.91	1.28 ± 0.07
72 hours pancreatectomy	166.13 ± 1.59 ***	1.12 ± 0.03 *
7 days pancreatectomy	118.37 ± 0.85	1.27 ± 0.03 *

Values are Mean ± S.E.M. of 4-6 separate experiments
*p<0.05 when compared to sham; ***p<0.001 when compared to sham
B_{max} – Maximal binding; K_d – Dissociation constant

Figure-18: Scatchard analysis of [^{3}H]dopamine binding against butaclamol in corpus striatum of pancretectomised rat



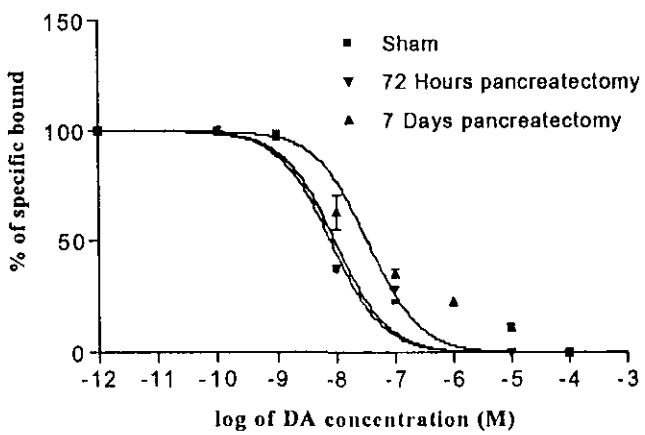
DISPLACEMENT ANALYSIS OF [^{3}H]DOPAMINE AGAINST DOPAMINE

Competition binding assay was carried out in the corpus striatum using 10^{-12}-10^{-3}M concentrations of unlabelled DA against [^{3}H] DA. All the experimental groups fitted best to a one-site model with the hill slope value within unity. The log (EC-50) value decreased with an increase in affinity during 72 hours pancreatectomy. The decreased log (EC-50) and Ki value partially reversed to near sham value after 7 days of pancreatectomy (Table-27; Fig.-19).

Table-27: Displacement of [^{3}H]dopamine with dopamine in corpus striatum

Animal status	Best-fit model	Log (EC_{50})	Ki	Hill slopes
Sham	One site	-7.512	8.45×10^{-9}	0.99
72 hours pancreatectomy	One site	-7.920	7.85×10^{-9}	0.98
7 days pancreatectomy	One site	-7.860	8.83×10^{-9}	0.99

Figure-19: Displacement of [3H]dopamine with dopamine in corpus striatum



Data were fitted with an interactive 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 specific binding.

SCATCHARD ANALYSIS USING [^{3}H]DOPAMINE IN BRAIN STEM

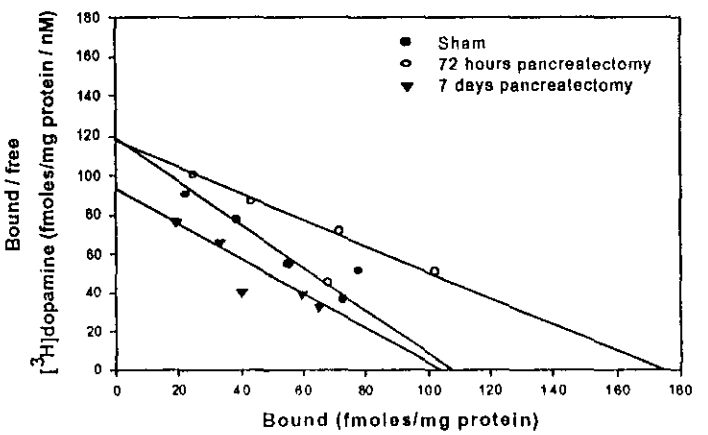
Scatchard analysis of [^{3}H] DA in brain stem of 72 hours pancreatectomised rats showed a significant increase in B_{max} ($p<0.001$) and K_d ($p<0.05$) compared to sham. The B_{max} and K_d reversed to near sham value after 7 days pancreatectomy (Table 28; Fig.-20).

Table-28: Scatchard analysis of [H]dopamine binding against butaclamol in brain stem of pancretectomised rat

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	108.71 ± 3.85	1.23 ± 0.12
72 hours pancreatectomy	171.86 ± 4.21 ***	1.63± 0.12*
7 days pancreatectomy	99.23 ± 3.49	1.19 ± 0.11

Values are Mean ± S.E.M. of 4-6 separate experiments
*p<0.05 when compared to sham; ***p<0.001 when compared to sham
B_{max} – Maximal binding; K_d – Dissociation constant

Figure-20: Scatchard analysis of [H]dopamine binding against butaclamol in brain stem of pancretectomised rat



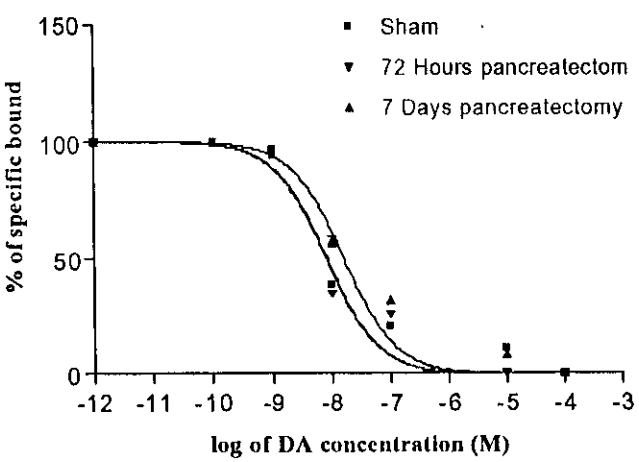
DISPLACEMENT ANALYSIS OF [^{3}H]DOPAMINE AGAINST DOPAMINE

Competition binding assay was carried out in the brain stem using 10^{-12}-10^{-3}M concentrations of unlabelled DA against [^{3}H] DA. All the experimental groups fitted best to a one-site model with the hill slope value within unity. The log (EC-50) increased during 72 hours after pancreatectomy. The log (EC-50) and Ki reversed to near sham value by 7 days after pancreatectomy (Table 29-; Fig.-21).

Table-29: Displacement of [^{3}H]dopamine with dopamine in brain stem

Animal status	Best-fit model	Log (EC_{50})	Ki	Hill slopes
Sham	One site	-6.617	5.74×10^{-9}	0.99
72 hours pancreatectomy	One site	-6.151	7.89×10^{-9}	1.00
7 days pancreatectomy	One site	-7.041	4.63×10^{-9}	1.04

Figure-21: Displacement of [^{3}H]dopamine with dopamine in brain stem



Data were fitted with an interactive 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 specific binding.

SCATCHARD ANALYSIS USING [^{3}H]DOPAMINE IN HYPOTHALAMUS

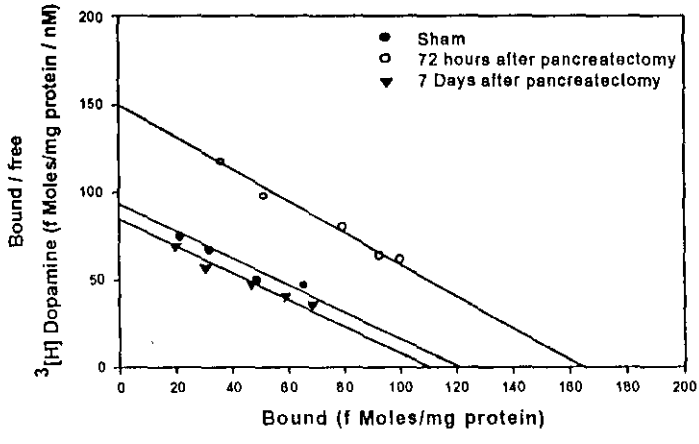
Scatchard analysis of [^{3}H] DA in hypothalamus of 72 hours pancreatectomised rats showed a significant increase in B_{max} ($p<0.001$) when compared to sham. The K_d in 72 hours pancreatectomised rats decreased significantly ($p<0.01$) when compared to sham. The B_{max} reversed to near sham value after 7 days of pancreatectomy. A further decrease in K_d was observed after 7 days of pancreatectomy (Table-30; Fig.-22)

Table-30: Scatchard analysis of [^{3}H]dopamine binding against butaclamol in hypothalamus of pancretectomised rat

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	120.03 ± 3.94	1.33 ± 0.012
72 hours pancreatectomy	165.83 ± 1.71 ***	0.13 ± 0.005**
7 days pancreatectomy	109.13 ± 2.28 **	1.36 ± 0.005 ***

Values are Mean ± S.E.M. of 4-6 separate experiments
**p<0.01 when compared to sham; ***p<0.001 when compared to sham
B_{max} – Maximal binding; K_d – Dissociation constant

Figure-22: Scatchard analysis of [^{3}H]dopamine binding against butaclamol in hypothalamus of pancretectomised rat



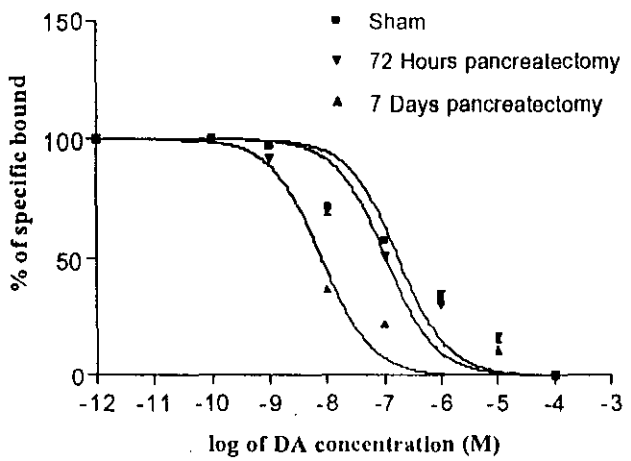
DISPLACEMENT ANALYSIS OF [^{3}H]DOPAMINE AGAINST DOPAMINE

Competition binding assay was carried out in the hypothalamus using 10^{-12}-10^{-3}M concentrations of unlabelled DA against [^{3}H]DA. All the experimental groups fitted best to a one-site model with the hill slope value within unity. The Log (EC_{50}) value during 72 hours pancreatectomy decreased. Both Log (EC_{50}) and Ki reversed to near sham value after 7 days after pancreatectomy. (Table-31; Fig.-23)

Table-31: Displacement of [^{3}H]dopamine with dopamine in hypothalamus

Animal status	Best-fit model	Log (EC_{50})	Ki	Hill slopes
Sham	One site	-7.098	4.51×10^{-9}	1.01
72 hours pancreatectomy	One site	-8.185	1.14×10^{-9}	1.00
7 days pancreatectomy	One site	-7.012	4.84×10^{-9}	1.00

Figure-23: Displacement of [^{3}H]dopamine with dopamine in hypothalamus



Data were fitted with an interactive 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 specific binding.

SCATCHARD ANALYSIS USING [^{3}H]DOPAMINE IN PANCREATIC ISLETS

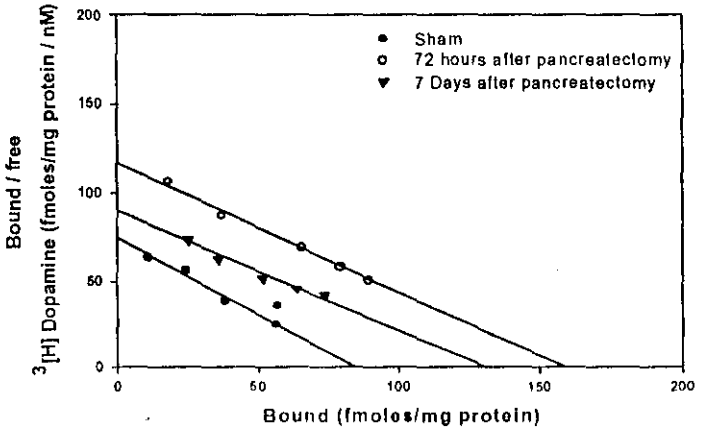
Scatchard analysis of [^{3}H] DA in pancreatic islets of 72 hours pancreatectomised rats showed a significant increase in B_{max} ($p<0.001$) and K_d ($p<0.001$) compared to sham. Both B_{max} and K_d to reversed to near sham value after 7 days of pancreatectomy (Table 32-; Fig.-24).

Table-32: Scatchard analysis of [^{3}H]dopamine binding against butaclamol in pancreatic islets of pancretectomised rat

Animal status	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	78.32 ± 2.96	0.218 ± 0.027
72 hours pancreatectomy	158.13 ± 4.87 ***	0.604 ± 0.030 ***
7 days pancreatectomy	135.84 ± 3.63 *	0.240 ± 0.004

Values are Mean ± S.E.M. of 4-6 separate experiments
*p<0.05 when compared to sham; ***p<0.001 when compared to sham
B_{max} – Maximal binding; K_d – Dissociation constant

Figure-24: Scatchard analysis of [^{3}H]dopamine binding against butaclamol in pancreatic islets of pancretectomised rat



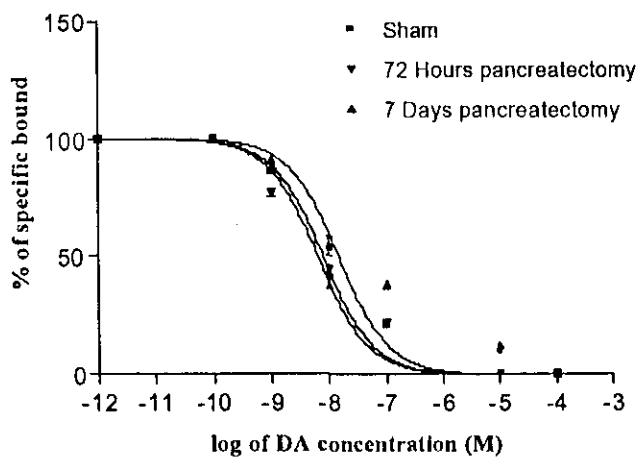
DISPLACEMENT ANALYSIS OF [^{3}H]DOPAMINE AGAINST DOPAMINE

Competition binding assay was carried out in the pancreatic islets using 10^{-12}-10^{-3}M concentrations of unlabelled DA against [^{3}H] DA. All the experimental groups fitted best to a one-site model with the hill slope value within unity. The log (EC-50) increased during 72hours after pancreatectomy. The log (EC-50) and Ki reversed to near sham value in 7 days after pancreatectomy (Table 33; Fig.-25).

Table-33: Displacement of [^{3}H]dopamine with dopamine in pancreatic islets

Animal status	Best-fit model	Log (EC_{50})	Ki	Hill slopes
Sham	One site	-7.811	3.28×10^{-9}	0.99
72 hours pancreatectomy	One site	-7.102	5.61×10^{-9}	1.00
7 days pancreatectomy	One site	-7.968	4.02×10^{-9}	1.02

Figure-25: Displacement of [^{3}H]dopamine with dopamine in pancreatic islets



Data were fitted with an interactive nonlinear regression software (Prism, GraphPad, San Diego, CA).

Ki-The affinity of the receptor for the competing drug.

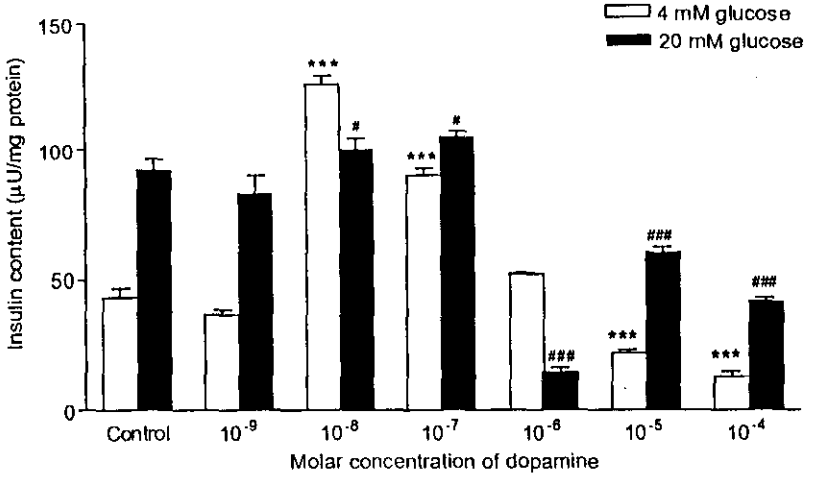
EC_{50} is the concentration of the competitor that competes for half specific binding.

INSULIN SECRETION STUDIES IN PANCREATIC ISLETS

Effect of dopamine on glucose induced insulin secretion in 24 hours *in vitro* culture

Pancreatic islets cells were incubated with 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M concentrations of DA and two different concentrations of glucose, 4mM and 20mM in 24 hours *in vitro* culture. DA significantly increased insulin secretion at 10^{-8}M ($p<0.001$) and 10^{-7} M ($p<0.001$) while decreased insulin secretion at 10^{-9}M ($p<0.001$), 10^{-5}M ($p<0.001$) and 10^{-4}M ($p<0.001$) in the presence of 4mM glucose. There was no significant change in insulin secretion at 10^{-6}M DA and 10^{-9}M DA in the presence of 4mM glucose (Figure - 35). DA significantly stimulated 20mM glucose induced insulin secretion at 10^{-8} ($p<0.05$) and 10^{-7} ($p<0.05$) while inhibited at 10^{-6}M ($p<0.001$), 10^{-5}M ($p<0.001$) and 10^{-4}M ($p<0.001$) concentrations (Figure -26).

Figure-26: Effect of dopamine on glucose induced insulin secretion from pancreatic islets in 24 hours in vitro culture

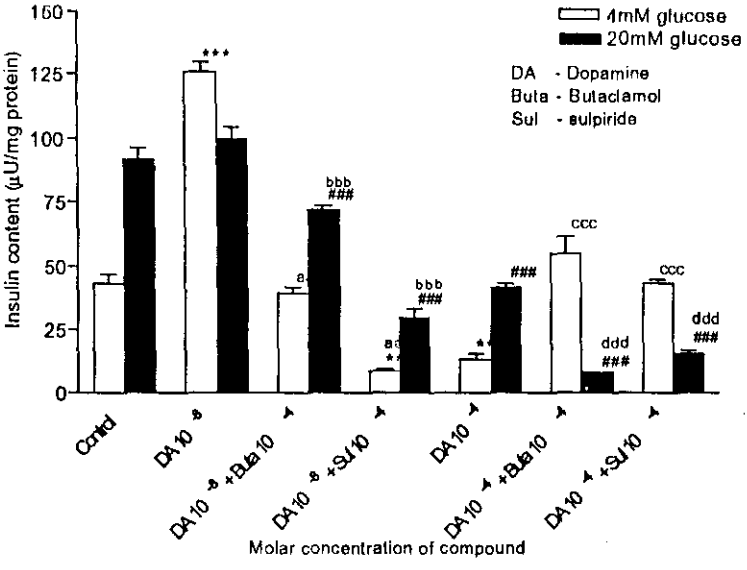


* P<0.05 when compared with control in the presence of 4mM glucose
*** P<0.001 when compared with control in the presence of 4mM glucose
# P<0.05 when compared with control in the presence of 20mM glucose

Effect of dopamine and its antagonists on glucose induced insulin secretion in 24 hours in vitro culture

DA antagonist - butaclamol and DA D_2 receptor specific antagonist- sulpiride at 10^{-4}M concentration was added to pancreatic islets cells incubated with 10^{-8}M DA and 10^{-4}M DA in the presence of 4mM and 20mM glucose. Presence of butaclamol and sulpiride at, 10^{-4}M concentration significantly ($p<0.001$) inhibited stimulatory effect of 10^{-8} M DA in the presence of 4mM and 20mM glucose. Inhibitory effect of 10^{-4}M DA on 4mM and 20mM glucose induced insulin secretion was reversed by the addition of 10^{-4}M butaclamol. Presence of 10^{-4}M sulpiride made a further significant decrease in 4mM and 20mM glucose induced insulin secretion (Figure -27).

Figure-27: Effect of dopamine and its antagonists on glucose induced insulin secretion from pancreatic islets in 24 hours in vitro culture



*** P<0.001 when compared with control in the presence of 4mM glucose
P<0.001 when compared with control in the presence of 20mM glucose
aaa P<0.001 when compared with 10^{-8} in the presence of 4mM glucose
bbb P<0.001 when compared with 10^{-8} in the presence of 20mM glucose
ccc P<0.001 when compared with 10^{-8} in the presence of 4mM glucose
ddd P<0.001 when compared with 10^{-8} in the presence of 20mM glucose

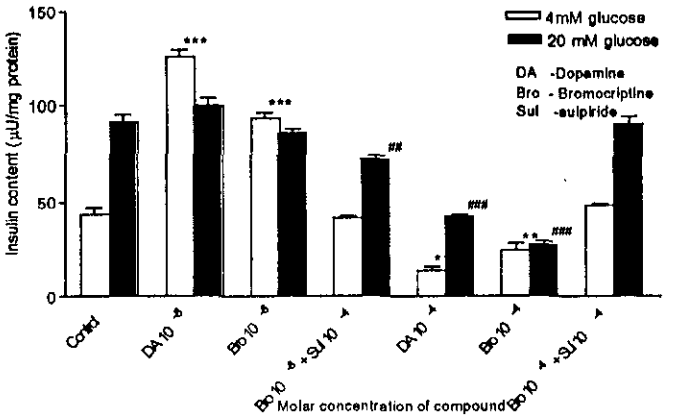
Effect of dopamine and its agonists on glucose induced insulin secretion in 24 hours *in vitro* culture

DA D_2 specific agonist- bromocriptine at 10^{-8}M and 10^{-4}M was added to pancreatic islets cell culture containing 10^{-8}M and 10^{-4}M DA in the presence of 4mM and 20mM glucose. DA D_2 receptor specific antagonist sulpiride was also added to block the DA D_2 receptors. DA at 10^{-8} M concentration significantly ($p<0.001$) increased 4mM glucose induced insulin secretion while no change was observed in 20mM induced insulin secretion compared to 4mM glucose induced insulin secretion. DA agonist bromocriptine at 10^{-8}M significantly ($p<0.001$) increased 4mM glucose induced insulin secretion while no change was observed in 20mM glucose induced insulin secretion. Addition of DA D_2 receptor specific antagonist- sulpiride at 10^{-4}M reversed the stimulatory effect of 10^{-8}M DA and bromocriptine on 4mM and 20mM glucose induced insulin secretion. There was a significant stimulation on insulin secretion by the addition of 10^{-8}M DA ($p<0.001$) and 10^{-8}M bromocriptine ($p<0.01$) in the presence of 4mM glucose. Also presence of 10^{-4}M DA ($p<0.001$) and 10^{-4}M bromocriptine ($p<0.001$) significantly inhibited 20mM glucose induced insulin secretion. Presence of sulpiride did not reverse the inhibition (Figure-28).

Effect of dopamine and norepinephrine on insulin secretion from pancreatic islets in 24 hours *in vitro* culture

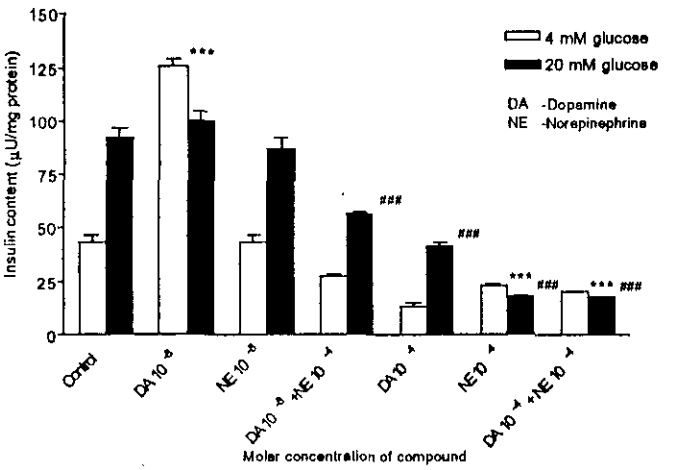
NE at 10^{-4}M was added to pancreatic islets cell culture containing 10^{-8}M DA and 10^{-4}M DA in the presence of 4mM and 20mM glucose. NE at 10^{-8}M had no effect on 4mM and 20mM glucose induced insulin secretion. NE at 10^{-4}M significantly inhibited 10^{-8}M DA mediated insulin secretion at 4mM ($p<0.001$) and 20mM ($p<0.001$) glucose concentrations. NE at 10^{-4}M ($p<0.001$) separately and in combination 10^{-4}M DA ($p<0.001$) significantly inhibited 4mM ($p<0.001$) and 20mM ($p<0.001$) glucose induced insulin secretion compare to sham. (Figure-29)

Figure-28: Effect of dopamine and its agonists, antagonists on glucose induced insulin secretion from pancreatic islets in 24 hours in vitro culture



*** P<0.001 when compared with control in the presence of 4mM glucose
** P<0.01 when compared with control in the presence of 4mM glucose
P<0.001 when compared with control in the presence of 20mM glucose
P<0.01 when compared with control in the presence of 20mM glucose

Figure-29: Effect of dopamine and norepinephrine on insulin secretion from pancreatic islets in 24 hours in vitro culture

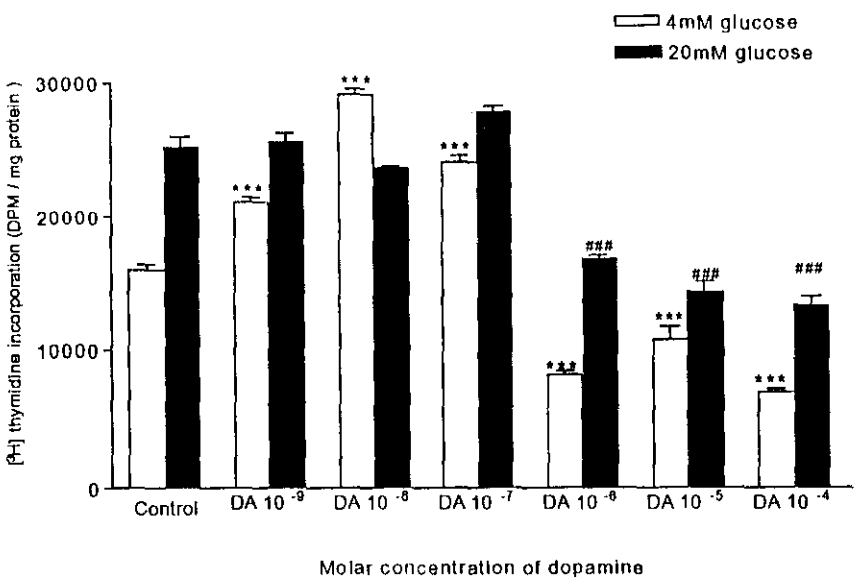


*** P<0.001 when compared with control in the presence of 4mM glucose
P<0.001 when compared with control in the presence of 20mM glucose

Effect of dopamine on DNA synthesis in pancreatic islets cells in 24 hours *in vitro* culture

DA at 10^{-9}M - 10^{-4}M was added to pancreatic islets cells cultured in the presence of 4mM and 20mM glucose. DA at 10^{-9}M - 10^{-7}M significantly ($p<0.001$) induced thymidine incorporation in cells cultured with 4mM glucose. Maximum thymidine incorporation was observed in the presence of 10^{-8}M DA with 4mM glucose. DA at 10^{-9} M - 10^{-7} M could not make any change in thymidine incorporation in the presence of 20mM glucose. DA 10^{-6} M - 10^{-4}M, significantly ($p<0.001$) inhibited thymidine incorporation in the presence of 4mM and 20mM glucose (Figure-30).

Figure-30: Effect of dopamine on DNA synthesis in pancreatic islets cells in 24 hours in vitro culture



*** P<0.001 when compared with control in the presence of 4mM glucose
P<0.001 when compared with control in the presence of 20mM glucose

DNA SYNTHESIS STUDIES IN PANCREATIC ISLETS

Effect of dopamine and its antagonists on DNA synthesis in pancreatic islets cells in 24 hours *in vitro* culture

DA receptor antagonist - butaclamol and DA D_2 receptor antagonist - YM-09151-2 at 10^{-4}M concentration were added to pancreatic islets cell culture containing 10^{-4}M DA and 10^{-8}M DA along with 4mM and 20mM glucose. DA at 10^{-8}M showed significant ($p<0.001$) increase in thymidine incorporation in the presence of 4mM glucose while no change was observed in the presence of 20mM glucose compare to control. Stimulatory effect of 10^{-8}M DA in the presence of 4mM glucose was reversed to control value in the presence of 10^{-4}M butaclamol while no change was observed in the presence of 20mM glucose. Addition of YM-09151-2 at 10^{-4}M also showed similar pattern of inhibition as that of 10^{-4}M butaclamol. DA at 10^{-4}M significantly ($p<0.001$) inhibited thymidine incorporation in the presence of 20mm glucose concentration while no change was observed in the presence of 4mm glucose. Also, this inhibitory effect was reversed by the addition of 10-4M butaclamol. Addition of YM-09151-2 at 10^{-4}M also reversed the inhibitory effect of 10^{-4}M DA (Figure-31).

Effect of dopmaine, its antagonist and norepinephrine on DNA synthesis in pancreatic islets cells in 24 hours in vitro culture

NE at 10^{-4}M was added to pancreatic islets cell culture containing 10^{-8}M and 10^{-4}M DA along with 4mM and 20mM glucose. DA at 10^{-8}M showed significant ($p<0.001$) increase in thymidine incorporation in the presence of 4mM glucose while no change was observed in the presence of 20mM glucose compare to control. Presence of 10^{-4}M NE along with 10^{-8}M DA in culture medium significantly inhibited thymidine incorporation compare to 10^{-8}M DA alone in the presence of both 4mM ($p<0.001$) and 20mM ($p<0.001$) glucose. Presence of 10^{-4}M YM-09151-2 in the culture medium containing 10^{-8}M DA and 10^{-4}M NE also significantly inhibited thymidine incorporation

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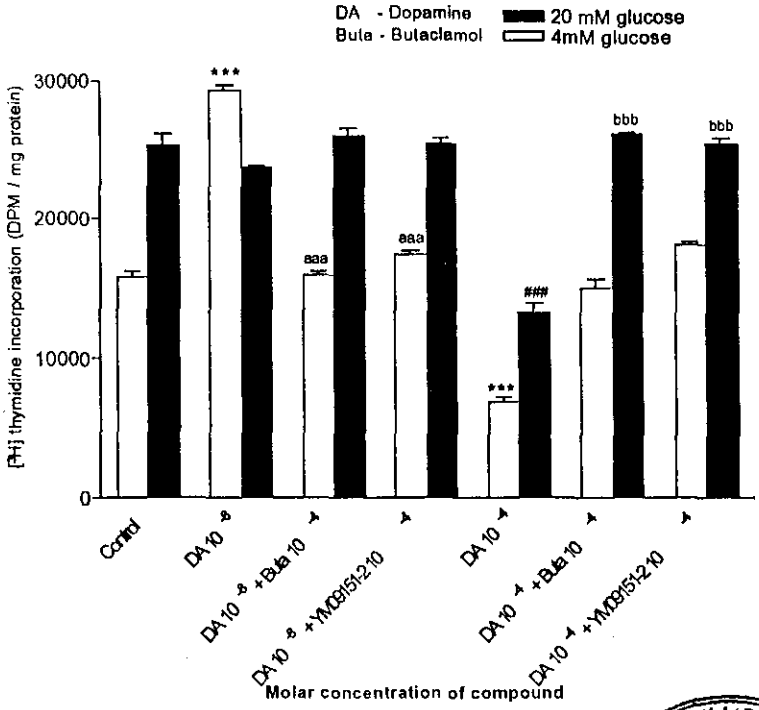
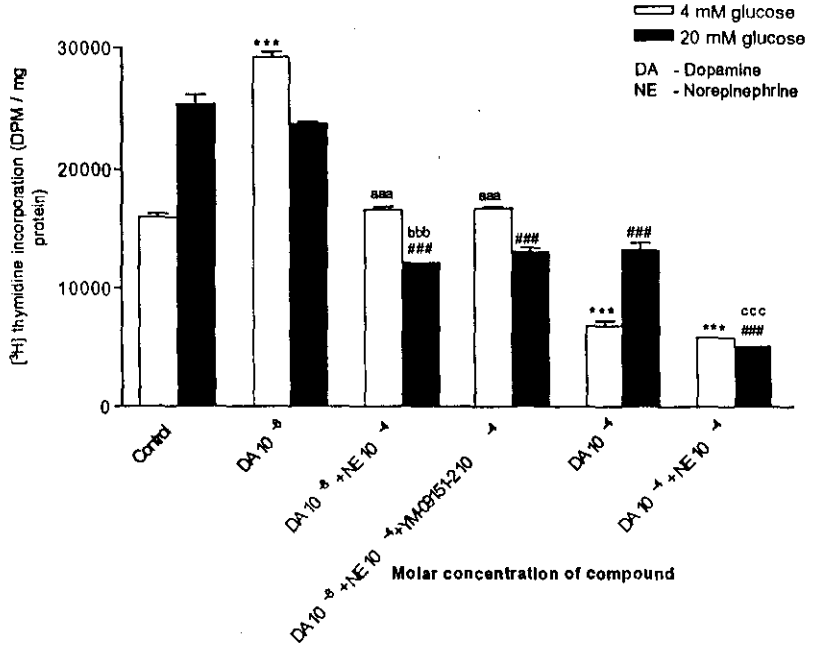
in the presence of both 4mM and 20mM glucose. Also, there was no significant change compare to cells cultured in the presence of 10^{-8}M DA and 10^{-4}M NE along with 4mM and 20mM glucose. DA at 10^{-4}M concentration significantly ($p<0.001$) inhibited thymidine incorporation compare to control in the presence of both 4mM and 20mM glucose. Addition of NE at 10^{-4}M concentration inhibited thymidine incorporation compare to 10^{-4}M DA alone (Figure-32).

Figure-31: Effect of dopamine and its antagonists on DNA synthesis in pancreatic islets cells in 24 hours in vitro culture



*** P<0.001 when compared with control in the presence of 4mM glucose
P<0.001 when compared with control in the presence of 20mM glucose
aaa P<0.001 when compared with 10^{-8} in the presence of 4mM glucose
bbb P<0.001 when compared with 10^{-4} in the presence of 20mM glucose

Figure-32: Effect of dopmaine, its antagonist and norepinephrine on DNA synthesis in pancreatic islets cells in 24 hours in vitro culture



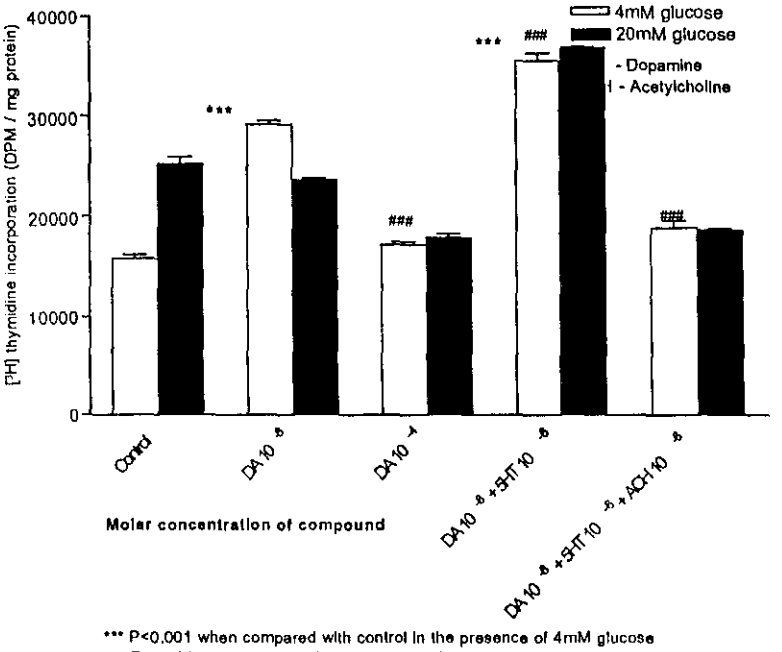
*** P<0.001 when compared with control in the presence of 4mM glucose
P<0.001 when compared with control in the presence of 20mM glucose
aaa P<0.001 when compared with 10^{-8}M DA in the presence of 4mM glucose
bbb P<0.001 when compared with 0^{-8}M DA in the presence of 20mM glucose
ccc P<0.001 when compared with 10^{-4} DAin the presence of 20mM glucose

Effect of dopamine, 5HT and acetylcholine on DNA synthesis in pancreatic islets cells in 24 hours *in vitro* culture

Effect of neurotransmitter like DA, 5HT, acetylcholine and its combinations were added to pancreatic islets cells were cultured in the presence of 4mM and 20mM glucose. DA 10^{-8}M significantly ($p<0.001$) increased thymidine incorporation in the presence of 4mM glucose. DA 10^{-4}M significantly ($p<0.001$) inhibited thymidine incorporation in the presence of 20mM glucose. DA at 10^{-8}M in combination with 10^{-6}M 5HT significantly ($p<0.001$) stimulated thymidine incorporation in the presence of both 4mM and 20mM

glucose. A combination of 10-8M DA, 10-6M 5HT and 10-6M acetylcholine significantly ($p<0.001$) inhibited the thymidine incorporation in the presence of 4mM glucose while thymidine incorporation un altered in the presence of 20mM glucose (Figure-33).

Figure-33: Effect of dopamine, 5HT and acetylcholine on DNA synthesis in pancreatic islets cells in 24 hours in vitro culture



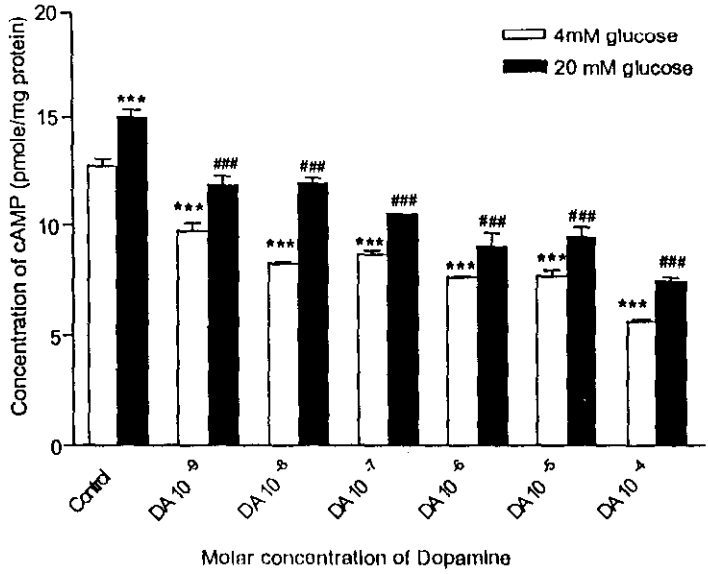
*** P<0.001 when compared with control in the presence of 4mM glucose
P<0.001 when compared with control in the presence of 20mM glucose

EFFECT OF DOPAMINE ON cAMP CONTENT IN PANCREATIC ISLETS IN ONE HOUR IN VITRO PANCREATIC ISLETS CELL CULTURE.

There was a significant ($p<0.001$) increase in cAMP content in cells cultured in the presence of 20mM glucose compare to 4mM glucose. DA at 10^{-9}M to 10^{-4}M was added to pancreatic islets incubated in the presence of 4mM and 20mM glucose. A dose

dependent significant (P<0.001) inhibition was observed from 10^{-9}M to 10^{-4}M DA in the presence of both 4mM and 20mM glucose concentrations (Figure-34)

Figure-34: Effect of dopamine on cAMP content in pancreatic islets in one hour in vitro culture



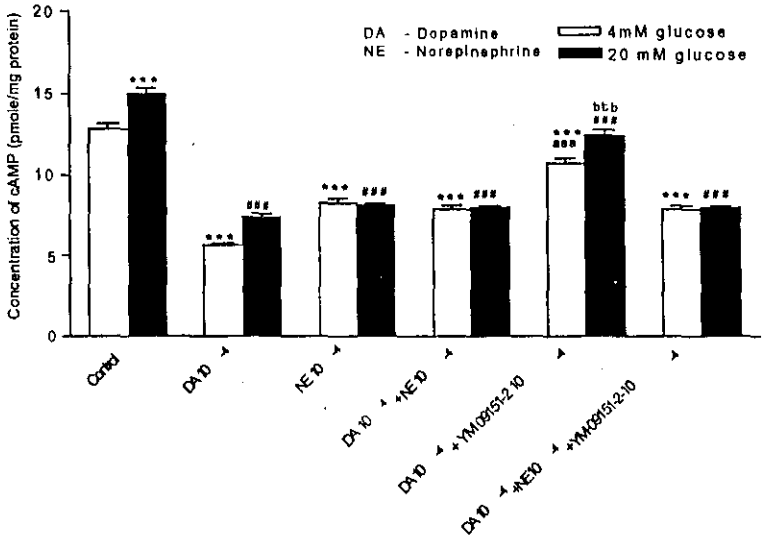
*** P<0.001 when compared with control in the presence of 4mM glucose
P<0.001 when compared with control in the presence of 20mM glucose

Effect of dopamine and norepinephrine on cAMP level in pancreatic islets in one hours *in vitro* culture.

cAMP level was significantly inhibited by 10^{-4}M DA (P<0.001), 10^{-4}M NE DA (P<0.001) and 10^{-4}M DA + 10^{-4}M NE (p<0.001) in the presence of 4mM and 20mM glucose. Presence of 10^{-4}M YM 09151-2 significantly (p<0.001) reversed the inhibitory effect of 10^{-4}M DA on insulin secretion in the presence of 4mM and 20mM glucose.

Presence of 10-4M NE inhibited cAMP level in the presence of 10^{-4}M DA and its antagonist YM 09151-2 at 10^{-4}M (Figure-35).

Figure-35: Effect of dopamine and norepinephrine on cAMP content in pancreatic islets in one hour in vitro culture

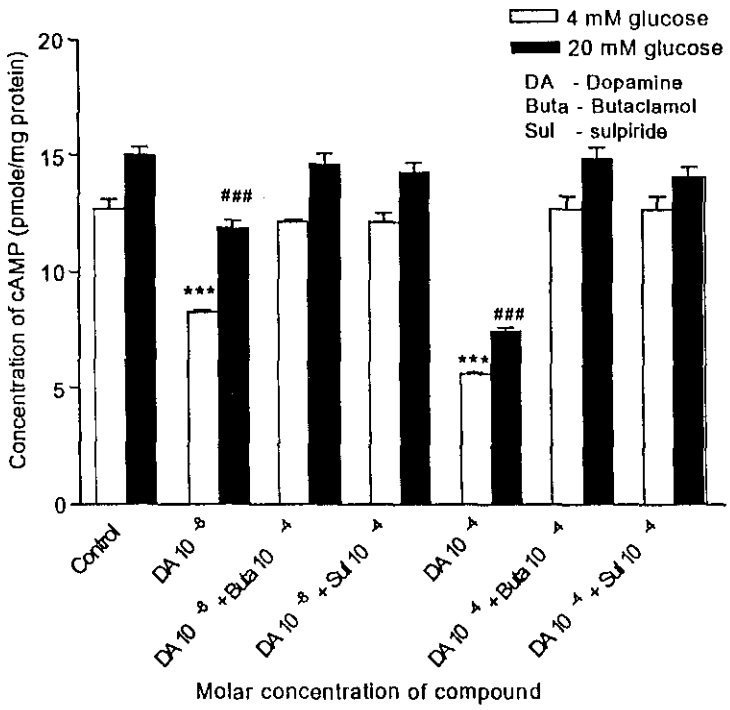


*** P<0.001 when compared with control in the presence of 4mM glucose
P<0.001 when compared with control in the presence of 20mM glucose
aaa P<0.001 when compared with DA 10^{-4}M in the presence of 4mM glucose
bbb P<0.001 when compared with DA 10^{-4}M in the presence of 20mM glucose

Effect of dopamine and its antagonists on cAMP level in pancreatic islets in one hour *in vitro* culture

Pancreatic islets cells were incubated in the presence of 10^{-8}M DA and 10^{-4}M DA and its agonist 10^{-4}M Butaclamol and 10^{-4}M sulpiride. DA 10^{-8}M ($p<0.001$) and DA 10^{-4}M ($p<0.001$) significantly inhibited cAMP level compare to control in the presence of both 4mM and 20mM glucose. This inhibited was reversed and cAMP level was restored by the addition of 10^{-4}M butaclamol and 10^{-4}M sulpiride (Figure-36).

Figure-36: Effect of dopamine and its antagonists on cAMP level in pancreatic islets in one hour in vitro culture



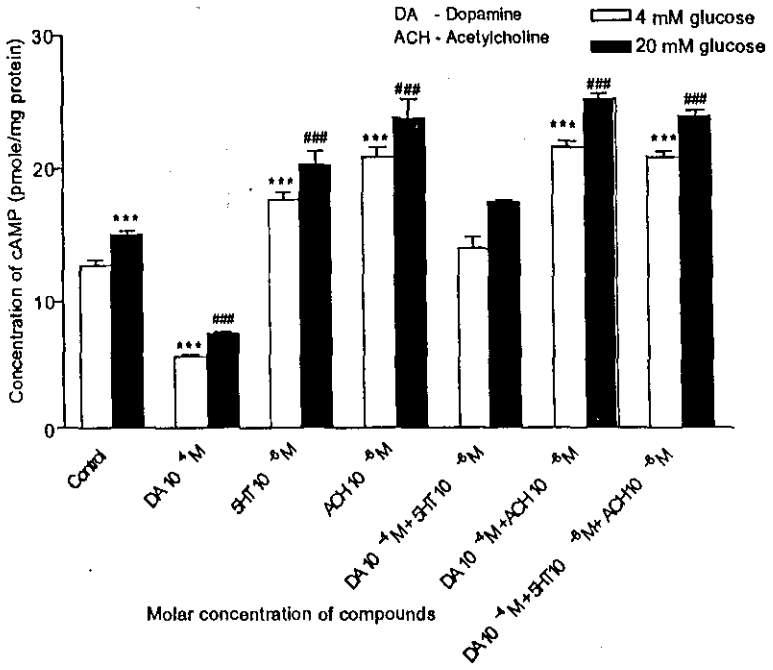
*** P<0.001 when compared with control in the presence of 4mM glucose
P<0.001 when compared with control in the presence of 20mM glucose

Effect of dopamine, 5HT and acetylcholine on cAMP level in pancreatic islets cells in one hour *in vitro* culture

Pancreatic islets cells were cultured in the presence of DA, 5HT, acetylcholine and its combinations. DA at 10^{-4}M significantly inhibited cAMP level while 10^{-6}M 5HT and 10^{-6}M acetylcholine significantly increased cAMP content in islets cells cultured in the presence of both 4mm and 20mm glucose. There was no change in cAMP content in cells cultured in the presence of 10^{-4}M DA + 10^{-6}M 5HT along with 4mM and 20mM

glucose. DA at 10^{-4}M in combination with 10^{-6}M acetylcholine significantly ($p<0.001$) increased cAMP content in cells cultured along with 4mM and 20mM glucose. DA at 10^{-4}M in combination with 10^{-6}M acetylcholine and 10^{-6}M 5HT significantly ($p<0.001$) increased cAMP content in cells cultured along with 4mM and 20mM glucose (Figure-37).

Figure-37: Effect of dopamine, 5HT and acetylcholine on cAMP level in pancreatic islets cells in one hour in vitro culture



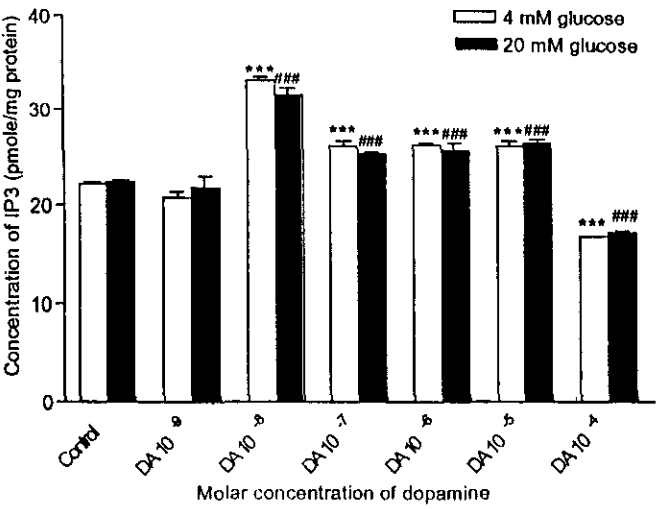
*** P<0.001 when compared with control in the presence of 4mM glucose
P<0.001 when compared with control in the presence of 20mM glucose

Effect of dopamine on IP3 content in pancreatic islets in one hour *in vitro* culture

There was no significant difference in the IP3 level in islets cells cultured in the presence of 4mM and 20mM glucose. DA at 10^{-8}M to 10^{-4}M was added to pancreatic

islets incubated in the presence of 4mM and 20mM glucose. DA at 10^{-8}M - 10^{-5} M significantly ($p<0.001$) increased IP3 level in the presence of both 4mM and 20mM glucose. Maximum increase in IP3 level was observed in the presence of 10^{-8}M DA along with 4mM and 20mM glucose. DA at 10^{-4}M significantly ($p<0.001$) inhibited IP3 level in the presence of both 4mM and 20mM glucose (Figure-38)

Figure-38: Effect of dopamine on IP3 content in pancreatic islets in one hour in vitro culture



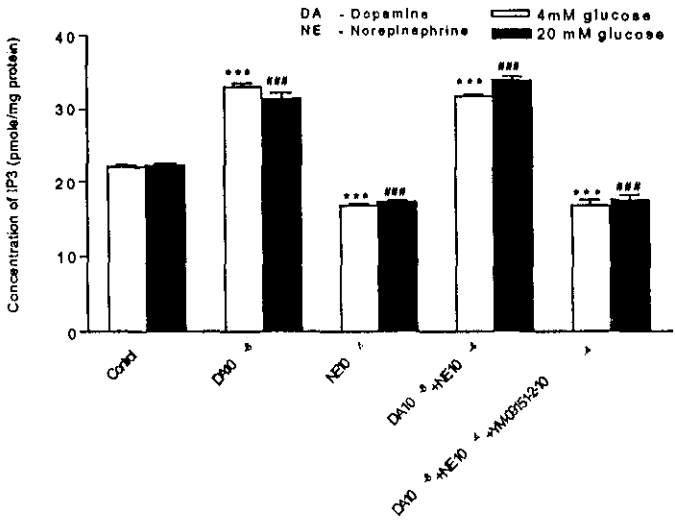
*** P<0.001 when compared with control in the presence of 4mM glucose
P<0.001 when compared with control in the presence of 20mM glucose

Effect of dopamine, its antagonists and norepinephrine on IP3 level in pancreatic islets in one hour *in vitro* culture

NE 10^{-4}M was added to pancreatic islets cells and incubated with media containing DA 10^{-8}M and 10^{-4}M along with 4mM and 20mM. DA 10^{-4}M ($p<0.001$) produced significant inhibition while DA 10^{-8}M ($p<0.001$) increased IP3 level in the presence of both 4mM and 20mM glucose. Presence of NE at 10^{-4}M reversed the inhibitory effect of 10^{-4}M DA. Presence of 10^{-4}M NE significantly ($p<0.001$) increased IP3 level compared to control and 10^{-4}M DA in the presence of 4mM and 20mM glucose.

DA 10^{-8}M produced significant ($p<0.001$) increase in IP3 level in the presence of both 4mM and 20mM glucose and addition of NE10^{-4}M made a further significant ($p<0.001$) increase in IP3 level in the presence of both 4mM and 20mM glucose compare to control and 10^{-8}M DA (Figure-39)

Figure-39: Effect of dopamine, its antagonists and norepinephrine on IP3 level in pancreatic islets in one hour in vitro culture.



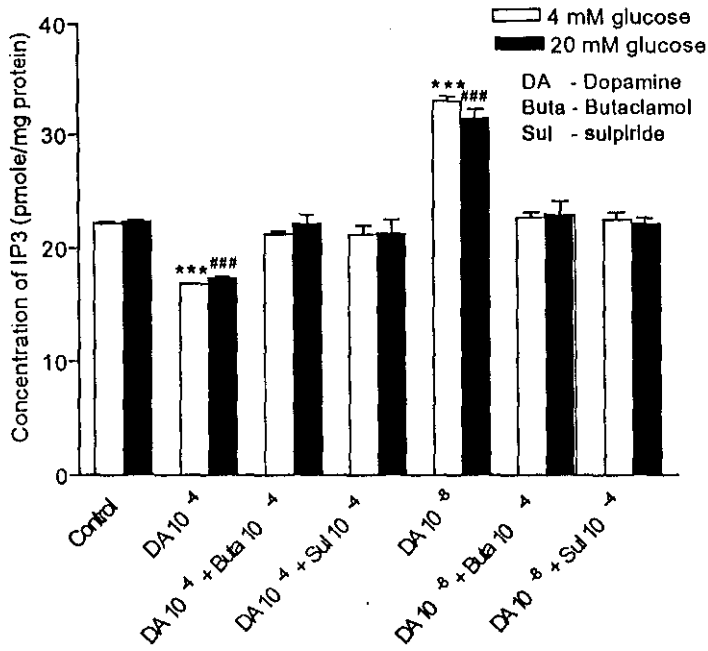
*** P<0.001 when compared with control in the presence of 4mM glucose
P<0.001 when compared with control in the presence of 20mM glucose

Effect of dopamine and its antagonists on IP3 level in pancreatic islets in one hour *in vitro* culture.

Pancreatic islets cells were incubated with DA 10^{-8}M, DA 10^{-4}M and DA antagonist - butaclamol 10^{-4}M and DA D_2 receptor specific antagonist sulpiride 10^{-4}M in the presence of 4mM and 20mM glucose. DA at 10^{-4}M significantly ($p<0.001$) inhibited IP3 content in the presence of both 4mM and 20mM. Presence of antagonist reversed the effect of 10^{-4}M DA on IP3 level in the presence of 4mM and 20mM glucose. DA 10^{-8}M significantly ($p<0.001$) increased IP3 level but addition of 10^{-4}M butaclamol and

sulpiride reversed the stimulatory and inhibitory effect of DA on IP3 content compare to control in the presence of 4mM and 20mM glucose (Figure-40).

Figure-40: Effect of dopamine and its antagonists on IP3 level in pancreatic islets in one hour in vitro culture



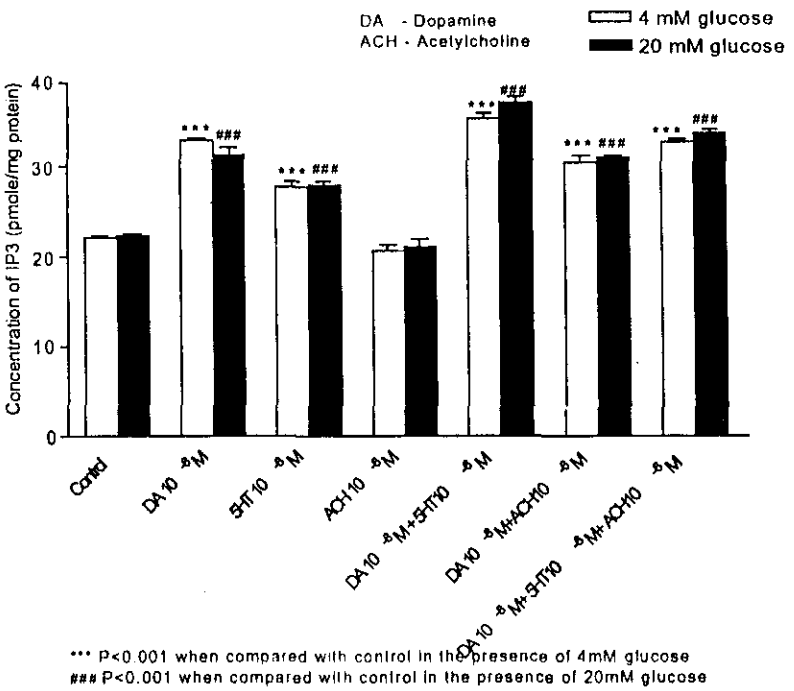
*** P<0.001 when compared with control in the presence of 4mM glucose
P<0.001 when compared with control in the presence of 20mM glucose

Effect of dopamine, 5HT and acetylcholine on IP3 content in pancreatic islets cells in one hour *in vitro* culture.

Pancreatic islets cells were cultured in the presence of DA, 5HT, acetylcholine and its combination. DA at 10^{-8}M and 5HT at 10^{-6}M significantly increased IP3 content in pancreatic islets cells cultured in the presence of 4mM and 20mM glucose. Acetylcholine 10^{-6}M did not make any change in the presence of 4mM and 20mM glucose. DA at 10^{-8}M in combination with 10^{-6}M 5HT significantly ($p<0.001$) increased IP3 content in cell cultured along with 4mM and 20mM glucose. DA at 10^{-8}M in

combination with 10^{-6}M acetylcholine significantly ($p<0.001$) increased IP3 content in cell cultured along with 4mM and 20mM glucose. DA at 10^{-8}M in combination with 10^{-6}M acetylcholine and 10^{-6}M 5HT significantly ($p<0.001$) increased IP3 content in cell cultured along with 4mM and 20mM glucose (Figure-41).

Figure-41: Effect of dopamine, 5HT and acetylcholine on IP3 content in pancreatic islets cells in one hour in vitro culture

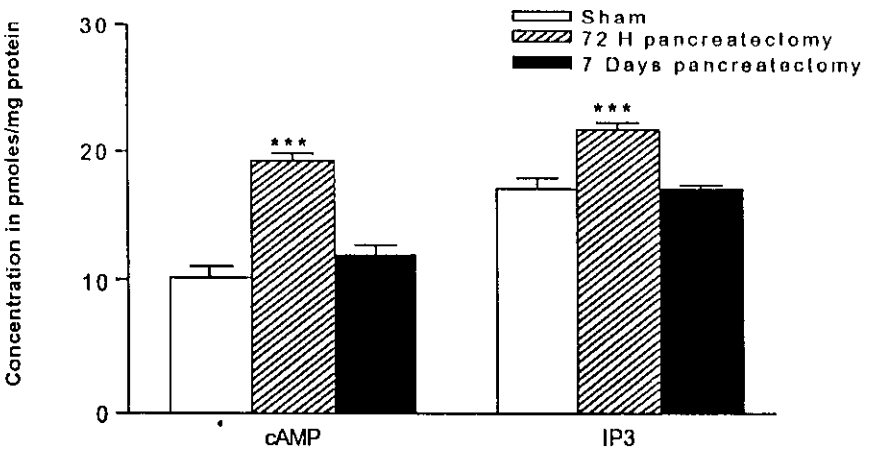


Changes in cAMP and IP3 content in pancreatic islets during pancreatic regeneration *in vivo*

cAMP and IP3 level was estimated in sham, 72 hours pancreatectomy and 7 days pancreatectomy. cAMP level increased significantly ($p<0.001$) during 72 hours pancreatectomy and reversed to near sham value after 7 days of panceratectomy. IP3

level increased significantly ($p<0.001$) during 72 hours pancreatectomy and reversed to near sham value after 7 days of pancreatectomy (Figure-42).

Figure-42: Changes in cAMP and IP3content in pancreatic islets during pancreatic regeneration in vivo



*** P<0.001 when compared with control in the presence of 4mM glucose

INTRACELLULAR CALCIUM IMAGING USING CONFOCAL MICROSCOPY

Pancreatic islets cells were cultured in the presence of 4mM glucose. DA at 10^{-8}M significantly ($p<0.001$) increased intracellular calcium concentration in the presence of 4mM glucose (Figure-43). Addition of 10^{-4}M YM09151-2 concentration did not increase the intracellular calcium level. (Figure-44).

Figure-43: Effect of 10^{-8}M DA on intracellular calcium level in pancreatic islets cells

(Confocal image at 0 second)

(Confocal image at 72^{nd} second)

Table-34: Pixel intensity analysis of Confocal image(figure-43)

Time	Area	Mean	IntDen
Initial	130000	227.172	29532317
At 72^{nd} seconds	130200	473.492	51063632

Figure-44: Effect of 10^{-4}M YM-09151-2 on 10^{-8}M DA induced intracellular calcium level in pancreatic islets cells.

Confocal image at 0 second

Confocal image at 72^{nd} second

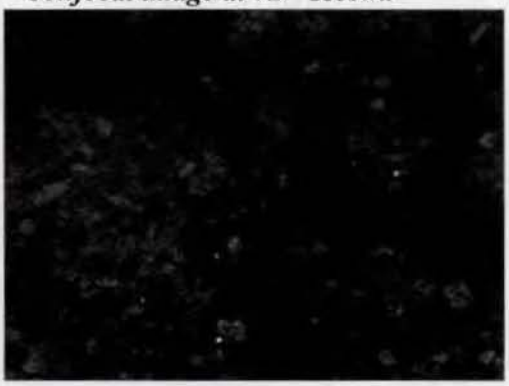


Table-35: Pixel intensity analysis of Confocal image(figure-44)

Time	Area	Mean	IntDen
Initial	227101	204.892	28746571
At 72^{nd} seconds	226998	210.746	28732869

DISCUSSION

Pancreas is an organ of limited regenerative capacity. Partial pancreatectomy is an established model to study the pancreatic regeneration (Pearson *et al.*, 1977). Pancreatic islets β-cell mass is dynamic. It increases and decreases both in function and mass to maintain the glycaemic level with in a narrow physiological range. The changes in mass can be in both number and individual volume of the β-cells. When the mass cannot increase adequately, diabetes ensues (Bonner-Weir, 2000). The pancreatic islets are richly innervated by parasympathetic, sympathetic and sensory nerves. Several neurotransmitters- DA, acetylcholine, NE, and neuropeptides are stored within the terminals. Stimulation of autonomic neurotransmitters and treatment with neurotransmitters affect insulin secretion. Regeneration is a complex interplay of several factors - growth factors, hormones and neurotransmitters. Nutrients including glucose are reported to stimulate ß-cell replication (Swenne., 1982; Hellerstrom *et al.*, 1985). The stimulatory effect of growth hormone on insulin production and ß-cell replication are well documented (Nielsen, 1986; Swenne *et al.*, 1987; Sjoholm *et al.*, 2000). *In vitro* and *in vivo* studies have established the role of insulin in ß-cell replication (Chick *et al.*, 1973). Insulin interacts with type1 IGF receptor and stimulates ß-cell proliferation.

CHANGES IN DOPAMINE AND HOMOVALINIC ACID CONTENT IN BRAIN REGIONS DURING PANCREATIC REGENERATION.

Experiments have demonstrated the neural regulation of glucose induced insulin secretion (Curry & McDonald, 1991). But the previously reported studies have not given any insight to the regulatory role of central nervous system dopaminergic signiling on pancreatic cell proliferation and insulin secretion. DA content decreased significantly in brain regions such as cerebral cortex, corpus striatum, brain stem and hypothalamus during pancreatic regeneration. Alteration in HVA has always been considered as an

index of DA metabolism in the brain (Lambert, *et al.*, 1991; Sternberg, *et al.*, 1983). DA turnover ratio decreased in cerebral cortex and hypothalamus while it was found to be increased in corpus striatum and brain stem. An increase in DA level in the brain regions attributes to the decreased release of DA (Lim & Lee, 1994). Dopaminergic action is important in the regulation of the hypothalamic-pitutary hormone release. The central vagal connection with dopaminergic innervation is reported to reach the pancreatic islets through the parahypothalamic ventricular (PHV) nucleus while aderenergic and serotonergic innervations reach the pancreas through the brain stem (Smith & Davis, 1983; Lowey *et al.*, 1994). It is reported that administration of DA through lateral brain ventricle inhibited pancreatic secretion dose dependently (Masuda *et al.*, 1998). Thus our results suggest that the decrease in DA content in brain help to decrease its inhibitory effect on pancreatic insulin secretion so as to secret more insulin for the regulation of blood glucose during pancreatic regeneration.

CHANGES IN DOPAMINE AND HOMOVALINIC ACID CONTENT IN PANCREAS, ADRENALS AND PLASMA DURING PANCREATIC REGENERATION

DA at high concentrations reported to inhibit glucose stimulated insulin secretion from pancreatic islets (Tabeuchi *et al.*, 1990). Low concentrations of DA can stimulate insulin secretion. DA content decreased in pancreas during pancreatic regeneration and reversed to near sham value after 7 days. HVA content in pancreas decreased during pancreatic regeneration. Alteration in HVA has always been considered as an index of DA metabolism in the brain (Lambert, *et al.*, 1991; Sternberg, *et al.*, 1983). Our results suggest that decrease in DA content in pancreas contribute for increased insulin secretion from pancreatic islets.

Reports show that DA D_2 receptors are present at the level of the adrenal medulla and that their activation could mediate an inhibitory modulation on the adrenal

catecholamine release (Foucart, 1988; Mannelli *et al*, 1997). DA content in plasma and adrenals was found to be increased during pancreatic regeneration. Epinephrine when used in high doses *in vivo* or *in vitro*, reduces the insulin response to stimulators (Malaisse, 1972). EPI and NE have an antagonistic effect on insulin secretion and glucose uptake (Porte, 1966). Our results show that there was a significant decrease in NE ($p<0.001$) and EPI ($p<0.001$) content in pancreas during pancreatic regeneration. Earlier studies from our lab reported a decrease in the adrenergic activity during pancreatic regeneration. The decrease in the NE and EPI stimulate the β-adrenergic receptors which are stimulatory to insulin secretion (Ani, 2000). Our result suggest that the increased DA content in plasma has an inhibitory effect on adrenal catecholamines such as DA, epinephrine and nor epinephrine release and thereby decrease in sympathetic activity. This decreased sympathetic activity will enhance insulin secretion from pancreatic islets.

BRAIN DOPAMINE D_2 RECEPTOR ALTERNATIONS

DA containing neurons arise mainly from DA cell bodies in the substantia nigra and ventral tegmental area in mid-brain region (Florijn, *et. al.*,). 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 patho-physiology 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 behavior. 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.

Hypothalamus is an important brain region with neuro-endocrine function. The central vagal connection with dopaminergic innervation is reported to reach the pancreatic islets through the parahypothalamic ventricular (PHV) nucleus (Smith and Davis, 1983, Lowey *et al.*, 1994). Total DA receptor number in hypothalamus was found to be increased during pancreatic regeneration but the affinity of total DA receptor increased significantly during pancreatic regeneration. The receptor number partially returned to near normal after 7 days. But a further decrease in affinity was observed after 7 days. DA D_2 receptor number also increased significantly during pancreatic regeneration. There was an increase in affinity of the receptor during pancreatic regeneration. The DA D_2 receptor number in hypothalamus returned to near sham value after 7 days of pancreatectomy but there was a further increase in affinity was observed after 7 days. Even though there was an increase in receptor number, DA content in hypothalamus was found to be decreased during pancreatic regeneration and returned to near sham value after 7 days of pancreatectomy. Inhibition of DA synthesis by alpha Mpt produced significant elevations in plasma GH concentration (Buonomo *et al.*, 1984). The growth hormone (GH), prolactin (PRL) and placental lactogen (PL) were found to stimulate proliferation of normal rat β-cells (Nielsen, 1982). Among the large number of

protein hormones, GH, lactogenic peptides, PRL and PL have an important role in β-cell proliferation. GH has been reported to stimulate the *in vitro* replication of foetal (Dudek *et al.*, 1984), neonatal (Brelje *et al.*, 1989) and adult rat β-cells (Swenne & Hill, 1989). Thus our results suggest that the decreased dopaminergic activity in hypothalamus will contribute for increased pancreatic islets cell proliferation mediated through the GH.

DA receptor kinetics in corpus striatum showed a significant increase DA receptor number during pancreatic regeneration as indicated by the increased B_{max}. The affinity of the receptor increased significantly during pancreatic regeneration as indicated by the decrease in Ki and log (EC_{50}) value. Changes in receptor number and affinity were reversed to near sham value after 7 days of pancreatectomy. There was a decrease in DA D_2 receptor number during pancreatic regeneration. Affinity of DA D_2 receptor increased significantly during pancreatic regeneration. There was no change in DA D_2 mRNA level during pancreatic regeneration. This may be explained by the decreased translational process resulted in decreased production of DA D_2 receptor protein.

DA receptor kinetics in cerebral cortex showed a significant increase in DA receptors number in cerebral cortex during pancreatic regeneration as indicated by increased Bmax after 72hours pancreatectomy. The affinity of the receptor decreased after 72 hours of pancreatectomy as indicated by an increase in Ki and log (EC_{50}) value. Real Time PCR analysis also confirmed the data. It is reported that the dopaminergic activity changes in cerebral during acute experimental pancreatitis (Kopieniak *et al.*, 2004). Our results suggest that any leasons in the pancreas will make receptor changes in cerebral cortex.

DA receptor number in brain stem showed a significant increase during pancreatic regeneration and returned to near sham value after 7 days. There was a decrease in affinity during pancreatic regeneration returned to near normal value after 7

days. A decrease in DA and its metabolite content was observed during pancreatic regeneration. DA D_2 receptor number was found to be decreased during pancreatic regeneration while there was an increase in its affinity. The DA D_2 receptor alterations were trend to reverse after 7 days of pancreatectomy. The dopaminergic signaling was decreased in brain stem during pancreatic regeneration. Real time PCR analysis also confirmed the DA D_2 receptor data in brain stem. Chemical activation of Rpa neurons increases pancreatic insulin release through medullary TRH and vagal-mediated pathways (Yang *et al.*, 2005). It is postulated that DA, either from projections from the substantia nigra or the ventral tegmental area or from the DA-containing neurons of the DRN, may increase 5-HT release in the DRN, which, through autoreceptor stimulation, can profoundly influence the activity of ascending serotoninergic neurons (Ferre & Artigas, 1993). DA D_2-like receptor activation excites rat dorsal raphe 5-HT neurons (Haj-Dahmane, 2001).

DA receptors in pancreas increased during pancreatic regeneration and reversed to near sham value after 7 days of pancreatectomy. There was a decrease in affinity of DA receptors during pancreatic regeneration and returned to near sham value after 7 days. DA D_2 receptor number in pancreas was found to be increased during pancreatic regeneration. There was a decrease in affinity of the receptor during pancreatic regeneration. Receptor number and affinity returned to near sham value after 7days of pancreatectomy. DA content in pancreas decreased significantly during pancreatic regeneration and returned to near sham value after 7 days. There is a report that shows that DA D_2-like receptors are expressed in pancreatic ß-cells and mediate inhibition of insulin secretion (Rubi *et al.*, 2005) but the experiment is done in the presence of 10µM of DA and 5µM of DA D_2 receptor specific antagonist. We conducted *in vitro* experiment to study the involvement of DA and DA D_2 receptor. It revealed that DA at 10^{-8}M induced insulin secretion while the µM range inhibited the insulin secretion. Studies

using DA D_2 specific agonist and antagonists reveal that the stimulatory effect of DA at 10^{-8}M and inhibitory effect of DA at μM range are mediated through DA D_2 receptor. There was a significant difference in IP3 level in the pancreatic islets cultured in the presence of 10μM DA and 10nM DA. The later made significant induction of IP3 which can contribute positively for increased insulin secretion by an increase in intracellular calcium level from intracellular calcium stores. DA D_2 receptor activation causes mitogenesis via p44/42 mitogen-activated protein kinase in opossum kidney cells (Narkar *et al.*, 2001). So our results suggest that the up regulation of DA D_2 receptor in pancreas contribute positively for increased insulin secretion and pancreatic cell proliferation.

DOPAMINERGIC STIMULATION ON INSULIN SYNTHESIS AND SECRETION FROM PANCREATIC ISLETS DURING 24 HOURS *IN VITRO* CULTURE

Neurotransmitters especially catecholamines play an important role in insulin synthesis and secretion from pancreatic β cells. In the ß cells, DA might be released from neurons innervating pancreatic islets and exocrine pancreas is an important source of DA. (Ahren & Lundquist, 1985; Éva Mezey *et al.*, 1996) DA is stored in the secretary granules of the pancreatic islets along with serotonin and calcium and could also be generated in pancreatic islets from its precursor L-dopa (Borelli *et al.*, 1987; Takayanagi & Watanabe, 1996; Ericson *et al.*, 1997; Blanca Rubí *et al.*, 2005). Glucose induced insulin secretion was found to be significantly increased in the presence of DA at a concentration 10^{-8}M and 10^{-7}M in the presence of 4mM glucose. DA 10^{-4}M completely inhibited the glucose induced insulin secretion. Maximum stimulation of insulin secretion was seen in 10^{-8}M DA This is suggested to have an implication in insulin secretion as high concentrations of DA in the presence of glucose causes a reduction in insulin secretion. DA 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. The function of islet ß-cells is controlled by a glucose sensor that operates at

physiological glucose concentrations and acts in synergy with signals originating from hypothalamic neurons. Our *in vitro* studies on pancreatic islets suggest that the DA exerts a differential regulatory role in glucose induced insulin secretion.

To determine the modulation of insulin synthesis and secretion by DA through its specific receptors, antagonists - butaclamol and sulpiride were used. Butaclamol, a general antagonist of DA receptors blocked the stimulatory effect of 10^{-8}M DA and inhibitory effect of 10^{-4}M DA on glucose induced insulin secretion. Sulpiride a potent DA D_2 receptor antagonist at 10^{-4}M in pancreatic islets culture effectively blocked the dopaminergic action on insulin secretion in the pancreatic islets. Identification of DA D_2 receptors in the pancreatic islets of rodents suggests that these receptors would play an important role in insulin secretion (Blanca Rubí *et al.*, 2005). Our results suggest that DA differentially regulate the pancreatic islets insulin secretion and it is mediated through DA D_2 receptors. Earlier studies from our laboratory have shown that addition of forskolin an activator of cAMP antagonized the inhibitory effect of DA on insulin secretion (Abraham, 1998). DA at a concentration of 10^{-8}M increased insulin synthesis and secretion while 10^{-4}M inhibited insulin synthesis and secretion. The stimulatory and inhibitory effect of bromocriptine was reversed by DA D_2 receptor specific antagonist sulpiride.

Increased NE level is reported to inhibit the pancreatic islet function (Adeghate, *et al.*, 2001). The hypothalamic neuronal messages and the DA presence in the pancreatic islets cause the inhibition of insulin secretion during diabetes (Nogueira *et al.*, 1994). Also, high concentrations of NE, DA, and 5HT in the pancreatic islets are reported to decrease glucose-stimulated insulin secretion (Zern., 1980) It has been previously reported that high concentrations of NE inhibited the glucose induced insulin secretion (Zern., 1980). NE is reported to have an inhibitory effect on insulin secretion in the

pancreatic islets (Porte, 1967). Our results show that a high concentration of NE was inhibitory to the stimulatory effect of 10^{-8}M DA on insulin secretion. Low concentration of NE did not affect the stimulatory effect of DA on insulin secretion. The α_{2A} adrenergic receptors respond to endogenous NE and EPI to elicit a variety of physiological responses, including inhibition of neurotransmitter release and suppression of insulin release from pancreatic β-cells (MacMillan *et al.*, 1998). α_2-adrenergic receptor activation leads to inhibition of insulin release by a mechanism distal to those regulating β-cell cyclic AMP production and $[Ca^{2+}]_i$ (Ullrich & Wollheim, 1985). Our results suggest that 10^{-8}M DA, low concentration enhanced insulin secretion which is blocked by 10^{-4}M NE.

Thus from our *in vitro* results, it is suggested that DA concentration is influential on glucose induced insulin secretion. The inhibitory and stimulatory effect on insulin secretion by DA is mediated through the DA D_2 receptors in the pancreatic islets. Also, it is suggested that the increased NE inhibit the dopaminergic effect on insulin synthesis and secretion.

DOPAMINERGIC STIMULATION ON DNA SYNTHESIS IN PANCREATIC ISLETS *IN VITRO*

There was an increase in DNA synthesis in the presence of 20mM glucose compared to normal glucose concentration (4mM). Low concentrations of DA (10^{-9}M to 10^{-7}M) stimulated DNA synthesis at physiological glucose concentration. It did not make any effect at high glucose concentrations. Higher concentrations DA (10^{-6}M to 10^{-4}M) inhibited DNA synthesis in pancreatic islets in the presence of both normal and high glucose concentrations. Maximum stimulation was seen in the presence of 10^{-8}M DA at physiological glucose concentration and it was reversed by the addition of DA general receptor antagonist - butaclamol and DA D_2 receptor specific antagonist - YM09151-2.

Inhibitory effect of high concentration of DA was also reversed by the addition of DA general antagonist - butaclamol and DA D_2 receptor specific antagonist - YM09151-2. Presence of NE at 10^{-4}M decreased the stimulatory and inhibitory effect of DA on DNA synthesis. Thus our results suggest that DA exerts a differential regulation on DNA synthesis in pancreatic islets through its DA D_2 receptors. Even though glucose itself has a stimulatory effect on DNA synthesis in pancreatic islets, the stimulatory of effect DA is observed only in normal glucose concentrations otherwise the stimulatory action of DA is not active in hyperglycemic conditions. Stimulatory and inhibitory effect will be blocked by the presence of higher concentration of NE.

DOPAMINERGIC EFFECT ON CAMP LEVEL IN PANCREATIC ISLETS *IN VITRO*

Glucose induced insulin secretion is accompanied by an increase in the islet cAMP content (Rabinovitch, 1976). High glucose significantly increased cAMP content in cells incubated compare to physiological glucose concentration. Increased glucose concentration will increase the cAMP level which then trigger the mitogenesis in the β-cells (Sjoholm, 1997). DA dose dependently inhibited cAMP content in pancreatic islets *in vitro*. Maximum inhibition was seen at 10^{-4}M DA. Presence of DA general antagonist-butaclamol and DA D_2 receptor specific antagonist - YM09151-2 reversed the inhibitory effect of DA on cAMP content. It suggests that the inhibition was mediated through DA D_2 receptor. Pancreatic islets cells are equipped with DA D_2 receptors (Rubi *et al.*, 2005). DA D_2 receptors are coupled with Gi protein of G protein complex (Senogles *et al.*, 2004). Our results suggested that the inhibitory effect of DA on cAMP content in pancreatic islets is mediated through its DA D_2 receptors.

cAMP content was significantly inhibited by the presence of 10^{-4}M DA, 10^{-4}M NE and 10^{-4}M DA + 10^{-4}M NE. At low concentration NE 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 (Lacey et al., 1993). α_2-adrenergic receptor activation leads to inhibition of insulin release by a mechanism distal to those regulating β-cell cAMP production and $[Ca^{2+}]_i$ (Ullrich & Wollheim, 1985). The alpha 2-adrenoceptor agonist clonidine markedly inhibited cAMP content in isolated pancreatic islets (Sjoholm, 1991) A potent and specific α_2-adrenoceptor agonist, medetomidine decreased intracellular cAMP concentrations that were elevated by phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) administration. Also, it abolished IBMX induced insulin release from pancreatic islets (Chen & Hsu, 1994). So our results suggest that higher concentration of NE can inhibit cAMP content in pancreatic islets possibly by the α_2-adrenoceptor activation. *In vitro* insulin secretion data also shows that the presence of high concentration of NE (10^{-4}M) is inhibitory to insulin secretion. Also, inhibitory effect of 10^{-4}M NE on glucose induced insulin secretion is due to its inhibitory effect on cAMP concentration.

The effect of other neurotransmitters - 5HT and acetylcholine separately and in combination with DA, on cAMP content were studied. 5HT at 10^{-6}M and acetylcholine at 10^{-6}M stimulated cAMP content in the presence of both 4mM and 20mM glucose. Combination of 10^{-4}M DA and 10^{-6}M 5HT did not make any effect on cAMP content in pancreas. But combination of DA 10^{-4}M, 5HT 10^{-6}M and acetylcholine 10^{-6}M significantly stimulated cAMP level. It is reported that acetylcholine directly stimulates insulin secretion from the islet ß-cells through activation of muscarinic receptors (Ahren, 2000). Reports show that 5HT dose dependently induce hypoglycemia and serum insulin levels (Sugimoto *et al.*, 1990).). Increase in calcium and cAMP can stimulate insulin release from the pancreatic ß cells of islets of langerhans (Siegel *et al.*, 1980). Our results show that DA content in pancreas decreased during pancreatic regeneration and insulin secretion. So the decreased DA content in pancreas will decrease the dopaminergic

inhibition on cAMP level while serotonergic activity and cholinergic activity during pancreatic regeneration will enhance cAMP level. This will contribute for enhanced insulin secretion and pancreatic cell proliferation.

The effect of DA on IP3 content was checked in pancreatic islets in *in vitro*. There was no difference in IP3 content in the presence of high glucose compare to normal physiological glucose concentrations. DA at 10^{-9}M significantly inhibited IP3 content in pancreas in the presence of both normal and hyperglycemic glucose concentrations. There was an increase in IP3 content in pancreatic islets in the presence of 10^{-8}M DA, 10^{-7}M DA and 10^{-6}M DA. Maximum stimulation was seen in the presence of 10^{-8}M DA in the presence of both normal and hyperglycemic glucose concentrations. An inhibition was produced by 10^{-4}M DA in the presence of both the concentrations of glucose. To check the possible receptor specific effect of IP3 content change in pancreatic islets, DA general antagonist-butaclamol and DA D_2 receptor specific antagonist-sulpiride was added. Stimulatory and inhibitory effect of DA was reversed due to the presence of 10^{-4}M butaclamol and 10^{-4}M sulpiride. Our results suggest that the stimulatory and inhibitory effect of DA on IP3 content was mediated through DA D_2 receptors in pancreatic islets. Reports shows that the activation of DA D_2 receptor will increase IP3 content in cells. Also, a differential regulation on IP3 by DA through DA D_2 receptors was observed. Reports shows that the increased IP3 can mobilize calcium fro intracellular calcium stores which in turn can enhance insulin secretion from pancreatic islets. Our data from *in vitro* studies on pancreatic islets show, DA exert a differential regulation by inducing insulin secretion at 10^{-8}M DA while inhibiting at 10^{-4}M DA. This differential regulation of DA may be possible due to the changes in IP3 content in the cell by the activation of DA D_2 receptors.

NE at 10^{-4}M was added to incubation media to check the effect on IP3 content in pancreatic islets *in vitro*. NE at 10^{-4}M significantly inhibited IP3 level *in vitro*. NE could not inhibit the DA D_2 mediate stimulation of IP3 in pancreatic islets. DA D_2 receptor specific antagonist YM09151-2 was added along with 10^{-4}M NE and 10^{-8}M DA. The stimulatory effect was inhibited significantly by the blockade of DA D_2 receptor. Our results suggest that the inhibition of NE on insulin secretion is not mediated through the signaling cascade of DA.

The effect of other neurotransmitters such as 5HT and acetylcholine were studied on IP3 content in pancreatic islets separately and in combination with 10^{-8}M DA. Presence of 5HT at 10^{-6}M significantly increased IP3 content in the pancreatic islets while acetylcholine at 10^{-6}M did not make any effect in the presence of normal and hyperglycemic glucose concentrations. Combination of 10^{-8}M DA with 5HT and acetylcholine stimulated IP3 content in pancreatic islets in the presence of both the glucose concentrations. But none of these were significantly higher or lower than the 10^{-8}M DA induced IP3 change. Our results suggest that DA at lower concentration is essential for glucose induced insulin secretion mediated through IP3 and it can be inhibited by higher concentrations of NE.

CHANGES IN CAMP AND IP3 DURING PANCREATIC REGENERATION AND INSULIN SECRETION *IN VIVO*

Increased cAMP can contribute to increased cell proliferation and the increased IP3 content can contribute positively for increased glucose stimulated insulin secretion. Pertussis toxin or the stimulatory cAMP analog Sp-cAMPS increased DNA synthesis and insulin secretion (Sjoholm, 1991; Rabinovitch *et al.*, 1980). There was an increase in cAMP and IP3 level during pancreatic regeneration and it was reversed to near sham

value after 7days. Our results suggest that the increased cAMP and IP3 content in pancreas contribute for the pancreatic cell proliferation and insulin secretion.

EFFECT OF DOPAMINE ON INTRACELLULAR CALCIUM LEVEL IN PANCREATIC ISLETS CELL CULTURE *IN VITRO*.

The effect of DA on intracellular calcium level was studied. It is reported that a raise in intracellular calcium level will result in increased exocytosis of insulin secretary granules from pancreatic β-cells. DA at 10^{-8}M was added to incubation media and calcium imaging was done using confical microscopy. There was a significant increase in intracellular calcium level after the addition of 10^{-8}M DA. Maximum intracellular calcium level was observed at 72^{nd} second after the addition of DA. The observation was terminated after 80 seconds. The significant increase in IP3 content in pancreatic islets in the presence of 10^{-8}M DA mobilize calcium from intracellular calcium stores which in turn lead to the increase in intracellular calcium level. The increased intercellular calcium level is suggested to induce insulin secretion from pancreatic islets.

SUMMARY

1. Pancreatic regeneration after partial pancreatectomy was used as a model system to study pancreatic β-cell proliferation in rats

2. Primary cultures of pancreatic islets were used as an *in vitro* system to study pancreatic islets cell proliferation and insulin secretion.

3. [^{3}H]thymidine incorporation was used as an index for pancreatic DNA synthesis.

4. DA content was analysed using HPLC. It decreased in the brain regions and pancreas during active cell proliferation and insulin secretion.

5. EPI and NE content was analysed using HPLC. It decreased in plasma and pancreas during active cell proliferation and insulin secretion.

6. Dopamine and DA D_2 receptor functional status was analysed using Scatchard analysis using [^{3}H]DA and [^{3}H]YM-09151-2 . Receptor analysis was confirmed by studying mRNA expression status using Real Time PCR analysis. Differential regulation of DA D_2 receptor was observed in brain regions. DA D_2 receptor was up regulated in pancreas.

7. *In vitro* insulin secretion and [^{3}H]thymidine incorporation study using DA agonists and DA D_2 specific antagonist showed that dopamine exerts a differential regulation in pancreatic insulin secretion and DNA synthesis. Lower

concentration 10^{-8}M showed a maximum stimulation while 10^{-4}M showed inhibition. These effects are mediated through DA D_2 receptors.

8. *In vitro* studies on pancreatic islets cell cAMP content using DA agonists and DA D_2 specific antagonist showed that DA inhibits cAMP. Lower concentration 10^{-8}M showed a maximum stimulation while 10^{-4}M showed inhibition. It is mediated through DA D_2 receptors.

9. *In vitro* studies on pancreatic islets cell IP3 content using DA agonists and DA D_2 specific antagonist showed that DA stimulate IP3. Lower concentration 10^{-8}M showed a maximum stimulation while 10^{-4}M showed inhibition. It is mediated through DA D_2 receptors.

10. cAMP and IP3 content in the pancreas increased during pancreatic regeneration and insulin secretion.

11. Intracellular calcium imaging on pancreatic islets in the presence of 10^{-8}M DA and 10^{-4}M DA D_2 receptor specific antagonist showed that there was an increase in calcium level during 72^{nd} second after the addition of DA. It is mediated through DA D_2 receptors.

Thus the results suggest that the there is a differential functional regulation of DA and DA D_2 receptors in brain and pancreas during pancreatic regeneration. *In vitro* studies confirmed a concentration depend functional regulation of DA through DA D_2 receptors on pancreatic islets cell proliferation and insulin secretion mediated through increased cAMP, IP3 and intracellular Ca^{2+} level.

CONCLUSION

Our findings demonstrate that the differential regulation of DA content in pancreas and functional regulation of DA D_2 receptor in brain regions such as hypothalamus, brain stem, cerebral cortex and corpus striatum play an important role during pancreatic islets cell proliferation and insulin secretion. Though may reports are there implicating the functional interaction between DA receptor and pancreatic islets cell insulin secretion, the involvement of specific DA D_2 receptors and changes in second messenger system during insulin secretion and pancreatic islets cell proliferation were not given emphasis. Down regulation of DA content in brain regions and pancreatic islets were observed during pancreatic regeneration. Up regulation of DA content in plasma and adrenals down regulated sympathetic activity in pancreas which cause an increase in insulin secretion and pancreatic islets cell proliferation during pancreatic regeneration. There was a differential regulation of DA D_2 receptor in brain regions. The pancreatic islets DA D_2 receptors were up regulated during pancreatic regeneration. DA D_2 receptor activation at specific concentration has accounted for increased pancreatic islets cell proliferation. *In vitro* experiments have proved the differential regulation of DA on insulin synthesis and pancreatic islets cell proliferation. Inhibitory effect of DA on cAMP and stimulatory effect of DA on IP3 through DA D_2 receptors were observed in *in vitro* cell culture system. These effects are correlating with the DA, cAMP and IP3 content during pancreatic regeneration and islets cell proliferation. Up regulation of intracellular Ca^{2+} was also observed at 10^{-8}M DA, a specific concentration of DA which showed maximum increase of IP3 content in pancreatic islets through DA D_2 receptor activation in *in vitro* culture. These *in vitro* data was highly correlating with the changes in DA, cAMP and IP3 content in pancreas during pancreatic regeneration and insulin secretion. Thus we conclude that there is a differential functional regulation of DA and DA D_2 receptors in brain and pancreas during pancreatic regeneration. *In vitro* studies confirmed

a concentration depend functional regulation of DA through DA D_2 receptors on pancreatic islets cell proliferation and insulin secretion mediated through increased cAMP, IP3 and intracellular Ca^{2+} level. This will have immense clinical significance in the management in diabetes mellitus.

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