5-HT_{1A} AND 5-HT_{2C} RECEPTOR GENE EXPRESSION: THEIR FUNCTIONAL ROLE IN PANCREATIC REGENERATION AND INSULIN SECRETION IN RATS

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MOHANAN V.V.

DEPARTMENT OF BIOTECHNOLOGY
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
COCHIN - 682 022, KERALA, INDIA.

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CERTIFICATE

This is to certify that the thesis entitled "**5-HT_{1A} AND 5-HT_{2C} RECEPTOR GENE EXPRESSION: THEIR FUNCTIONAL ROLE IN PANCREATIC REGENERATION AND INSULIN SECRETION IN RATS**" is a bonafide record of the research work carried out by Mr.Mohanan.V.V. 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.

Cochin - 682 022

Date: 22 April, 2004

Dr. C.S. Paulose

Director, Centre for Neuroscience

Reader, Department of Biotechnology

Cochin University of Science & Technology.

CENTRE FOR NEUROSCIENCE
UNIVERSITY OF SCIENCE AND TECHNOLOGY
COCHIN
682 022
KERALA
INDIA
DEPARTMENT OF BIOTECHNOLOGY
COCHIN UNIVERSITY OF SCIENCE & TECHNOLOGY

Dr. C.S. PAULOSE M.Sc., Ph.D., FIMSA.
DIRECTOR
CENTRE FOR NEUROSCIENCE
DEPARTMENT OF BIOTECHNOLOGY
Cochin University of Science and Technology
Cochin-682 022, Kerala, India

DECLARATION

I hereby declare that this thesis entitled "**5-HT_{1A} AND 5-HT_{2C} RECEPTOR GENE EXPRESSION: THEIR FUNCTIONAL ROLE IN PANCREATIC REGENERATION AND INSULIN SECRETION IN RATS**" is based on the original research carried out by me at the Department of Biotechnology, Cochin University of Science and Technology, under the guidance of **Dr. C. S. Paulose**, Director, Centre for Neuroscience, Reader, 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.

Cochin - 682 022
Date: 22 April, 2004

MOHANAN.V.V.
Reg. No. 2249

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(Mohanan V. V.)

Dedicated to My Mother

ABBREVIATIONS USED IN THE TEXT

5-CT	5-carbaxamidotryptamine
5-HIAA	5-hydroxy indole - 3 acetic acid
5-HT	5-Hydroxy tryptamine
5-HTP	5-Hydroxy tryptophan
8-OH DPAT	8-Hydroxy-2(di-n-propylamino)-tetralin
Ach	Acetylcholine
ACII	Adenylyl cyclase typeII
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BP	Blood pressure
B_{max}	Maximal binding
BS	Brain stem
cAMP	Cyclic adenosine mono phosphate
CC	Cerebral cortex
CHO	Chinese hamster ovary cells
CRH	Corticotropin-releasing hormone
CNS	Central nervous system
CREB	cAMP regulatory element binding protein
DAG	Diacylglycerol
dATP	Deoxy adenosine triphosphate
DOI	1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane
dCTP	Deoxy cytosine triphosphate
DEPC	Di ethyl pyro carbonate
dGTP	Deoxy guanosine triphosphate
DNTP	Deoxynucleotide triphosphate
DOI	1-(2,5-di-methoxy-4-iodophenyl)-2-aminopropane
dTTP	Deoxynucleotide thymidine triphosphate

ECD	Electrochemical detector
EGF	Epidermal growth factor
EPI	Epinephrine
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
FGF	Fibroblast growth factor
GABA	Gamma aminobutyric acid
GAP	GTPase activating protein
GFC	Glass microfiber filters : Type C
GRP	Gastrin releasing peptide
GTP	Guanosine triphosphate
HBSS	Hank's balanced salt solution
HGF	Hepatocyte growth factor
HPA	Hypothalamic-pituitary-adrenal
HPLC	High performance liquid chromatography
HYPO	Hypothalamus
IAPP	Islet amyloid polypeptide
IGF	Insulin like growth factor
IL	Interleukin
i.p	Intraperitoneally
IP_3	Inositol triphosphate
K_d	Dissociation constant
KRB	Krebs Ringer Bicarbonate
LN	Lead nitrate
mCPP	(3-chlorophenyl) piperazine
MAPK	Mitogen-activated protein kinase
MIF	Macrophage migration inhibiting factor
MuMLV	Murine moloney leukemia virus reverse transcriptase
NADH	Nicotinamide adenine dinucleotide, reduced form

NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NE	Norepinephrine
NO	Nitric oxide
P	Level of significance
PACAP	Pituitary adenylate cyclase activating protein
PBS	Phosphate buffered saline
PDGF	Platelet Derived Growth Factor
PDX-1	Pancreas duodenum homeobox gene-1
PEG	Polyethylene glycol
PH	Partially hepatectomised
PIP_2	Phosphatidylinositol-4,5-biphosphate
PKC	Protein kinase C
PLC	Phospholipase C
POD	Peroxidase
PTX	Pertussis toxin
PRL	Prolactin
RIA	Radioimmunoassay
RT-PCR	Reverse-transcription-polymerase chain reaction
S.E.M.	Standard error of mean
SMC	Smooth muscle cells
SV40	Simian virus 40
STAT	Signal transducer and activator of transcription
T_3	Tri-iodo thyronine
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TR	Thyroid hormone receptor
VIP	Vasoactive intestinal peptide

CONTENTS

INTRODUCTION

Brain neurotransmitters through their receptors or hormonal pathway can regulate physiological functions in diabetes and cell proliferation. Serotonin, also known as 5-hydroxytryptamine (5-HT), is a monoamine neurotransmitter found in cardiovascular tissue, the peripheral nervous system, blood cells and the central nervous system. 5-HT has been implicated in the regulation of diverse physiological processes, including cellular growth and differentiation (Tecott *et al.*, 1995), neuronal development (Eaton *et al.*, 1995) and regulation of blood glucose concentration (Smith & Pogson, 1977). Since the early seventies, the hypothesis for a control of circulating glucose and insulin levels by 5-HT system has been the matter of numerous works. There are reports on reduction in central nervous system (CNS) 5-HT synthesis and turn over in chronically hyperglycaemic rats (Trulson *et al.*, 1978). Specific 5-HT receptor agonists and antagonists can modulate circulating levels of blood glucose in rodents. 5-HT promotes hyperglycaemia by a mechanism that may involve increased renal catecholamine release (Wozniak & Linnoila, 1991). There is a prevailing view that blood glucose is lowered by 5-HT and that this response can be suppressed by 5-HT receptor antagonists (Furman & Wilson, 1980). All these evidences show that 5-HT has a role in the regulation of insulin secretion.

Recent observations indicate that insulin can stimulate pancreatic β-cell growth *in vivo*. The level to which β-cell proliferation increased is related to the degree to which insulin biosynthesis and/or release is enhanced (Chick *et al.*, 1975). Pancreatic regeneration after pancreatectomy has been well documented in animal models to study β-cell proliferation (Pearson *et al.*, 1977). Various hormones and growth factors have been shown to affect the proliferation of the endocrine and exocrine cell types of pancreas. The addition of new β-cells would increase the total insulin secretary potential. Studies have shown that insulin secretion is modulated by

the central nervous system, through its sympathetic and parasympathetic division, although glucose and other substrates are generally thought to be the principal regulators of insulin secretion. The pancreatic islets are richly innervated by parasympathetic, sympathetic and sensory nerves (Miller, 1981). Several different neurotransmitters are stored within the terminals of these nerves - serotonin, acetylcholine, noradrenaline and several neuropeptides. Stimulation of the autonomic nerves and treatment with neurotransmitters affect islet hormone secretion.

Most of the neurons synthesizing 5-HT in CNS are located in raphe nuclei of brain stem, but serotonergic nerve terminals can be found in virtually every brain region. These 5-HT containing cells give rise to ascending and descending pathways that innervate large areas of the brain and spinal cord. These pathways largely mediate the varied roles of 5-HT in sensory, motor and autonomic functioning. Dorsal motor nucleus of brain stem is connected to the endocrine pancreas exclusively via vagal fibres and has a role in neurally mediated insulin release (Azmitia & Gannon, 1986)

The effect of 5-HT is mediated in different tissues by different subclasses of receptors, each of which are coded by a distinct gene and possesses distinct pharmacological properties and physiological functions. In addition to its role as a neurotransmitter, 5-HT also has been shown to play a role in cell proliferation (Seuwen & Poussegur, 1990). The mitogenic action of 5-HT was first identified in bovine aortic smooth muscle cells (Nemeck *et al.*, 1986). There is a synergistic effect of 5-HT with traditional protein growth factors such as platelet derived growth factor, fibroblast growth factor, epidermal growth factor and insulin like growth factor (Crowley *et al.*, 1994). In aortic smooth muscle cells, 5-HT induced mitogenesis was comparable with that of human platelet derived growth factor. 5-HT rapidly elevates superoxide formation, stimulates protein phosphorylation, and

enhances proliferation of bovine pulmonary artery smooth muscle cells (SMCs). 5-HT_{1A} receptor is coupled through Gi to the activation of ERK1 and ERK2 in CHO cells (Daniel *et al.*, 1996). 5-HT has been increasingly recognised to be a mitogen for both vascular and non-vascular cells. 5-HT rapidly induces tyrosine phosphorylation of GTPase activating protein (GAP), possibly activates p21ras and produces cellular hyperplasia and hypertrophy in bovine pulmonary artery. All agents that block transport of 5-HT and 5-HT receptor antagonists inhibit proliferative response.

5-HT receptors can be classified into seven classes from 5-HT_1 to 5-HT_7 based upon their pharmacological profiles, cDNA-deduced primary sequences and signal transduction mechanisms of receptors (Hoyer *et al.*, 1994). The 5-HT_{1A} receptor was reported to exist in two isoforms in rat brain regions, i.e., a high affinity 5-HT_{1A} receptor and a low affinity 5-HT_{1A} receptor. These two isoforms can be labelled by high and low concentrations of [^{3}H]8-OH DPAT (Nenonene *et al.*, 1994). 8-Hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) is a 5-HT_{1A} receptor-selective agonist that has recently been reported to trigger adrenal catecholamine release and hyperglycaemia by the activation of 5-HT_{1A} receptors (Chauloff & Jeanrenaud, 1987). The EPI releasing effect of 8-OH DPAT are blocked by both 5HT_{1A} and β-adrenoreceptor antagonist (-)-pindolol (Chauloff *et al.*, 1990b). Neurotransmitters that elevate cAMP inhibit cell proliferation. 5-HT_{1A} receptor agonists promote adenylyl cyclase activity by activation of stimulatory G proteins (Gs), inhibit proliferation (Fanburg & Lee, 1997). Neurite outgrowth is inhibited by micromolar amounts of 5-HT added to dissociated cultures of cortical or raphe neurons. 5-HT_1 agonists could potentially be providing long term repression of G protein coupled receptors and other MAP kinase-responsive genes. There are also reports regarding 5-HT_{1A} receptor mediated stimulation of cell division in different cell types. The human 5-HT_{1A} receptor expressed in Chinese hamster ovary cells

promotes activation of ERK1 and ERK2 (Daniel *et al.*, 1996). 5-HT_1 mediates inhibitory signaling pathways primarily in neuroendocrine cells, and stimulatory pathways are mostly restricted to mesenchymal and/or immune cells, this cell specificity appears not to be absolute. In hippocampal membranes, the 5-HT_1 receptor mediates increased rather than reduced cAMP levels, presumably via activation of adenylyl cyclase type II (ACII). Furthermore, an inhibitory signal, such as a decrease in cAMP, might lead to a stimulation of cell proliferation in mesenchymal cells. Inhibitory effects of 5-HT have been linked to activation of 5-HT_{1A} receptors.

5-HT_{2C} receptor is one of the three closely related receptor subtypes in 5-HT_2 receptor family. Administration of 5-HT_{2C} receptor agonist, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) triggers adrenal catecholamine release and hyperglycaemia. The hyperglycaemic effect of DOI administration is mediated by centrally located 5-HT_{2C} receptors and, in turn, adrenal epinephrine release due to increase in sympathetic nerve discharge (Welch & Saphier, 1994). This suggests that insulin release is inhibited by 5-HT_{2C} receptor agonist and the activation of 5-HT receptors may affect glycaemia. The 5-HT_2 receptor subtype of 5-HT has been shown to mediate cell growth in fibroblasts. 5-HT enhances EGF-stimulated DNA synthesis of mature rat hepatocytes in primary culture and this effect of 5-HT is suggested to be mediated by the 5-HT_2 receptors. *In vivo* studies indicated that the 5-HT_2 receptors were activated in the regenerating rat liver during DNA synthetic phase. These receptors are coupled to phosphoinositide turnover and diacylglycerol formation, which activates protein kinase C, an important second messenger for cell division. The 5-HT_{2C} receptors activate phospholipase. This receptor functions as a protooncogene when expressed in NIH 3T3 fibroblasts (Julius *et al.*, 1989). The

presence of 5-HT_{2C} receptors may initiate tumorigenesis by facilitating the growth of fibroblasts in the mouse.

Several studies have described the role of 5-HT_{1A} and 5-HT_{2C} receptors in neuroendocrine regulation and cell proliferation. The involvement of these receptors in the regulation of catecholamine release by facilitating sympathetic system has been examined. However, there have not been many studies examining the role of central 5-HT_{1A} and 5-HT_{2C} receptors and their relationship between sympathoadrenal secretions and insulin secretion during pancreatic regeneration. In the present study, the changes in the brain and pancreatic 5-HT, its receptor subtypes, and their gene expression were investigated during pancreatic regeneration in rats. The work focuses on the role of 5-HT_{1A} and 5-HT_{2C} receptor changes and their regulatory role in pancreatic islet cell proliferation.

OBJECTIVES OF THE PRESENT STUDY

1. To induce pancreatic regeneration by partial pancreatectomy in weanling rats.
2. To study the DNA synthesis by [^{3}H]thymidine incorporation during pancreatic regeneration.
3. To study the changes in 5-HT content in various rat brain regions – cerebral cortex (CC), brain stem (BS) and hypothalamus (Hypo) during pancreatic regeneration using High Performance Liquid Chromatography.
4. To study the changes in epinephrine and norepinephrine content in plasma and adrenals during pancreatic regeneration using High Performance Liquid Chromatography.
5. To study the 5-HT content in plasma and pancreas of experimental rats.
6. To study the 5-HT_{1A} and 5-HT_{2C} receptor changes in CC, BS, Hypo and in the pancreas of different experimental groups of rats.
7. To study the effect of 5-HT, 5-HT_{1A} and 5-HT_{2C} receptor ligands in insulin secretion using rat primary islet culture.
8. To study the effect of 5-HT, 5-HT_{1A} and 5-HT_{2C} receptor ligands in DNA synthesis using rat primary islet culture.
9. To study the gene expression of 5-HT_{1A} and 5-HT_{2C} receptors in the brain and pancreas of different experimental groups of rats.

LITERATURE REVIEW

The pancreas is a complex organ composed of two different cell populations, exocrine and endocrine. 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 mantel of three other cell types, *i.e.*, glucagon-secreting - alpha cells, somatostatin secreting - delta cells and pancreatic polypeptide secreting cells (PP-cells).

The endocrine pancreas is derived from progenitor cells in the ducts of the exocrine portion of the pancreas. The pancreatic β-cell is characterized by a limited proliferative potential in man (Hellerstrom *et al.*, 1985). In animals there are, however indications of regenerative phenomena in the β-cell (Logothetopoulos, 1972). Studies showed that the rat pancreatic islet cells in allogeneic grafts are capable of regeneration (Socha *et al.*, 2003). The use of partial pancreatectomy as a research tool in studying pancreatic regeneration is well established and dates back to studies at the turn of the century. Pancreatectomy, or removal of pancreas, is a very useful approach to demonstrate the regenerative potential of β-cells. 60% partial pancreatectomy does not result in glucose intolerance or permanent diabetes. This maintenance of glucose homeostasis is due to regeneration among the remaining pancreatic β-cells (Leahy *et al.*, 1988; Lohr *et al.*, 1989). However, when 85-90% partial pancreatectomy is performed, mild hyperglycaemia ensues which is followed

by increased β-cell replication and a 40% increased β-cell mass (Bonner-Weir, 1983). Regeneration of endocrine cells started immediately after 90% pancreatectomy (Hayashi *et al.*, 2003). Interestingly, 95% pancreatectomy results in severe hyperglycaemia with non-existent or very minor signs of β-cell replication (Clark *et al.*, 1982). Based on the pancreatectomy models, it is evident that β-cells have a certain regenerative capacity.

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 *et al.*, 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.

NEURAL INNERVATION OF THE PANCREAS

The endocrine pancreas is richly innervated, but the abundance and organization of this innervation are highly variable between species (Kobayashi & Fujita, 1969). Most of the nerve fibres enter the pancreas along the arteries (Woods & Porte Jr. D, 1974; Miller, 1981). Well differentiated synapses with islet cells have rarely been observed (Orci *et al.*, 1973; Watanabe & Yasuda, 1977). The innervation of the islet is very plastic, as suggested by the observation that islets transplanted in

the portal vein of diabetic rats became reinnervated by hepatic nerves (Gardemann *et al.*, 1994)

The autonomic innervation of the endocrine pancreas has several origins. The autonomic nervous system uses two interconnected neurons to control effector functions and is divided into two systems, the sympathetic and the parasympathetic nervous systems, according to the location of the preganglionic cell bodies. However, there are indications suggesting that these two systems are not always independent of each other, but display anatomical interactions (Berthoud & Powley, 1993) or share similar neurotransmitters (Sheikh *et al.*, 1988; Verchere *et al.*, 1996). The endocrine pancreas also receives other types of nerves. These nerves are of peptidergic and nonpeptidergic nature (Brunicardi *et al.*, 1995; Ahrén, 2000).

The parasympathetic innervation

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. D, 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).

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: norepinephrine, 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).

CENTRAL NERVOUS SYSTEM REGULATION OF INSULIN SECRETION

The autonomic nervous system, acting through both its sympathetic and parasympathetic branches, has the potential to modulate the rate of insulin secretion over a wide range at a constant intermediate glucose concentration. 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 norepinephrine decreased glucose-induced insulin secretion. Sympathetic inhibition was observed at glucose concentrations greater than 5mM (Campfield *et al.*, 1976; Campfield *et al.*, 1980).

The recent 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). Also it shows the importance of central nervous system neurotransmitters in the pancreatic hormone secretion and their importance in glucose homeostasis.

Central nervous system borne hyperglycaemia is mediated via central noradrenergi pathways (McCaleb & Myers, 1982) by an activation of sympathoadrenal system Plasma glucose appears to be under separate serotonergic and dopaminergic contro exerted via 5-HT_{1A} and DA_{D3} receptors respectively (Alster & Hillegaart, 1996).

It is well established that the autonomic fibres supplying the pancreas trave via the vagus and splanchnic nerves (Helman *et al.*, 1982). These nerves are clearl related to the ventral hypothalamus. The hypothalamus plays a central role in th integration of neurohormonal function (Oommura & Yoshimatsu, 1984). The ventro medial hypothalamic nucleus is considered as the sympathetic centre and th stimulation of this area decreases insulin secretion (Helman *et al.*, 1982). Lesions i the ventro-medial hypothalamus, resulted in behavior alterations and morphologica changes in pancreatic islets (Scalfani, 1981). Ventro-lateral hypothalamus is th parasympathetic centre, stimulation of which increases the circulating level of insuli (Helman *et al.*, 1982). Lesions in ventro-lateral hypothalamus results in decrease body weight; food intake, plasma insulin levels and decrease in islet size (Powley & Opsahl, 1976). Hyperactivation of the HPA axis in diabetes is associated wit increased expression of hypothalamic corticotrophin-releasing hormone (CRH mRNA and hippocampal mineralocorticoid receptor (MR) mRNA (Chan *et al.*, 200

FACTORS AFFECTING INSULIN SECRETION FROM PANCREATI β-CELLS

Glucose

Insulin is secreted primarily in response to elevated blood gluco concentrations. The mechanism of glucose induced insulin release is not complete understood. Phosphorylation of glucose to glucose-6-phosphate serves as the ra limiting step in glucose oxidation (Schuit, 1996). Glucokinase acts as a gluco sensor during this process. The entry of glucose into β-cells is followed by

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). Glucose induced insulin secretion is also partly dependent upon the activation of typical isoforms of protein kinase C (PKC) within the β-cell (Harris *et al.*, 1996). Although intracellular Ca^{2+} activates protein kinases such as Ca^{2+} and calmodulin dependent protein kinase (Breen & Aschroft, 1997), it remains unclear how increases in intracellular Ca^{2+} leads to insulin release. 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 *et al.*, 1994).

Amino acids

Many amino acids increase insulin secretion. Amino acids like arginine increase insulin secretion from pancreatic β-cells (Holstens *et al.*, 1999). 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 and glucagon in mice. L-Tryptophan which is the precursor of 5-HT can act as a stimulator of insulin release (Bird *et al.*, 1980)

Fatty acids

Free fatty acids act as signaling molecules in various cellular processes, including insulin secretion (Haber *et al*, 2003). Short chain fatty acids and their derivatives are highly active stimulators of insulin release in sheep (Horino *et al.*, 1968). 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).

Glucagon

Glucagon is secreted by the α-cells of the pancreatic islets. It has been shown that glucagon has a striking stimulation of insulin release in the absence of glucose (Sevi & Lillia, 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 *et al.*, 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 *et al.*, 1996).

Substrates derived from nutrients

Substrates like pyruvate (Lisa *et al.*, 1994), citrate, ATP (Tahani, 1979), NADH and NADPH (Iain *et al.*, 1994) may involve indirect reflux stimulation triggered by food intake or local islet stimulation through the production of metabolites. Adenosine diphosphate 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 *et al.*, 1987). Glucose induced insulin secretion

is accompanied by an increase in the islet content of cAMP (Rabinovitch *et al.*, 1976).

Somatostatin

This hormone is secreted by the pancreatic δ-cells of the islets of Langerhans. Somatostatin inhibits insulin release (Ahren *et al.*, 1981). Its action is dependent on the activation of G-proteins but not associated with the inhibition of the voltage dependent Ca^{2+} currents or adenylate cyclase activity (Renstrom *et al.*, 1996).

Pancreastatin

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

Amylin

Amylin is a 37-amino acid peptide hormone co-secreted with insulin from pancreatic β-cells. Amylin appears to control plasma glucose via several mechanisms that reduce the rate of glucose appearance in the plasma. Amylin limits nutrient inflow into the gut to blood and by its ability to suppress glucagon secretion. It is predicted to modulate the flux of glucose from liver to blood. Amylin is absolutely or relatively deficient in type I - diabetes and in insulin requiring type II - diabetes (Young, 1997). Islet amyloid polypeptide (IAPP) or amylin 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 *et al.*, 1994). Pancreatic islets amylin play a role in isl enlargement, an important issue in the progression towards overt diabetes (Wooke & Cooper, 2001)

Adrenomedullin

Adrenomedullin is a novel hypotensive adrenal polypeptide isolated from human phaeochromocytoma and is structurally related to calcitonin gene relate peptide and islet amyloid polypeptide. It has been suggested that besides being a adrenal hypotensive peptide, adrenomedullin may be a gut hormone with potentia insulinotropic function (Mulder *et al.*, 1996).

Galanin

Galanin is a 29 amino acid neuropeptide localised in the intrinsic nervou system of the entire gastrointestinal tract and the pancreas of man and several anima species (Scheurink *et al.*, 1992). Among other functions galanin inhibits insuli release (Ahren *et al.*, 1991), probably via activation of G-proteins (Renstrom *et al.* 1996) by the mediation of activated galanin receptors. However, galanin receptor are not as effective as α_2-adrenergic receptors in activating G-proteins.

Macrophage migration inhibitory factor (MIF)

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 *et al.*, 1997).

Other agents

Coenzyme Q_{10} improved insulin release (Conget *et al.*, 1996) and it may also have a blood glucose lowering effect. Inositol hexa bisphosphate stimulates non-Ca^{+} mediated and purine-Ca^{2+} mediated exocytosis of insulin by activation of protein kinase C. (Efanov *et al.*, 1997). Small GTPases of the rab 3A family expressed in insulin secreting cells are also involved in the control of insulin release in rat and hamster (Regazzi *et al.*, 1996).

ROLE OF NEUROTRANSMITTERS IN INSULIN REGULATION

Epinephrine and Norepinephrine

Various neurotransmitters like NE, GABA, 5-HT, DA and ACh have important role in cell proliferation and insulin secretion (Paulose *et al.*, 2004). Epinephrine and Norepinephrine are secreted by the adrenal medulla. Norepinephrine (NE) is a principal neurotransmitter of sympathetic nervous system. These hormones inhibit insulin secretion, both *in vivo* and *in vitro* (Renstrom *et al.*, 1996; Porte, 1967). Epinephrine exerts opposite effects on peripheral glucose disposal and glucose stimulated insulin secretion (Avogaro *et al.*, 1996). NE and EPI, the flight and fright hormones are released in all stress conditions and are the main regulators of glucose turnover in strenuous exercise (Simartirkis *et al.*, 1990). In severe insulin-induced hypoglycaemia, a 15 to 40-fold increase of epinephrine plays a pivotal role in increasing glucose production independently of glucagon (Gauthier *et al.*, 1980). It is already known that, when used in high doses *in vivo* or

in vitro, epinephrine reduces the insulin response to stimulators (Malaisse, 1972). EPI and NE have an antagonistic effect on insulin secretion and glucose uptake (Porte *et al.*, 1966 a, b). They also inhibit insulin -stimulated glycogenesis through inactivation of glycogen synthase and activation of phosphorylase with consequent accumulation of glucose-6-phosphate. In addition, it has been reported that epinephrine enhances glycolysis through an increased activation of phospho-fructokinase. In humans, adrenaline stimulates lipolysis, ketogenesis, thermogenesis and glycolysis and raises plasma glucose concentrations by stimulating both glycogenolysis and gluconeogenesis. Adrenaline is, however, known to play a secondary role in the physiology of glucose counter-regulation. Indeed, it has been shown to play a critical role in one pathophysiological state, the altered glucose counter-regulation in patients with established insulin-dependent diabetes mellitus (Cryer, 1993). The inhibitory effect of EPI upon insulin secretion induced by glucose was reported by Coore and Randle (Coore *et al.*, 1964), who incubated pancreatic tissue from the rabbit. As judged by Malaisse *et al*, the inhibitory effect of EPI on glucose-induced insulin secretion is mediated through the activation of α-adrenoreceptors (Malaisse *et al.*, 1967). Adrenaline inhibits insulin release through α_{2A} - and α_{2C} - adrenoreceptors via distinct intracellular signaling pathways (Peterhoff *et al.*, 2003).

Acetylcholine

Acetylcholine is one of the principal neurotransmitters of the parasympathetic system. Acetylcholine increases insulin secretion (Tassava *et al.*, 1992) through vagal muscarinic and non-vagal muscarinic pathways (Greenberg *et al.*, 1994). They function through muscarinic receptors present on pancreatic islet cells (Ostenson *et al.*, 1993). Central muscrainic M1 and M3 receptors are involed in

the regulation of insulin secretion from pancreatic β-cells during pancreatic regeneration (Paulose & Renuka, 2004).

Dopamine

High concentrations of dopamine in pancreatic islets can decrease glucose stimulated insulin secretion (Tabeuchi *et al.*, 1990). L-DOPA the precursor of dopamine had similar effect to that of dopamine (Lindstrom *et al.*, 1983). Dopamine D_3 receptors are implicated in the control of blood glucose levels (Alster *et al.*, 1996). Dopamine D_1 receptors have also been reported to be present on pancreatic β-cells (Tabeuchi-*et al.*, 1990). These clearly indicate the role of dopamine in the regulation of pancreatic function.

γ-Aminobutyric acid

Gamma aminobutyric acid (GABA) is the main inhibitory neurotransmitter in central nervous system. GABA is reported to present in the endocrine pancreas at concentrations comparable with those found in central nervous system. The highest concentration of GABA within the pancreatic islet is confined to β-cells (Sorenson *et al.*, 1991). Glutamate decarboxylase, the primary enzyme that is involved in the synthesis of GABA, has been identified as an early target antigen of the T-lymphocyte mediated destruction of pancreatic β-cells causing insulin-dependent diabetes mellitus (Baekkeskov *et al.*, 1990). GABA through its receptors has been demonstrated to attenuate the glucagon and somatostatin secretion from pancreatic α-cells and δ-cells respectively (Gaskins *et al.*, 1995). GABA which is present in the cytoplasm and in synaptic-like microvesicles (Reetz *et al.*, 1991) is co-released with insulin from β-cells in response to glucose. The released GABA inhibits islet α-and δ-cell hormonal secretion in a paracrine manner. During diabetes the destruction of β-cells will lead to decrease in GABA release resulting in the enhancement of

glucagon secretion from α-cells leading to hyperglycaemia. The brain GABAergic mechanisms also play an important role in glucose homeostasis. Inhibition of central $GABA_A$ receptors increases plasma glucose concentration (Lang, 1995). Thus, any impairment in the GABAergic mechanism in central nervous system and/or in the pancreatic islets is important in the pathogenesis of diabetes.

Serotonin

Since the early seventies the hypothesis for a control of circulating glucose and insulin levels by 5-HT system has been the matter of numerous works. 5-HT content is increased in the brain regions and hypothalamic nuclei (Chen *et al.*, 1991; Lackovic *et al.*, 1990), but there are reports suggesting a decrease in brain 5-HT content during diabetes (Jackson *et al.*, 1999; Sumiyoshi *et al.*, 1997; Sandrini *et al.*, 1997). Ohtani *et al.*, 1997 have reported a significant decrease in extracellular concentrations of NE, 5-HT and their metabolites in the ventro medial hypothalamus (VMH). The ratio of 5-HIAA/5-HT was increased. A similar observation was reported by (Ding *et al.*, 1992) with a decrease in 5-HT in cortex (19%) and 5-HT turnover (5-HIAA/5-HT) that increased by 48%. Chu *et al* (1986) has reported lower 5-HT levels in both hypothalamus and brain stem but not in corpus striatum. Insulin treatment brought about an increase in the cerebral concentration of 5-HIAA and accelerated the cerebral 5-HT turnover (Juszkiewicz, 1985). The 5-HIAA concentration was reported to be approximately twice as high as the controls regardless of duration of treatment. Brain tryptophan, the precursor of 5-HT, was also reduced in brain regions during diabetes (Jamnicky *et al.*, 1991). Insulin treatment was reported to reverse this reduced tryptophan content to normal (Jamnicky *et al.*, 1993).

5-HT IN THE PANCREATIC ISLETS

In the pancreas, 5-HT is mainly present in β-cells of the islet of Langerhans (Ekholm *et al.*, 1971; Cetin, 1992). 5-HT containing nerves were also observed in the periacinar and periinsular regions of normal pancreas. 5-HT may help in the maintenance of the blood sugar level in normal pancreas by increasing insulin secretion and decreasing glucagon secretion (Adeghate *et al.*, 1999). In response to 5-HT receptor activation, both decreased and increased insulin levels, respectively with hyperglycaemia and hypoglycaemia, have been reported in animals (Bjorkstrand, 1996; Chaouloff *et al.*, 1987c). Insulin resistance has been reported to vary inversely with brain serotonergic activity (Horacek *et al.*, 1999), and genetic variation in two 5-HT receptors has been associated with abdominal obesity and diabetes (Yuan *et al.*, 2000). Pancreatic islets receive innervation from both divisions of the autonomic nervous system, and pancreatic endocrine secretion is partly controlled by the autonomic nervous system (Holst *et al.*, 1986). Pharmacological manipulations of pancreatic islet 5-HT and dopamine content *in vitro* and *in vivo* systems have resulted in evidence for monoaminergic inhibition (Feldman *et al.*, 1972) and stimulation of insulin secretion (Telib *et al.*, 1968). Several amino acids are able to synthesis insulin release in the presence of glucose. 5-HTP is readily taken up into the islet in the presence of glucose and stimulates insulin secretion. But the enzyme 5-hydroxytryptophan decarboxylase readily converts it into 5-HT that inhibits insulin secretion (Sundler *et al.*, 1990). When 5-HTP was tested in conjunction with a decarboxylase inhibitor, the glucose stimulated insulin release from rabbit pancreas was significantly enhanced (Gylfe *et al.*, 1973). Tryptophan a precursor of 5-HTP, has a stimulating effect on insulin release from hamster pancreas. The presence of monoamine oxidase enzyme that catabolises 5-HT within the β-cells shows an effective 5-HT metabolism within the islets (Feldman & Chapman, 1975). 5-HT can also act as a marker for insulin secretion. 5-HT is

taken up into insulin granules and co-released with insulin on stimulation of pancreatic β-cells by glucose (Zhou & Misler, 1996).

PANCREATIC REGENERATION AND β-CELL GROWTH

The adult pancreas has a capacity to respond to changing physiological needs such as the requirement for increased β-cell mass/function during pregnancy, obesity or insulin resistance and an ability to regenerate cells including β-cells that has been convincingly demonstrated in animal models of pancreatic injury and diabetes (Rosenberg, 1995 & 1998). Animal models in which pancreatic endocrine and exocrine regeneration can be observed include chemically induced models of pancreatic injury following administration of alloxan (Davidson *et al.*, 1989; Waguri *et al.*, 1997), streptozotocin (Like &-Rossini, 1976) or cerulein (Elsasser *et al.*, 1986) and hemipancreatectomy (Bonner-Weir *et al.*, 1993; Sharma *et al.*, 1999). Although the triggers may differ, in each of these models pancreatic regeneration is thought to occur through the expansion of progenitor cells present either in, or closely associated with, the ductal epithelium. In these models, both endocrine and exocrine cells have been observed to arise from duct cells (Waguri *et al.*, 1997; Bonner-Weir *et al.*, 1993). Supporting this observation, 'transitional' cells have been identified that co-express ductal markers with endocrine or exocrine cell-specific markers, suggesting a reprogramming of duct-like cells (Wang *et al*, 1985). In the 90% pancreatectomy model, regeneration has been suggested to mimic embryonic pancreogenesis with proliferation occurring initially from expansion of the common pancreatic duct epithelium followed by branching of smaller ductules and subsequent regeneration of exocrine, endocrine and mature duct cells (Bonner-Weir *et al.*, 1993)

Islet cells in regeneration

The endocrine cell mass in the adult pancreas is maintained through a slow turnover of cells involving a balance of replication from existing differentiated cells, apoptosis and neogenesis from less-differentiated progenitor cells. Morphometric analysis, combined with mathematical modelling, has shown that the turnover of adult rat β-cells is 1 to 4% per day (Bonner-Weir, 2000a; Finegood *et al.*, 1995). In situations of increased demand, this rate may be increased through changes in the rate(s) of replication, apoptosis or neogenesis. Although there is little evidence for islet-derived progenitors, mitotic analysis indicates that islet cells contribute to the regeneration observed in animal models of diabetes and pancreas injury. Islet cells may increase their rate of replication in times of stress (Waguri *et al.*, 1997), although this is usually accompanied by neogenesis that appears to derive from the ducts (Waguri *et al.*, 1997). Three-dimensional reconstruction of histological sections has revealed that all cells within rat islets are 'differentiated', inferring that there is not an easily discernible, and discrete progenitor cell population in the islet (Bonner-Weir, 2000b). While this does not necessarily preclude the possibility that a sub-population of 'differentiated' islet cells possesses a more multipotent phenotype or retains the capacity to de-differentiate and assume a new fate, there is presently little data to support this. Some evidence for islet-derived progenitors is provided by three studies in which β-cells apparently reverted to a more primitive insulin- Pdx1+ phenotype. When cultured as a monolayer (Beattie *et al.*, 1999) adopted a duct-like phenotype in a collagen matrix (Yuan *et al.*, 1996), streptozotocin-treated, normoglycaemic mice, exhibited enhanced neogenesis (Guz *et al.*, 2001).

Mechanism of β-cell growth

Beta-cell growth is a cumulative effect of the following three phenomena during β-cell development (i) differentiation of β-cells from precursors, a process

referred to as neogenesis (ii) changes in the size of individual β-cells and (ii replication capacity of existing β-cells (Swenne, 1992). The relative contribution replication, neogenesis or increased β-cell size to the increased β-cell mass is n very clear at this time. The ability of the pancreas to regenerate and the effects trophic hormones on regeneration of the pancreas after partial pancreatectomy are n completely understood. There is strong evidence to the existence of neogenesis as plausible mechanism for changes in β-cell mass based on studies in rat mode (Swenne, 1982; Swenne & Eriksson, 1982). In contrast, changes in size of individu β-cells is not very well documented, even though, glucose, which is the prim stimulator of β-cell replication, increases β-cell size and apparently leads to increase insulin synthesis (Hakan Borg *et al.*, 1981). Several studies pioneered b Hellerström and Bonner-Weir have lead to an improved understanding c mechanisms associated with β-cell proliferation (Bonner-Weir 1994; Hellerstrom 1984). Swenne performed the initial cell cycle characterization of β-cells and pave the way for further investigations into the replication capacity of β-cells. Islet ce replication has been determined by standard thymidine incorporation assays an more recently using antibody-based bromodeoxyuridine assays.

Upon receiving stimulatory influences from either cytokines or growt factors, mammalian cells undergo a regulated cell cycle progression. Every phase q the cell cycle is under regulatory influences of different cell cycle proteins. Change in cell cycle progression modulate the rate of proliferation and growth. Moreove the decision made by a cell to exit the cell cycle to undergo an irreversible post mitotic differentiation state or a state of irreversible cellular senescence is dictated b changes in the cell cycle. Finally, the decision of putting an end to the cellular life span by undergoing apoptosis is also a reflection of decisions made by protein regulating the cell cycle machinery (Sherr, 1996; Grana *et al.*, 1995). The cell cycl

is typically divided into the following phases, G0 (reversible quiescence), G1 (first gap phase), S (DNA synthesis), G2 (second gap phase) and M (mitosis).

Pancreatic β-cells, similar to other cell types, pass through several distinct phases of the cell cycle. Studies pioneered by Ingemar, Swenne, Claes and Hellerström have elucidated the replication capacity of β-cells. Swenne maintained β-cell enriched fetal rat pancreatic islets in tissue culture at various glucose concentrations (Swenne, 1982). The observations prompted two inferences, (a) glucose stimulated β-cell proliferation by increasing the number of cells entering the cell cycle and (b) only a limited fraction of the total β-cell population is capable of entering the active cell cycle. Furthermore, these studies allowed an estimation of the rate of new β-cell formation per 24 hrs, which indicated that 4.2% new β-cells were formed in the presence of 2.7 mM glucose, whereas, 10.4% new β-cells were formed in the presence of 16.7 mM glucose. Furthermore, an age-dependent study of cell cycle progression of β-cells isolated from fetal, 1-week, 3-week and 3-month old rats revealed that the cell cycle was similar in all age groups (Swenne, 1983).

The growth of β-cells is determined by the number of β-cells entering the cell cycle rather than changes in the rate of the cycle. The β-cell passes through the cell cycle at a relatively high rate but the fraction of proliferating cells is low. During fetal life, the β-cell exhibits a poor insulin response to glucose. In late fetal life, glucose is a strong stimulus to β-cell replication and the metabolism of glucose is a pre-requisite for this process. Glucose stimulates proliferation by recruiting β-cells from a resting G0 state, into the proliferative compartment composed of cells in an active cell cycle. The drastic reduction of β-cell proliferation with increasing age is, most likely, due to a gradual withdrawal of cells from the active cell cycle into an irreversible G0 state. However, the observations that a very small fraction of β-cells are capable of entering the cell cycle argues that β-cells have replication potential.

This fraction can be potentially increased by recruitment of β-cells, which are in the quiescent G0 phase to re-enter the cell cycle and undergo replication.

Brelje *et al* (1994) studied the regulation of islet β-cell proliferation in response to prolactin (PRL). Insulin secretion and β-cell proliferation increased significantly in neonatal rat islets in response to prolactin. Initial PRL mitogenic stimulus occurred by a limited procurement of non-dividing β-cells into the cell cycle followed by majority of the daughter cells proceeding directly into additional cell division cycles. The maximal PRL stimulatory affect was maintained by a continued high rate of recruitment of β-cells into the cell cycle with only about one-fourth of the daughter cells continuing to divide. This study suggested that instead of a limited pool of β-cells capable of cell division, β-cells are transiently entering the cell cycle and dividing infrequently in response to PRL, indicating that the majority of β-cells are not in an irreversible G0-phase. This observation partly contradicts the initial islet cell cycle studies and prompts a careful analysis of the cell cycle machinery active in β-cells.

The re-entry of resting β-cells into the active cell cycle requires the knowledge of proteins involved in regulation of cell cycle progression of β-cells. At this time, we have very little knowledge of the molecules which determine the cell cycle kinetics of β-cells. The low proliferative capacity of β-cells has also been proposed to result from a low expression of p34CDC2 Serine/threonine kinase and cyclin B1 which are necessary for normal progression of the cell cycle (Mares *et al.*, 1993). Several other studies highlight the role of cell cycle proteins in controlling the replication capacity of β-cells. Expression of growth promoting genes such as SV40 large T antigen and the oncogenes v-src, myc and ras have been altered either in transgenic mice or in islet cells in culture. Transgenic mice with insulin promoter driven β-cell specific expression of SV40 large T antigen developed insulinomas (Hanahan, 1985). However, the observation that expression of large T antigen was

not sufficient to form β-cell tumors indicated that transformation of β-cells is a rare event requiring multiple co-operating mutations. β-cells derived from the SV40 large T antigen transgenic mice maintained elevated DNA synthetic rates compared with control islets in which the DNA synthesis gradually decreased with age (Teitelman *et al.*, 1988). Beta cells from transgenic mice harbored elevated levels of p53 protein, which can bind to SV40 large T antigen. The interaction is thought to inactivate the anti-proliferative activity of p53 (Marshall, 1991)

Transfection of activated v-src oncogene, a cytoplasmic tyrosine kinase, into β- cells stimulated DNA synthesis and substrate phosphorylation (Welsh *et al.*, 1988). Similarly, transfection of activated myc and ras oncogenes also led to increased rates of DNA synthesis (Welsh *et al.*, 1988). DNA synthesis in β-cells was also stimulated by overexpression 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.

FACTORS REGULATING β-CELL GROWTH

Development of pancreatic endocrine cells in the rat fetus reveals the presence of insulin-positive β-cells by gestational day 13 (Fujii, 1979). Measurement of changes in the alpha, β-and delta-endocrine cell population in post-natal rodent islets indicates a continuous increase of β-cell mass throughout post-natal life (McEvoy, 1981). Morphological quantification of endocrine cells in human fetal pancreas reveals the presence of insulin-positive β-cells by the eighth fetal week (Clark & Grant, 1983) with almost a 130-fold increase in β-cell mass between the 12th week in utero and the fifth post-natal month (Stefan *et al.*, 1983). New

pancreatic exocrine and islet cells are formed by differentiation of pre-exist embryonic ductal cells, which is referred to as neogenesis, or by replication of cells. While neogenesis is the primary mode of increase in β-cell mass dur gestation, after birth most of the β-cells are formed by replication.

Studies with rodent islets have been the basis of much of our information factors influencing β-cell replication. Among the various factors, glucose is a pri regulator of β-cell replication and is known to stimulate replication in both fetal a adult rodent islets (Hellerstrom, 1984). In addition, glucose leads to an increased cell proliferative compartment (Swenne, 1982). Insulin and IGF-1 stimulate islet cell replication in neonatal rodent pancreatic cells in culture providing evidence t insulin itself can regulate the replication capacity of β-cells in an autocrine fashi (Rabinovitch *et al.*, 1982).

This study prompted the examination of several other growth factors for th role in regulating β-cell replication (Hill *et al.*, 1998). Thus, growth hormone (GI prolactin and the related placental lactogen, IGF-1, IGF-2 and platelet-deriv growth factor (PDGF) have been recognized as stimulators of β-cell replicati (Brelje & Sorenson, 1991.). Growth hormone has been reported to stimulate the *vitro* replication of foetal, neonatal and adult rat β-cells. The stimulation replication activity resulted in an increased insulin content and secretion where t effects of GH were mimicked by prolactin and its related peptide, placental lactoge

Growth hormone elicits many of its actions by inducing local production IGFs in target cells. Studies aimed at investigating a similar paracrine pathw operative in islet cells have yielded confusing results. GH, but not gluco stimulated the release of IGF-1 from fetal and adult rat islets leading to mitogene which could be partially negated by addition of monoclonal antibodies to IGF (Swenne *et al.*, 1987). The presence of high-affinity IGF-1 receptors on β-cells a the finding that exogenous IGF-1 stimulates β-cell replication (Van Schravendi

et al., 1987), supported a concept that GH mitogenic activities might be mediated, at least in part, by a paracrine regulation involving IGF-1. This theory has been challenged by several studies, which failed to demonstrate an intermediary role for IGF-1 in mitogenic activities of GH in β-cells (Romanus *et al.*, 1985). Romanus *et al* failed to detect increased IGF-1 secretion from islets after GH stimulation. Other factors which lead to a stimulation of β-cell replication include, amino acids (Swenne *et al.*, 1980), lithium (Sjoholm *et al.*, 1992), the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) (Sjoholm, 1991a), nicotinamide (Sandler & Andersson, 1986), amniotic fluid (Dunger *et al.*, 1990) and serum (Hellerstrom & Swenne, 1985).

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, 1991b). 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.*, (1991c) 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

Neurotransmitters act as growth regulatory signals in primitive organisms, embryos and the developing nervous system. They exert these effects by activating receptors and signal transduction mechanisms similar to those used in

neurotransmission. Neurotransmitters and their receptors linked to second messengers mediate growth responses in neuronal and non-neuronal cells. Stimulation of proliferation is most often associated with activation of G-proteins negatively coupled to adenylate cyclase (Gi), or positively coupled to phospholipase C (Gq) or to pertussis toxin–sensitive pathways (Go, Gi) (Lauder, 1993).

Norepinephrine

Norepinephrine is reported to amplify the mitogenic signals of both EGF and HGF by acting through the α_1 adrenergic receptors. It induces the production of EGF and HGF at distal sites and also enhances the response to HGF at target tissues (Broten *et al.*, 1999). Norepinephrine rises rapidly in the plasma within one hour after PH (Knopp *et al.*, 1999). NE also enhances the mito-inhibitory effects of TGF-β1 on cultured hepatocytes isolated from the early stages of regeneration (Michalopoulose & DeFrancis, 1997). 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). Studies from our lab have shown that α_1.receptors expressed altered affinity in hypothalamus and brain stem of diabetic rats (Pius, 1996). α_1-adrenergic receptors are inhibitory β-adrenergic receptors are stimulatory to islet DNA synthesis (Ani Das, 2000)

Gamma amino butyric acid

Gamma amino butyric acid (GABA) is the principal inhibitory neurotransmitter of the mammalian brain. 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).

Acetylcholine

The mitogenic effect of acetylcholine has been studied in different cell types. Acetylcholine analogue carbachol stimulated DNA synthesis in primary astrocytes derived from perinatal rat brain (Ashkenazi *et al.*, 1989). Acetylcholine esterase kinetic parametes in brain stem during pancreatic regeneration in pancratectomised rats showed a decrease in the cholinergic activity (Renuka *et al.*, 2004). Acetylcholine is reported to induce proliferation of rat astrocytes and human astrocytoma cells (Guzzetti *et al.*, 1996). Muscarinic M1 and M3 receptors were up regulated in the pancreas at the time of pancreatic regeneration (Renuka, 2003).

SEROTONIN

Serotonin has been known for the last half century to influence vasoactivity and to participate in neurotransmission. More recently this compound has been recognized to cause proliferation of a variety of cells in culture, including those of vascular smooth muscle. Furthermore, the proliferative effect is synergistic with that of more conventional growth-producing polypeptides. A hypertrophic, as well as a proliferative response, has been shown to occur in some smooth muscle cells. There

is a synergistic effect of urotensin II with 5-HT on vascular smooth muscle c proliferation (Watanabe *et al.*, 2001).

5-HT is synthesised in situ from tryptophan through the actions of t enzymes tryptophan hydroxylase and aromatic L-amino acid decarboxylase. Bo dietary and endogenous 5-HT are rapidly metabolized and inactivated by monoami oxidase and aldehyde dehydrogenase to the major metabolite, 5-hydroxyindoleace acid (5-HIAA). 5-HT is produced in and released from neurons that originate with discrete regions, or nuclei, in the brain (Cooper *et al.*, 1991). Many serotoner neurons are located at the base of the brain in an area known as the raphe nucle which influences brain functions related to attention, emotion, and motivation. T axons of the neurons in the raphe nucleus extend, or project, throughout the brain numerous regions with diverse functions. These brain regions include the amygda an area that plays an important role in the control of emotions, and the nucle accumbens, a brain area involved in controlling the motivation to perform certa behaviors, including the abuse of alcohol and other drugs. In these brain regions, t axon endings of the serotonergic neurons secrete 5-HT when activated. T neurotransmitter then traverses the small space separating the neurons from ea other (i.e., the synaptic cleft) and binds to specialized docking molecules (i. receptors) on the recipient cell. The binding of 5-HT to its receptors initiates a seri of biochemical events that converts the extracellular, chemical signal into a intracellular signal in the recipient cell. 5-HT can influence mood states; thinki patterns; and even behaviors, such as alcohol drinking.

The actions of 5-HT are terminated by three major mechanisms: diffusio metabolism; and uptake back into the synaptic cleft through the actions of specif amine membrane transporter systems.

CLASSIFICATION OF 5-HT RECEPTORS

The various effects of 5-HT on the central nervous system and peripheral organs are mediated through activation of multiple types of receptors (Hoyer & Martin, 1997). 5-HT receptors can be classified into seven classes from 5-HT_1 to 5-HT_7, based upon their pharmacological profiles, cDNA-deduced primary sequences and signal transduction mechanisms of receptors (Bradley *et al.*, 1986; Zifa & Fillion, 1992). All 5-HT receptors belong to the superfamily of G-protein coupled receptors containing a seven transmembrane domain structure except 5-HT_3 receptor, which forms a ligand-gated ion channel.

5-HT_1 Receptor

At least five 5-HT_1 receptor subtypes have been recognised, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F}. All are seven transmembrane, G-protein coupled receptors (via Gi or Go), encoded by intronless genes, between 365 and 422 amino acids with an overall sequence homology of 40%. 5-HT_{1A} receptor subtype, which is located on human chromosome 5cenq11, is widely distributed in the CNS, particularly hippocampus (Hoyer *et al.*, 1994). The 5-HT_{1B} receptor is located on human chromosome 6q13 and is concentrated in the basal ganglia, striatum and frontal cortex. The receptor is negatively coupled to adenylyl cyclase. The 5-HT_{1D} receptor has 63% overall structural homology to 5-HT_{1B} receptor and 77% amino acid sequence homology in the seven transmebrane domains. The receptor is located on human gene 1p36.3-p34.3 and is negatively linked to adenylyl cyclase. 5HT_{1D} receptor mRNA is found in the rat brain, predominantly in the caudate putamen, nucleus accumbens, hippocampus, cortex, dorsal raphe and locus ceoruleus (Hoyer *et al.*, 1994). The 5-HT_{1E} receptor was first characterised in man as a [^{3}H]5-HT binding site in the presence of 5-carboxyamidotryptamine (5-CT) to block binding to the 5-HT_{1A} and 5-HT_{1D} receptors. Human brain binding studies have reported that 5-HT_{1E} receptors are concentrated in the caudate putamen with lower levels in the amygdala,

frontal cortex and globus pallidus (Hoyer *et al.*, 1994). This is consistent with the observed distribution of 5-HT_{1C} mRNA (Hoyer *et al.*, 1994). The receptor has been mapped to human chromosome 6q14-q15, is negatively linked to adenylyl cyclase and consists of a 365 amino acid protein with seven transmembrane domains. 5-HT_{1F} receptor subtype is most closely related to the 5-HT_{1E} receptor with 70% sequence homology across the 7 transmembrane domains. mRNA coding for the receptor is concentrated in the dorsal raphe, hippocampus and cortex of the rat and also in the striatum, thalamus and hypothalamus of the mouse (Hoyer *et al.*, 1994). The receptor is negatively linked to adenylyl cyclase.

5-HT$_2$ Receptor

The 5-HT_2 receptor family consists of three subtypes namely 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}. 5-HT_{2C} was previously termed as 5-HT_{1C} before its structural similarity to the 5-HT_2 family members was recognised. All three are single protein molecules of 458-471 amino acids with an overall homology of approximately 50% rising to between 70-80% in the seven transmembrane domains. All three are thought to be linked to the phosphoinositol hydrolysis signal transduction system via the α subunit of Gq protein. In human pulmonary artery endothelial cells, 5-HT_{2C} receptor stimulation causes intracellular calcium release via a mechanism independent of phosphatidylinositol hydrolysis (Hagan *et al.*, 1995). 5-HT_{2A} receptor previously termed as 5HT_2 receptor is located on human chromosome 13q14-q21 and is widely distributed in peripheral tissues. It mediates contractile responses of vascular, urinary, gastrointestinal and uterine smooth muscle preparations, platelet aggregation and increased capillary permeability in both rodent and human tissue (Hoyer *et al.*, 1994). The 5-HT_{2B} receptor located on chromosome 2q36-2q37.1 mediates contraction of the rat stomach fundus and endothelium dependent relaxation of the rat and cat jugular veins and possibly of the pig pulmonary artery, via nitric oxide release (Choi & Maroteaux, 1996). 5-HT_{2B} receptor mRNA has been detected

throughout the mouse, rat and guinea pig colon and small intestine. 5-HT_{2C} specific antibodies have recently used to show the presence of the receptor protein in the choroid plexus (highest density), and at a lower level in the cerebral cortex, hippocampus, striatum, and substantia nigra of rat and a similar distribution in man. The receptor has been mapped to human chromosome Xq24. No splice variants have been reported but the receptor is capable of post translational modification whereby adenosine residues can be represented as guanosine in the second loop to yield 4 variants.

5-HT_3 Receptor

The 5-HT_3 receptor binding site is widely distributed both centrally and peripherally and has been detected in a number of neuronally derived cells. The highest densities are found in the area postrema, nucleus tractus solitarius, substantia gelatinosa and nuclei of the lower brainstem. It is also found in higher brain areas such as the cortex, hippocampus, amygdala and medial habenula, but at lower densities. Unlike other 5-HT receptors, 5-HT_3 receptor subunits form a pentameric cation channel that is selectively permeable to Na^+, K^+ and Ca^{++} ions causing depolarisation. The 5-HT_3 receptor is a member of a superfamily of ligand-gated ion channels, which includes the muscle and neuronal nicotinic acetylcholine receptor (AchR), the glycine receptor, and the γ-aminobutyric acid type A receptor (Karlin & Akabas, 1995; Ortells & Lunt, 1995). Like the other members of this gene superfamily, the $5HT_3$ receptor exhibits a large degree of sequence similarity and thus presumably structural homology with the AchR (Maricq *et al.*, 1991).

5-HT_4 Receptor

Receptor binding studies have established that the 5-HT_4 receptor is highly concentrated in areas of the rat brain associated with dopamine function such as the striatum, basal ganglia and nucleus accumbens. These receptors are also located on

GABAergic or cholinergic interneurons and/or on GABAergic projections to substantia nigra (Patel *et al.*, 1995). The receptor is functionally coupled to th protein.

5-HT$_5$ Receptor

Two 5-HT receptors identified from rat cDNA and cloned were found have 88% overall sequence homology, yet were not closely related to any other 5- receptor family (Erlander *et al.*, 1993). These receptors have thus been classified 5-HT$_{5A}$ and 5-HT$_{5B}$ and their mRNAs have been located in man (Grailhe *et* 1994). In cells expressing the cloned rat 5-HT$_{5A}$ site, the receptor was negativ linked to adenylyl cyclase and may act as terminal autoreceptors in the mouse fro cortex (Wisden *et al.*, 1993).

5-HT$_6$ Receptor

Like the 5-HT$_5$ receptor, the 5-HT$_6$ receptor has been cloned from rat cDN based on its homology to previously cloned G protein coupled receptors. The receptor consists of 438 amino acids with seven transmembrane domains and positively coupled to adenylyl cyclase via the Gs G protein. The human gene h been cloned and has 89% sequence homology with its rat equivalent and is coupl to adenylyl cyclase (Kohen *et al.*, 1996). Rat and human 5-HT$_6$ mRNA is located the striatum, amygdala, nucleus accumbens, hippocampus, cortex and olfacto tubercle, but has not been found in peripheral organs studied (Kohen *et al.*, 1996).

5-HT$_7$ Receptor

5-HT$_7$ receptor has been cloned from rat, mouse, guinea pig and hum cDNA and is located on human chromosome 10q23.3-q24.4. Despite a high degr of interspecies homology (95%) the receptor has low homology (<40%) with other

HT receptor subtypes. The human receptor has a sequence of 445 amino acids and appears to form a receptor with seven transmembrane domains.

5-HT AND CELL PROLIFERATION

Growth-regulatory effects of 5-HT mediated by specific 5-HT receptor subtypes have been linked to a number of signal transduction pathways that either promote or inhibit cell proliferation (Lauder, 1993). 5-Hydroxytryptamine has been implicated as a potential mitogen (Seuwen & Poussegur, 1990) and was shown to have effects on morphogenesis and neuronal development (Lauder, 1990). 5-Hydroxytryptamine has been recognised to cause proliferation of a variety of cells in culture including vascular smooth muscle cells and hepatocytes (Fanburg & Lee, 1997). In pancreatic cell line, activation of pertussis toxin sensitive $5HT_{1A/1B}$ receptors stimulate proliferation through the activation of PLC and PKC that resulted in the down regulation of cAMP (Ishizuka *et al.*, 1992). In cultured rat pulmonary artery smooth mucsle cells (SMC), 5-HT induces DNA synthesis and potentiates the mitogenic effect of platelet-derived growth factor (Eddahibi *et.al.*, 1999). Studies from our lab reported the involvement of 5-HT, S2 receptors in the DNA synthesis of primary culture of rat hepatocytes (Sudha *et al.*, 1997). $5HT_{1A}$ receptor agonist 8-OHDPAT inhibited the DNA synthesis in rat hepatocytes *in vitro*. Studies using mesulergine, $5HT_{2C}$ antagonist revealed that $5HT_{2C}$ receptors are stimulatory to hepatocyte cell division (Pyroja, 2002).

5-HT_{1A} Receptor and Cell Proliferation

Signal transduction cascades following the activation of $5\text{-}HT_{1A}$ receptors were found to involve activation of the adenylyl cyclase/PKA pathway (Lambert

et al., 2001). Treatment of cells with either the 5-HT_{1A} receptor agonist, 8-OH-DPAT, or forskolin (which directly activates adenylyl cyclase) increased cellular IGF-I. These treatments increased cAMP synthesis and caused phosphorylation of the cAMP response element binding protein, CREB, an effect that was blocked by a PKA inhibitor, Rp-cAMPs. Rp-cAMPS also blocked the increases in IGF-I caused by 8-OH-DPAT or forskolin, consistent with the presence of a cAMP response element (CRE) in the IGF-I promoter (Thomas *et al.*, 1996). Taken together, these findings suggest that the activation of 5-HT receptors positively coupled to the adenylyl cyclase/PKA pathway directly promote transcription of IGF-I, an important growth factor in craniofacial development.

5-HT_{1A} is a "transiently expressed" intronless receptor, i.e., at specific times in development or during stress, very high amounts are expressed quickly. 5-HT_{1A} receptor develops early and the receptor levels can then be reduced as the cells or animal ages. The decrease in receptor number is probably due to increased 5-HT brain levels, since the 5-HT_{1A} receptor expression is sensitive to autoinhibition (Nishi & Azmitia, 1999). The transduction action of the 5-HT_{1A} receptor is usually associated with a decrease in adenylyl cyclase activity. In cultures of hippocampal neurons, 5-HT_{1A} agonists block the forskolin-induced formation of p-CREB, an important transcription factor increased by cAMP (Nishi & Azmitia, 1999). In adult neurons, the 5-HT_{1A} receptor also is associated with a hyperpolarisation of the membrane potential, attributed to opening a K^+ current (Baskys *et al.*, 1989). The 5-HT_{1A} receptor uses these cellular mechanisms to differentiate its target cells. The 5-HT_{1A} receptor is found on serotonergic neurons and nonserotonergic neurons (Hamon, 1997). In the presence of a phosphodiesterase inhibitor 5-HT elevates bovine smooth muscle cellular cAMP and this elevation correlates with an inhibition of cellular proliferation (Assender *et al.*, 1992). Similarly other agents such as forskolin, histamine, isoproterenol and choleratoxin, which elevate cellular cAMP,

inhibit the proliferation. This activity of 5-HT is mimicked by 8-OH DPAT a reputed, 5-HT_{1A} agonist (Fanburg & Lee, 1997). 5-HT inhibits cellular growth of pulmonary artery smooth muscle cells (SMC) through its action on 5-HT_{1A} or 5-HT_4 receptors (Lee *et al.*, 1997).

The involvement of the 5-HT_{1A} receptor in cell proliferation is assumed to be inhibitory given its stimulatory effects on cell differentiation (Lauder *et al.*, 1983). However, some studies indicate a direct and indirect role for 5-HT_{1A} receptors in cell proliferation. 5-HT_{1A} agonists given in culture accelerate cell division, generate cell foci, and increase DNA synthesis in transfected NIH-3T3 cells (Varrault *et al.*, 1992). The early studies of 5-HT and cell proliferation in culture appear to argue that 5-HT may be important for cell differentiation and the inhibition of cell division in the CNS. The 5-HT_{1A} receptor is uniquely positioned during the early development of the brain to influence neuronal mitosis, in the maturation and the assembly of the spindle apparatus in the cell body which promotes cell division. In the 5-HT cell line, RN46A, the 5-HT_{1A} receptor is 20-fold higher in the undifferentiated cell than in the differentiated cell. It is suggested that the cell body of 5-HT_{1A} receptors may mediate autoregulation of serotonergic neuron development (Eaton *et al.*, 1995).

The 5-HT_{1A} receptors in the adult brain have clearly been shown to be involved in maintaining the mature state of neurons in the mammalian brain (Azmitia, 1999). Liu and Albert (1991) have demonstrated with transfection of the rat 5HT_{1A} receptor into a variety of cells that the receptor, acting through pertussis toxin (PTX) sensitive G proteins, can change its inhibitory signalled phenotype into a stimulatory one, depending on cell type, differentiation and culture medium.

5-HT_{1A} receptor is prominently expressed in neuronal cells (e.g. hippocampal CA-1, dorsal raphe nuclei) where it also opens potassium channels via activation of a PTX-sensitive G protein (Colino & Halliwell, 1987) and closes calcium channels (Penington & Kelly, 1990 & Ropert, 1988). This results in hyperpolarisation of the

membrane potential, closing of voltage-dependent calcium channels, and decrease Ca^{2+}. The expression of the rat 5-HT_{1A} receptor in pituitary GH4C1 cells (GH4ZD cells) resulted in a 5-HT-induced inhibition of Ca^{2+}; and cAMP accumulation simil to that observed in neurons (Liu *et al.*, 1991). Thus opposite effects occur with t use of 5-HT depending on whether or not cellular cAMP is elevated and this in tu depends on the activity of cellular phosphodiesterase (Fanburg & Lee, 1997).

The cells exposed to apoptotic-inducing conditions may actually up-regula 5-HT_{1A} receptors. Neuronal cell lines stably transfected with a promoter-le segment (G-21) of the human 5-HT_{1A} receptor gene (Singh *et al.*, 1996) show a to15-fold increase in the receptor when deprived of nutrient. 5-HT_2 receptor dru are not effective in these models of apoptosis. Conversely, reduced 5-HT levels in t hippocampus potentiate ischemic-induced neuronal damage (Nakata *et al.*, 1997).

5-HT_{2C} Receptor and Cell Proliferation

5-HT_{2C} receptors [formerly termed 5-HT_{1C}] are widely expressed in the bra and spinal cord, are particularly enriched in the choroid plexus, and appear mediate many important effects of 5-HT (Blier *et al.*, 1990). Previous studies ha shown that 5-HT_{2C} receptor undergoes RNA editing with the potential for produci 14 different receptor isoforms (Niswender *et al.*, 1998; Burns *et al.*, 1997). The rat HT_{2C} receptor is one of the three 5-HT_2 subtype receptors linked to phospholipase via G-protein coupling and is regulated by RNA editing (Burns *et al.*, 1997). Parr *et al* (1991) and Pakala *et al* (1994) proposed a 5-HT_2 receptor to be responsible f 5-HT induced proliferation of porcine smooth aortic muscle cells and canine ar bovine aortic endothelial cells. Similarly, Pitt *et al* (1994) and Corson *et al* (199 suggested that a 5-HT_2 receptor is responsible for proliferation of rat vascular smoo muscle cells caused by 5-HT through an increase in intracellular Ca^{2+}. Crowley *et* (1994) also concluded that stimulation of proliferation of bovine aortic smoo muscle cells by 5-HT occurs through a 5-HT_2 receptor.

5-HT_2 receptor can be referred to as a programmable receptor i.e., events during development may affect the number, affinity, or function of these receptors in the adult brain (Meaney *et al.*, 1994). For example, both prenatal and postnatal stress to the mother significantly increases the number of 5-HT_2 receptors in the offspring, which leads to activation of protein kinase C (PKC) and the activation of several important transcription factors including c-Fos, Jak, and STAT. 5-HT stimulates the turnover of phosphoinositide in primary cultures of astroglia from the cerebral cortex, striatum, hippocampus, and brain stem. 5-HT_2 receptors in glioma cells appear to regulate proliferation, migration, and invasion. 5-HT was found to positively modulate these three processes *in vitro* (Merzak *et al.*, 1996).

5-HT_2 receptor antagonists, ketanserin (10^{-6}M) and spiperone (10^{-6}M), blocked stimulation of DNA synthesis by 5-HT. Displacement studies on [^{3}H]5-HT binding to crude membranes from control and regenerating liver tissue, using cold ketanserin and spiperone, showed an increased involvement of 5-HT_2 receptors of 5-HT in the regenerating liver during the DNA-synthetic phase. 5-HT enhanced the phosphorylation of a 40-kd substrate protein of protein kinase C (PKC) in the regenerating liver during the DNA synthetic phase of the hepatocyte cell cycle. This was blocked by ketanserin, indicating that 5-HT_2 receptor activates PKC, an important second messenger in cell growth and division, during rat liver regeneration. 5-HT can act as a potent hepatocyte co-mitogen and induce DNA synthesis in primary cultures of rat hepatocytes, which is suggested to be mediated through the 5-HT_2 receptors of hepatocytes (Sudha & Paulose, 1997).

The predominant 5-HT_2 receptor in the neonatal period is the 5-HT_{2C} receptor (Ike *et al.*, 1995). The 5-HT_{2C} receptors activate phospholipase C (Conn *et al.*, 1986), whereas 5-HT_{1A} receptors modulate adenylyl cyclase activity (Siegelbaum *et al.*, 1982). In neurons that express the 5-HT_{1C} receptor activation by 5-HT is likely to generate inositol polyphosphates that release intracellular Ca^{2+} (Conn *et al.*, 1986).

NIH-3T3 cells that express high levels of 5-HT_{2C} receptor form foci in cell culture. Moreover the formation of foci is dependent on activation of the 5-HT_{1C} receptor by 5-HT. In addition the introduction of transformed foci into nude mice results in the rapid appearance of tumours. In fibroblasts this receptor alters the growth properties of cells and results in malignant transformation (Julius *et al.*, 1989).

In the present study the role of brain and pancreatic 5-HT_{1A} and 5-HT_{2C} receptors on regulation of pancreatic β-cell proliferation and insulin release during pancreatic regeneration have been investigated using rat models. *In vitro* studies were conducted to confirm the involvement of 5-HT_{1A} and 5-HT_{2C} receptors in the regulation of pancreatic β-cell proliferation using specific ligands in primary cultures

MATERIALS AND METHODS

BIOCHEMICALS AND THEIR SOURCES

Biochemicals used in the present study were purchased from SIGMA Chemical Co., St. Louis, U.S.A. All other reagents were of analytical grade purchased locally. HPLC solvents were of HPLC grade obtained from SRL and MERCK, India.

Important Chemicals Used for the Present Study

i) *Biochemicals*

5-Hydroxytryptamine (5-HT), 8-Hydroxy dipropylaminotetraline (8-OH DPAT), Mesulergine, (±) Norepinephrine, Sodium octyl sulphonate, Ethylenediamine tetra acetic acid (EDTA), HEPES (N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid. 2-Methane 2-propyl thiol]), Tris buffer, Fetal calf serum (heat inactivated), Collagenase type XI, Pertussis toxin, RPMI-1640 medium, Epidermal Growth Factor (EGF), Transforming Growth Factorβ1 (TGFβ1).

ii) *Radiochemicals*

8-Hydroxy-DPAT [propyl-2,3-ring-1,2,3-^{3}H] (Sp. activity – 127.0 Ci/mmol), was purchased from NEN Life Sciences products, Inc., Boston, USA.

[N^6-methyl-^{3}H]Mesulergine (Sp. activity - 79.0 Ci/mmol) and [^{3}H]Thymidine (Sp.activity 25Ci/mmol), were purchased from Amersham Life Science, UK.

iii) *Molecular biology chemicals*

Random hexamers, Taq DNA polymerase, Human placental RNAse inhibitor and DNA molecular weight markers were purchased from Bangalore Genei Pvt. Ltd., India. MuMLV and dNTPs were obtained from Amersham Life Science, UK. Tri-

reagent was purchased from Sigma Chemical Co., USA. RT-PCR primers used this study were synthesised by Sigma Chemical Co., USA.

ANIMALS

Wistar weanling rats of 80-100g-body weight purchased from Ker Agriculture University, Mannuthy and Amrita Institute of Medical Sciences, Coc were used for all experiments. They were housed in separate cages under 12 hr li and 12 hr dark periods and were maintained on standard food pellets and water *libitum*.

PARTIAL PANCREATECTOMY

Male Wistar weanling rats, 4-5 weeks old, were anaesthetised under asep conditions, the body wall was cut opened and 60-70% of the total pancreas, near the spleen and duodenum, was removed (Pearson *et al.*, 1977). The removal of m of the pancreas was done by gentle abrasion with cotton applications, leaving t major blood vessels supplying other organs intact (Zangen *et al.*, 1997). The sh operation was done in an identical procedure except that the pancreatic tissue w only lightly rubbed between fingertips using cotton for a minute instead of bei removed. All the surgeries were done between 7 and 9 A.M to avoid diur variations in responses. The rats were maintained for different time intervals (12 h 24 hrs, 48 hrs, 72 hrs, 7 days and 14 days). Body weight and blood glucose lev were checked routinely.

Sacrifice and Tissue Preparation

The rats were sacrificed at various intervals after surgery by decapitatic Pancreas and brain were rapidly dissected into different regions (Glowinski Iverson, 1966). The brain dissection was carried out on a chilled glass plate i hypothalamus (Hypo), brain stem (BS) and cerebral cortex (CC). These regions w

immediately immersed into liquid nitrogen and stored at -70°C for various experiments.

Estimation of Blood Glucose

Blood glucose was estimated using Glucose estimation kit (Merck). The spectrophotometric method using glucose oxidase-peroxidase reactions is as follows:

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

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

$$H_2O_2 + \text{Phenol} \quad \text{4-aminoantipyrene} \xrightarrow{\text{(POD)}} \text{Coloured complex} + H_2O$$

The hydrogen peroxide formed in this reaction reacts with 4-aminoantipyrine and 4-hydroxybenzoic acid in the presence of peroxidase (POD) to form N-(-4-antipyryl)-p-benzo quinoneimine. The addition of mutarotase accelerates the reactions. The amount of dye formed is proportional to the glucose concentration. The absorbance was read at 510nm in a spectrophotometer (Milton Roy Genesys 5 Spectronic).

***IN VIVO* DNA SYNTHESIS STUDIES IN PANCREAS**

5μCi of [^{3}H]thymidine was injected intra-peritoneally into partially pancreatectomised rats to study DNA synthesis at 24, 36, 72 hrs, 7 days and 14 days of pancreatic regeneration. [^{3}H]thymidine was injected 2 hrs before sacrifice. DNA was extracted from pancreatic islets according to (Schneider, 1957). A 10% trichloroacetic acid (TCA) homogenate was made and DNA was extracted from the lipid free residue by heating with 5% TCA at 90°C for 15minutes. DNA was estimated by diphenylamine method (Burton, 1955). DNA extract was counted in a

liquid scintillation counter (WALLAC 1409) after adding cocktail-T containing Triton-X 100. The amount of DNA synthesised was measured as DPM/mg DNA.

ESTIMATION OF CIRCULATING INSULIN BY RADIOIMMUNOASSAY

Principle of the assay

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

Assay Protocol

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

A standard curve was plotted with %B/B$_0$ on the Y-axis and insulin concentration/ml on the X-axis of a log-logit graph. %B/B$_0$ was calculated as:

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

[redacted]in concentration in the samples was determined from the standard curve plotted [redacted] MultiCalc™ software (Wallac, Finland).

ISOLATION OF PANCREATIC ISLETS

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

The pancreas was aseptically dissected out into a sterile petridish containing ice cold HBSS and excess fat and blood vessels were removed. The pancreas was cut into small pieces and transferred to a sterile glass vial containing 2ml collagenase type XI solution (1.5 mg/ml in HBSS), pH 7.4. The collagenase digestion was carried out for 15 minutes at 37°C in an environmental shaker with vigorous shaking (300rpm/minute). The tissue digest was filtered through 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.

5-HT QUANTIFICATION BY HPLC

Brain 5-HT HPLC determinations were done by electrochemical detection (Paulose *et al.*, 1988). 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 (Kubota Refrigerated Centrifuge, Japan) and the clear supernatant was filtered through 0.22 μm HPLC grade filters and used for HPLC analysis in Shimadzu HPLC

system with electrochemical detector fitted with C18-CLC-ODS reverse pha column. Mobile phase was 75mM sodium dihydrogen orthophosphate buff containing 1mM sodium octyl sulphonate, 50mM EDTA and 7% acetonitrile (p 3.25), filtered through 0.22μm filter delivered at a flow rate of 1.0 ml/minu Quantification was by electrochemical detection, using a glass carbon electrode set +0.80 V. The peaks were identified by relative retention time compared wi standards and concentrations were determined using a Shimadzu integrator interfac with the detector.

Adrenal monoamines

The monoamines were assayed according to Paulose *et al.*, (1988). Th adrenals were homogenised in 1N perchloric acid. The homogenate was centrifug at 5000xg for 10 minutes at 4°C (Kubota refrigerated centrifuge) and the cle supernatant was filtered through 0.45 μm HPLC grade filters and used for HPL analysis.

Norepinephrine (NE) and epinephrine (EPI), were determined in hi performance liquid chromatography (HPLC) with electrochemical detector (HPL ECD) (Shimadzu, Japan) fitted with CLC-ODS reverse phase columns of 5 μ particle size. The mobile phase consisted of 75 mM sodium dihydrog orthophosphate, 1mM sodium octyl sulfonate, 50mM EDTA and 7% acetonitril The pH was adjusted to 3.25 with orthophosphoric acid, filtered through 0.45 μ filter (Millipore) and degassed. A Shimadzu (model 10 AS) pump was used deliver the solvent at a rate of 1 ml/minute. The catecholamines were identified b amperometric detection using an electrochemical detector (Model 6A, Shimadz Japan) with a reduction potential of + 0.80 V. The range was set at 16 and a tim constant of 1.5 seconds. Twenty microlitre aliquots of the acidified supernatant we injected into the system for detection. The peaks were identified by relative retentio times compared with external standards and quantitatively estimated using a

integrator (Shimadzu, C-R6A -Chromatopac) interfaced with the detector. Data from adrenals of the experimental and control rats were tabulated and statistically analysed.

Analysis of circulating Norepinephrine

Plasma Norepinephrine (NE) was extracted from 1ml of plasma and diluted twice with distilled water. To it 50µl of 5mM sodium bisulphite was added, followed by 250µl of 1M Tris buffer, pH 8.6. 20mg of Acid alumina was added, shaken in the cold for 20 minutes and was washed with 5mM sodium bisulphite. Catecholamines were extracted from the final pellet of alumina with 0.1 N perchloric acid, mixed well and 20µl of filtered sample was analysed (Jackson *et al.*, 1997).

5-HT RECEPTOR STUDIES USING [^{3}H] RADIOLIGANDS

5-HT$_{1A}$ Receptor Binding Assays in Brain

5-HT$_{1A}$ receptor assay was done by using specific agonist [^{3}H]8-OH DPAT binding to the 5-HT$_{1A}$ receptors (Nenonene *et al.*, 1994). Brain tissues were homogenised in a polytron homogeniser with 50 volumes of 50mM Tris-HCl buffer, pH 7.4. After first centrifugation at 40,000 x g for 15 minutes, the pellets were resuspended in buffer and incubated at 37°C for 20 minutes to remove endogenous 5-HT. After incubation the homogenates were centrifuged and washed twice at 40,000 x g for 15 minutes and resuspended in appropriate volume of the buffer.

Binding assays were done using different concentrations i.e., 0.20nM - 100nM of [^{3}H]8-OH DPAT in 50mM Tris buffer, pH 7.4 in a total incubation volume of 250µl. Specific binding was determined using 100µM unlabelled 5-HT. Competition studies were carried out with 1.0nM [^{3}H]8-OH DPAT in each tube with unlabelled ligand concentrations varying from 10^{-12} – 10^{-4}M of 5-HT.

Tubes were incubated at 25°C for 60 minutes and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive

washing with 3ml of ice-cold 50mM Tris buffer, pH 7.4. Bound radioactivity was determined with cocktail-T in a Wallac 1409 liquid scintillation counter. Specific binding was determined by subtracting non-specific binding from total binding.

5-HT_{2C} Receptor Binding Assays in Brain

Tritiated mesulergine binding to 5-HT_{2C} receptor in the synaptic membrane preparations were assayed as previously described (Herrick-Davis *et al.*, 1999). Crude synaptic membrane preparation was suspended in 50mM Tris-HCl buffer (pH 7.4) and used for assay. In saturation binding experiments, 0.1nM - 6nM of [^{3}H]mesulergine was incubated with and without excess of unlabelled 5-HT (100μM) and in competition binding experiments the incubation mixture contained 1nM of [^{3}H]mesulergine with and without 5-HT at a concentration range of 10^{-12}M to 10^{-4}M. Tubes were incubated at 25°C for 60 minutes and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 3ml of ice-cold 50mM Tris buffer, pH 7.4. Bound radioactivity was determined with cocktail-T in a Wallac 1409 liquid scintillation counter. Specific binding was determined by subtracting non-specific binding from total binding.

BINDING STUDIES IN THE PANCREATIC ISLETS

5-HT_{1A} Receptor Binding Assays

Islets were isolated from rats by collagenase digestion method. Islets were then homogenised for 30 seconds in a polytron homogeniser with 50 volumes of 50mM Tris-HCl buffer, pH 7.4. After first centrifugation at 40,000 x g for 15 minutes, the pellets were resuspended in buffer and incubated at 37°C for 20 minutes to remove endogenous 5-HT. The homogenates were again centrifuged and washed twice at 40,000 x g for 15 minutes and resuspended in appropriate volume of the buffer.

Binding assays were done using different concentrations i.e., 1nM - 100nM of [^{3}H]8-OH DPAT in 50mM Tris buffer, pH 7.4 in a total incubation volume of 250µl. Specific binding was determined using 100µM unlabelled 5-HT. Competition studies were carried out with 5.0nM [^{3}H]8-OH DPAT in each tube with unlabelled ligand concentrations varying from 10^{-12} – 10^{-4}M of 5-HT.

Tubes were incubated at 25°C for 60 minutes and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 3ml of ice-cold 50mM Tris buffer, pH 7.4. Bound radioactivity was determined with cocktail-T in a Wallac 1409 liquid scintillation counter. Specific binding was determined by subtracting non-specific binding from total binding.

5-HT$_{2C}$ Receptor Binding Assays

The homogenate was prepared and the assay was done in a similar way as for the [^{3}H]8-OH DPAT binding with 0.2-6nM of [^{3}H]mesulergine in the incubation buffer. Non-specific binding was determined using 100µM unlabelled 5-HT. Competition studies were carried out with 0.5nM [^{3}H]mesulergine in each tube with unlabelled ligand concentrations varying from 10^{-12}-10^{-4}M of 5-HT. The tubes were incubated at 22°C for 2 hrs and filtered rapidly through GF/C filters (Whatman). The filters were washed with ice cold phosphate assay buffer. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non-specific binding determined showed 30-40% in all our experiments.

Protein Estimation

Protein concentrations were estimated (Lowry *et al.*, 1951) using bovine serum albumin as the standard.

Receptor Binding Parameters Analysis

The receptor binding parameters determined using Scatchard analysis (Scatchard, 1949). The maximal binding (B_{max}) and equilibrium dissociation

constant (K_d) were derived by linear regression analysis by plotting the speci binding of the radioligand on x-axis and bound/free on y-axis using Sigma pl computer software. This is called a Scatchard plot. The B_{max} is a measure of t total number of receptors present in the tissue and the K_d represents affinity of t receptors for the radioligand. The K_d is inversely related to receptor affinity or t "strength" of binding. Competitive binding data were analysed using non-line regression curve-fitting procedure (GraphPad PRISM™, San Diego, USA). T concentration of competitor that competes for half the specific binding was defin as EC_{50}. It is same as IC_{50}. The affinity of the receptor for the competing drug designated as K_i and is defined as the concentration of the competing ligand that w bind to half the binding sites at equilibrium in the absence of radioligand or oth competitors (Chen & Prusoff, 1973).

Displacement Curve analysis

The data of the competitive binding assays are represented graphically wi the negative log of concentration of the competing drug on x axis and percentage the radioligand bound on the y axis. The steepness of the binding curve can quantified with a slope factor, often called a Hill slope. A one-site competiti binding curve that follows the law of mass action has a slope of -1.0. If the curve more shallow, the slope factor will be a negative fraction (i.e., -0.85 or -0.60). T slope factor is negative because curve goes downhill. If slope factor diff significantly from 1.0, then the binding does not follow the law of mass action with single site, suggesting a two-site model of curve fitting.

REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

Isolation of RNA

RNA was isolated from the pancreas and brain regions of sham and pancreatectomised rats using Tri reagent (Sigma Chemical Co., USA). Brain tissues and islets isolated by collagenase digestion procedure (25-50 mg) were homogenised in 0.5 ml Tri Reagent. The homogenate was centrifuged at 12,000 *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, shaken vigorously for 15 seconds and allowed to stand at room temperature for 15 minutes. The tubes were centrifuged at 12,000 *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 allowed to stand at room temperature for 10 minutes. The tubes were centrifuged at 12,000 *g* for 10 minutes 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 *g* for 5 minutes at 4°C. The pellet was semi dried and dissolved in minimum volume of DEPC-treated water. 2µl of RNA was made up to 1ml and optical density was measured at 260nm and 280nm. For pure RNA preparation the ratio of optical density at 260/280 was ≥ 1.7. The concentration of RNA was calculated as one optical density$_{260}$ = 42µg.

RT-PCR Primers

The following primers were used for 5-HT_{1A}, 5-HT_{2C} receptors and β-actin mRNA expression studies.

5'- TGG CTT TCT CAT CTC CAT CC -3' 5'- CTC ACT GCC CCA TTA GTG C –3' PRODUCT SIZE: 357bp	5-HT_{1A}
5'- CCA ACG AAC ACC TTC TTT CC -3' 5'- GCA TTG TGC AGT TTC TTC TCC -3' PRODUCT SIZE: 252bp	5-HT_{2C}
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 5-HT_{1A}, 5-HT_{2C} Receptor and β-Actin

RT-PCR was carried out in a total reaction volume of 20µl in 0.2ml tubes. RT-PCR was performed on an Eppendorf Personal thermocycler. cDNA synthesis of 2µg RNA was performed in a reaction mixture containing MuMLV reverse transcriptase (40units/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. After incubation heating at a temperature of 95°C inactivated the reverse transcriptase enzyme, MuMLV.

Polymerase Chain Reaction (PCR)

PCR was carried out in a 20µl volume reaction mixture containing 4µl of cDNA, 0.25mM dNTPs (dATP, dCTP, dGTP and dTTP), 0.5units of Taq DNA

Polymerase and 10 picomoles of specific primer. The three primers used have the same annealing temperature

Following is the thermocycling profile used for PCR

94°C – 5 minutes --- Initial Denaturation

94°C – 30 seconds --- Denaturation

56°C – 30 seconds --- Annealing 30 cycles

72°C – 30 seconds --- Extension

72°C – 5 minutes --- Final Extension

Analysis of RT-PCR product

After completion of RT-PCR reaction 5µl of bromophenol blue gel-loading buffer was added to 10µl reaction mixture and the total volume was applied to a 2.0% agarose gel containing ethidium bromide. The gel was run at 60V constant voltage with 0.5 x TBE buffer. The image of the bands was captured using an Imagemaster VDS gel documentation system (Pharmacia Biotech) and densitometrically analysed using Imagemaster ID software to quantitate the 5-HT_{1A} receptor, 5-HT_{2C} receptor mRNA expression in sham, 72 hrs and 7 days pancreatectomised rats.

INSULIN SECRETION STUDIES WITH 5-HT, 8-OH DPAT AND MESULERGINE

Pancreatic islets were isolated by collagenase digestion method and islets were incubated in RPMI-1640 medium for 16 hrs in 5%CO_2 at 37°C for fibroblast attachment. Islets were harvested after 16 hrs and used for secretion studies.

Insulin secretion study - 1 hour

Islets were harvested after removing the fibroblasts and resuspended in Krebs Ringer Bicarbonate buffer, pH 7.3 (KRB). The isolated islets were incubated for 1hour in KRB at 37°C with 4mM and 20mM glucose concentrations, different

concentrations of 5-HT (10^{-8} M - 10^{-4} M), 8-OH DPAT (10^{-8} M - 10^{-4} M) and 5-H (10^{-8} M - 10^{-4} M) with 10^{-4}M 5-HT_{2C} antagonist mesulergine. To study the effect 5-HT_{1A} and 5-HT_{2C} receptor subtypes islets were incubated with 5-HT_{1A} recept agonist 8-OH DPAT and 5-HT_{2C} receptor antagonist mesulergine. After incubati cells were centrifuged at 1,500xg for 10 minutes at 4^0C and the supernatant we transferred to fresh tubes for insulin assay by radioimmunoassay.

Insulin secretion study - 24 hrs

The islets were harvested after removing the fibroblasts and cultured for 2 hrs in RPMI-1640 medium. Insulin secretion study was carried out by preincubati the cells in 4mM and 20mM glucose concentrations with different concentrations 5-HT (10^{-8} M - 10^{-4} M), 8-OH DPAT (10^{-8} M - 10^{-4} M) and 5-HT (10^{-8} M - 10^{-4} M with 10^{-4}M 5-HT_{2C} antagonist mesulergine. The cells were then harvested a washed with fresh KRB and then incubated for another 1 hour in the presence same concentrations of glucose, 5-HT, 8-OH DPAT and mesulergine. At the end incubation period the medium was collected and insulin content was measured RIA method using kit from BARC, Mumbai.

PANCREATIC DNA SYNTHESIS STUDIES *IN VITRO*

Islets were isolated from adult male Wistar rats by collagenase digesti method as mentioned earlier. The isolated islets were then suspended in RPMI 16 medium containing 10% FCS, and incubated for 16 hrs at 37^0C and 5% CO_2 remove the fibroblasts. The cells were recultured for three days after fibrobl removal to remove all other non-endocrine tissue. The medium will be rich in β-ce after the incubation. Groups of 100 islets were transferred at the end of cultu period to 1ml fresh medium containing 5% FCS, antibiotics, different concentrati of glucose (4mM), and appropriate concentrations of 5-HT and 5- HT ligands (10^{-} 10^{-4}). EGF (10 ng/ml) and TGF (1ng/ml) were also added and cultured free floati

for an additional 24 hrs in the presence of 2.5μCi of [^{3}H]thymidine (Sjoholm, 1991). Effect of pertussis toxin was studied by adding 50 ng into 1ml. The cells were harvested and the protein was measured by method of Lowry *et al.*, (1951) using bovine serum albumin as standard. The radioactivity incorporated was determined by counting in a scintillation counter.

STATISTICS

Statistical evaluations were done by ANOVA using InStat (Ver.2.04a) computer programme. Linear regression Scatchard plots were made using SIGMA PLOT (Ver 2.03).

RESULTS

Body Weights and Blood Glucose Levels

The body weights and blood glucose levels of rats showed no significant change in sham operated and pancreatectomised rats (Table - 1).

DNA Synthesis in the Regenerating Pancreas

Tritiated thymidine incorporation into replicating DNA was used as a biochemical index for quantifying DNA synthesis during pancreatic regeneration. DNA synthesis was negligible in the pancreatic islets of sham operated rats. A significant increase ($p<0.01$) in the [^{3}H]thymidine incorporation was observed at 36 hrs and 48 hrs after partial pancreatectomy. The DNA synthesis was peaked at 72 hrs after partial pancreatectomy ($p<0.001$). It reversed to near normal levels by 7 days and reached the basal level by 14 days after partial pancreatectomy (Fig. 1).

Circulating Insulin Level

The insulin levels in the plasma of pancreatectomised rats showed a significant increase at 48 hrs ($p<0.05$) and peaked at 72 hrs ($p<0.01$) after partial pancreatectomy. The elevated insulin levels then reversed to basal levels by 7 and 14 days (Fig. 2).

5-HT Content in the Brain Regions (CC, BS and Hypo) of Experimental Rats

In the cerebral cortex and hypothalamus the 5-HT content was increased significantly ($p<0.01$) at 72 hrs after partial pancreatectomy when compared with control. 5-HT content was also increased significantly ($p<0.05$) in the brain stem during active DNA synthesis. The increased contents were reversed to near normal by 7 days after partial pancreatectomy in the cerebral cortex and hypothalamus while it remained unchanged in the brain stem (Table - 2).

5-HT and 5-HIAA Content in the Pancreas of Experimental Rats

There was a significant ($p<0.05$) decrease in the pancreatic 5-HT conte during active cell proliferation when compared with control. The decreased conte was reversed to near normal at 7 days after partial pancreatectomy. The 5-HIA content and the turnover rate of 5-HIAA/5-HT were significantly increased ($p<0.0$ $P<0.001$) in 72 hrs pancreatectomised rats when compared with control. Th increased 5-HIAA content and the turnover of 5-HIAA/5-HT were reversed by days after partial pancreatectomy (Table - 3).

5-HT, NE and EPI Levels in the plasma of Experimental Rats

There was a significant decrease in the plasma EPI ($p<0.001$) and N ($p<0.05$) levels in 72 hrs pancreatectomised rats compared with control. The plasm 5-HT level increased significantly ($p<0.01$). The decreased NE and EPI levels a the increased 5-HT level reversed to normal levels by 7 days after parti pancreatectomy (Table - 4).

NE and EPI contents in the Adrenals of Experimental Rats

NE and EPI contents decreased significantly ($p<0.001$) in the adrenals duri pancreatic regeneration. The decreased NE and EPI reversed to control levels at days after partial pancreatectomy (Table - 5).

Receptor Alterations in the Brain Regions of Experimental Rats

5-HT_{1A} Receptor Analysis

Cerebral Cortex

[^{3}H]8-OH DPAT Binding Parameters

Scatchard analysis in the cerebral cortex of rats showed that there were two affinity sites for [^{3}H]8-OH DPAT binding. The K_d value of the high affinity receptor significantly increased ($p<0.01$) in 72 hrs pancreatectomised rats. There was no significant change in the B_{max} of [^{3}H]8-OH DPAT high affinity receptor binding to the membrane preparation of 72 hrs pancreatectomised rats (Figure 3 - & Table - 6). The B_{max} of low affinity receptor binding was decreased significantly in 72 hrs pancreatectomised rats ($p<0.01$) compared with control. The K_d of the receptor increased significantly ($p<0.01$) in 72 hrs after partial pancreatectomy. The K_d of high affinity receptor and the B_{max} and K_d of low affinity receptor reversed to near normal in the 7 days pancreatectomised rats (Figure - 4 & Table - 7).

Displacement Analysis of [^{3}H] 8-OH DPAT by 5-HT

The competition curve for 5-HT against [^{3}H]8-OH DPAT fitted for two-sited model in all the groups with Hill slope value away from Unity. The $Ki_{(H)}$ increased in 72 hrs pancreatectomised rats along with an increase in the log (EC_{50})-1 indicating a shift in high affinity towards low affinity. $Ki_{(L)}$ also showed an increase in 72 hrs pancreatectomised rats with an increase in log (EC_{50})-2 denoting a shift in the low affinity site towards much lower affinity (Figure -5 & Table -8).

RT – PCR Analysis of 5 – HT_{1A} Receptor

5- HT_{1A} receptor mRNA expression decreased in 72 hrs pancreatectomised rats and it reversed to control level at 7 days after partial pacreatectomy (Figure – 6 & Table – 9).

Brain Stem

[^{3}H]8-OH DPAT Binding Parameters

The B_{max} of the high affinity receptor binding decreased ($p<0.01$) and th K_d increased significantly ($p<0.01$) in 72 hrs pancreatectomised rats compared wi control. The decreased B_{max} and increased K_d reversed to control level by 7 da after partial pancreatectomy (Figure - 7 & Table - 10). The B_{max} of low affini receptor binding was decreased significantly ($p<0.01$) without any change in K_d in 7 hrs pancreatectomised rats compared with control. The B_{max} partially reversed control level by 7 days after partial pancreatectomy (Figure - 8 & Table - 11).

Displacement Analysis of [^{3}H]8-OH DPAT by 5-HT

The competition curve for 5-HT against [^{3}H]8-OH DPAT fitted for two-site model in all the groups with Hill slope value away from Unity. The $Ki_{(H)}$ increas in 72 hrs with an increased log (EC_{50})-1. This indicates a shift of high affini towards low affinity. $Ki_{(L)}$ and the log (EC_{50})-2 value showed no change (Figure - & Table - 12).

RT-PCR Analysis of 5-HT_{1A} Receptor

RT-PCR analysis revealed a decreased expression of 5-HT_{1A} receptor mRN in 72 hrs and it reversed to near normal level in 7 days pancreatectomised ra (Figure - 10 & Table 13).

Hypothalamus

[^{3}H]8-OH DPAT binding parameters

A significant increase ($p<0.01$) in the K_d of the high affinity [^{3}H]8-OH DPAT receptor binding was observed in 72 hrs pancreatectomised rats compared with control. There was no significant change in the B_{max}. The increased K_d value reversed to normal level by 7 days after partial pancreatectomy (Figure - 11 & Table - 14). The B_{max} of the low affinity [^{3}H]8-OH DPAT receptor binding decreased significantly ($p<0.01$) and the K_d value increased significantly ($p< 0.05$) in 72 hrs pancreatectomised rats compared with control. The B_{max} and K_d value reversed to control level by 7 days after partial pancreatectomy (Figure - 12 & Table - 15).

Displacement Analysis of [^{3}H]8-OH DPAT by 5-HT

The competition curve for 5-HT against [^{3}H]8-OH DPAT fitted for two-sited model in all the groups with Hill slope value away from unity. The $Ki_{(H)}$ and log (EC_{50})-1 increased in 72 hrs pancreatectomised rats indicating a shift in affinity of the high affinity receptor binding site towards low affinity. $Ki_{(L)}$ and log (EC_{50})-2 increased in 72 hrs pancreatectomised rats indicating a decrease in the low affinity site towards much lower affinity (Figure - 13 & Table - 16).

RT-PCR Analysis of 5-HT$_{1A}$ Receptor

A decreased 5-HT$_{1A}$ receptor mRNA expression was observed at 72 hrs pancreatectomised rats and it reversed to control level at 7 days (Figure - 14 & Table - 17).

5-HT_{2C} Receptor Analysis

Cerebral cortex

[^{3}H]Mesulergine Binding Parameters

There was a significant decrease ($p<0.05$) in the B_{max} of [^{3}H]mesulergi binding without any change in K_d in 72 hrs pancreatectomised rats compared wi control. The decreased B_{max} reversed to control level by 7 days after parti pancreatectomy (Figure - 15 & Table - 18).

Displacement Analysis of [^{3}H]Mesulergine by 5-HT

The competition curve for 5-HT against [^{3}H]mesulergine fitted for one-sit model in all the groups with Unity as Hill slope value. The Ki and log (EC_{50}) val showed no change in 72 hrs pancreatectomised rats compared with control indicati no shift in affinity (Figure - 16 & Table - 19).

RT-PCR Analysis of 5-HT_{2C} Receptor

5-HT_{2C} receptor mRNA expression decreased in 72 hrs and it reversed control level in 7 days pancreatectomised rats compared with control (Figure - 17 Table - 20).

Brain stem

[^{3}H]Mesulergine Binding Parameters

The B_{max} of [^{3}H]mesulergine binding decreased significantly ($p<0.01$). T K_d of the receptor binding showed a significant increase ($p<0.01$) in 72 h pancreatectomised rats compared with control. The altered parameters reversed near normal in 7 days pancreatectomised rats (Figure - 18 & Table - 21).

Displacement Analysis of [^{3}H]Mesulergine by 5-HT

The competition curve for 5-HT against [^{3}H]mesulergine fitted for one-sited model in all the groups with Unity as Hill slope value. There was an increase in the Ki and log (EC_{50}) in 72 hrs pancreatectomised rats (Figure - 19 & Table - 22).

RT-PCR analysis of 5-HT$_{2C}$ receptor: RT-PCR analysis revealed a decreased mRNA in 72 hrs pancreatectomised rats (Figure - 20 & Table 23).

Hypothalamus

[^{3}H]Mesulergine Binding Parameters

There was a significant decrease ($p<0.01$) in the B_{max} of the [^{3}H]mesulergine binding to the membrane preparation of hypothalamus in 72 hrs pancreatectomised rats. The K_d of the receptor binding showed a significant increase ($p<0.01$) in 72 hrs pancreatectomised rats compared with control. The decreased B_{max} and increased K_d reversed to control level by 7 days after partial pancreatectomy (Figure - 21 & Table - 24).

Displacement Analysis of [^{3}H]Mesulergine by 5-HT

The competition curve for 5-HT against [^{3}H]mesulergine fitted for one-sited model in all the groups with Unity as the Hill slope value. There was an increase in the Ki and log (EC_{50}) in 72 hrs pancreatectomised rats compared with control indicating a shift in affinity of the receptor towards low affinity (Figure - 22 & Table - 25).

RT-PCR Analysis of 5-HT$_{2C}$ Receptor: 5-HT_{2C} receptor mRNA decreased in 72 hrs pancreatectomised rats and it reversed to near normal level by 7 days after partial pancreatectomy (Figure - 23 & Table - 26).

RECEPTOR ALTERATIONS IN THE PANCREATIC ISLETS DURING PANCREATIC REGENERATION

5-HT_{1A} Receptor Analysis

[^{3}H]8-OH DPAT Binding Parameters

There was a significant increase ($p<0.01$) in the B_{max} of [^{3}H]8-OH DPAT receptor binding to the pancreatic islet membrane preparation of 72 hrs and 7 days pancreatectomised rats compared with control (Figure - 24 & Table - 27). The K_d of the receptor binding was increased significantly in 7 days pancreatectomised rats ($p<0.05$) compared with control.

Displacement Analysis of [^{3}H] 8-OH DPAT by 5-HT

The competition curve for 5-HT against [^{3}H]8-OH DPAT fitted for one-sited model in all the groups with Unity as the Hill slope value. The Ki and log (EC_{50}) value showed no change in 72 hrs pancreatectomised rats compared with control indicating no shift in affinity. While the Ki and log (EC_{50}) value of 7 days pancreatectomised rats increased significanly (Figure - 25 & Table - 28).

RT-PCR analysis of 5-HT_{1A} receptor: 5-HT_{1A} receptor mRNA expression increased in 72 hrs and 7 days pancreatectomised rats (Figure - 26 & Table -29).

5-HT_{2C} receptor analysis in the pancreatic islets

[^{3}H]Mesulergine Binding Parameters

There was a significant increase ($p<0.01$) in the B_{max} of the [^{3}H]mesulergine binding to the membrane preparation of pancreatic islets in 72 hrs and 7 days pancreatectomised rats. The K_d of the receptor binding showed no significant change in 72 hrs and 7 days pancreatectomised rats compared with control (Figure - 27 & Table - 30).

Displacement Analysis of [^{3}H]Mesulergine by 5-HT

The competition curve for 5-HT against [^{3}H]mesulergine fitted for one-sited model in all the groups with Unity as the Hill slope value. The Ki and log (EC_{50}) values were unchanged in all the experimental groups (Figure - 28 & Table - 31).

INSULIN SECRETION STUDIES IN PANCREATIC ISLETS

One hour *in vitro* culture

Effect of 5-HT on Glucose Induced Insulin Secretion in vitro

The isolated islets incubated for 24 hrs with 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4}M concentrations of 5-HT and with two different concentrations of glucose, 4mM and 20mM. 5-HT at lower concentrations (10^{-8}, 10^{-7} and 10^{-6}M) significantly increased ($p<0.01$, $p<0.001$, $p<0.01$ respectively) insulin secretion in the presence of 4mM glucose. But the insulin secretion significantly decreased at higher concentration (10^{-4}M) (Figure - 29). 5-HT dose dependently inhibited ($p<0.01$) insulin secretion from 10^{-7} to 10^{-4}M concentration in the presence of 20mM glucose (Figure - 30).

Effect of 8-OH DPAT on Glucose induced Insulin Secretion in vitro

The $5\text{-}HT_{1A}$ receptor agonist, 8-OH DPAT at lower concentrations, 10^{-7} & 10^{-6}M, significantly increased ($p<0.01$) glucose (4mM) induced insulin secretion. But at higher concentration (10^{-4}M) insulin secretion was significantly ($p<0.05$) inhibited (Figure - 31). 8-OH DPAT dose dependently inhibited ($p<0.01$, $p<0.05$) insulin secretion in the presence of 20mM glucose (Figure - 32).

Effect of Mesulergine on Glucose Induced Insulin Secretion in vitro

Mesulergine (10^{-4}M) decreased insulin secretion mediated by 5-HT. A significant decrease in insulin secretion was observed at 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4}M ($p<0.05$) concentrations of 5-HT in the presence of 4mM glucose (Figure - 33). There was also a significant decrease in insulin secretion at 10^{-6} ($P<0.01$), 10^{-5}

($p<0.05$) and 10^{-4}M ($p<0.01$) concentrations of 5-HT when incubated with 10^{-4} mesulergine in the presence of 20mM glucose (Figure - 34).

24 hrs *in vitro* culture

Effect of 5-HT on Glucose induced Insulin Secretion in 24 hrs Islet Cultures

Islets were incubated with 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M concentrations 5-HT and two different concentrations of glucose, 4mM and 20mM in 24 hrs *in vit* culture. 5-HT increased insulin secretion significantly at 10^{-8}M ($p<0.05$), 10^{-7} ($p<0.01$) and 10^{-6}M ($p<0.05$) concentration in the presence of 4mM glucose. But 10^{-4}M concentration 5-HT significantly inhibited ($p<0.01$) insulin secreti stimulated by 4mM glucose. There was no significant effect at 10^{-5}M concentrati (Figure - 35). 5-HT significantly inhibited glucose induced (20mM) insulin secreti at 10^{-7} ($p<0.01$), 10^{-6} ($p<0.01$), 10^{-5} ($p<0.05$) and 10^{-4}M ($p<0.01$) concentratio (Figure - 36).

Effect of 8-OH DPAT on Glucose induced Insulin Secretion in vitro

8-OH DPAT at 10^{-7} & 10^{-6}M concentrations significantly ($p<0.05$ & $p<0.0$ increased glucose (4mM) induced insulin secretion in the long term incubation stud Insulin secretion was slightly inhibited at 10^{-4}M concentration (Figure - 37). significant decrease in insulin secretion was observed at 10^{-6}, 10^{-5} and 10^{-4}M ($p<0.0$ concentrations in the presence of 20mM glucose. The inhibitory effect was n significant at 10^{-7} and 10^{-8}M concentrations (Figure - 38).

Effect of Mesulergine on Glucose Induced Insulin Secretion in vitro

Mesulergine (10^{-4}M) significantly decreased 5-HT induced insulin secreti at 10^{-7} ($p<0.05$), 10^{-6} ($p<0.05$), 10^{-5} ($p<0.05$) and 10^{-4}M ($p<0.01$) concentrations the presence of 4mM glucose (Figure - 39). A significant decrease in insul secretion was also observed at 10^{-7} ($p<0.01$), 10^{-6} ($P<0.01$), 10^{-5} ($p<0.01$) and 10^{-4}

($p<0.01$) concentrations of 5-HT with mesulergine (10^{-4}M) in the presence of 20mM glucose (Figure - 40).

IN VITRO DNA SYNTHESIS STUDIES IN PANCREATIC ISLETS

Effect of 5-HT on Islet DNA Synthesis

Isolated islets in culture medium exhibited very low levels of [^{3}H]thymidine incorporation into DNA. Addition of EGF (10ng) caused a significant increase ($p<0.01$) in the islet DNA synthesis. 5-HT at 10^{-4}M concentration caused no significant change in the DNA synthesis from basal level. But at lower concentration, 10^{-8}M, 5-HT significantly ($p<0.01$) increased DNA synthesis. Addition of 10^{-4}M and 10^{-8}M 5-HT along with EGF caused a significant increase ($p<0.01$) in DNA synthesis when compared with EGF alone group. Addition of TGFβ1 (1ng/ml) caused no significant change in the basal level of DNA synthesis, while addition of 10^{-4}M and 10^{-8}M 5-HT along with TGFβ1 caused a significant increase ($p<0.05$) in DNA synthesis when compared with TGFβ1 alone group. Addition of TGFβ1 along with EGF caused no significant change in DNA synthesis (Figure - 41).

Effect of 8-OH DPAT on Islet DNA Synthesis

Addition of 8-OH DPAT (10^{-4}M) caused a significant decrease ($p<0.01$) in the DNA synthesis when compared with control. Addition of 10^{-4}M 8-OH DPAT along with EGF caused a significant increase ($p<0.01$) in DNA synthesis when compared with EGF alone group. TGFβ1 mediated islet DNA synthesis was increased significantly ($p<0.05$) by the addition of 10^{-4}M 8-OH DPAT to the primary islet culture (Figure - 42).

Dose-dependent Response of Islet DNA Synthesis to 8-OH DPAT

8-OH DPAT at lower concentrations, 10^{-8} and 10^{-6}M, significantly increased ($p<0.01$ & $p<0.001$) the DNA synthesis of primary islet in culture. There was a significant decrease ($p<0.01$) in DNA synthesis at higher concentration (10^{-4}M) of 8-OH DPAT (Figure - 43).

Dose-dependent Response of EGF Induced islet DNA synthesis to 8-OH DPAT

Addition of 8-OH DPAT at a concentration from 10^{-8}M to 10^{-4}M significantly increased ($p<0.01$ & $p<0.001$) the EGF mediated DNA synthesis of cultured islets. Maximum DNA synthesis was observed at 10^{-6} M 8-OH DPAT (Figure - 44).

Dose-dependent Response of TGFβ1 Induced islet DNA Synthesis to 8-OH DPAT

TGFβ1 mediated DNA synthesis was increased significantly at 10^{-8} ($p<0.001$), 10^{-6} ($p<0.001$), 10^{-5} ($p<0.01$) and 10^{-4}M ($p<0.01$) 8-OH DPAT. Maximum DNA synthesis was found at 10^{-8}M 8-OH DPAT (Figure - 45).

Effect of pertussis toxin on 8-OH DPAT mediated DNA synthesis: Pertussis toxin significantly inhibited potentiation of EGF effect induced by 8-OH DPAT at 10^{-8}M ($p<0.001$) and 10^{-4}M ($p<0.01$) (Figure - 46).

Effect of Mesulergine on islet DNA Synthesis

Addition of 5-HT (10^{-4}M) with mesulergine (10^{-4}M) and EGF 1ng/ml with mesulergine (10^{-4}M) caused a significant decrease ($p<0.001$) in the basal and EGF mediated DNA synthesis. TGFβ1 mediated islet DNA synthesis decreased significantly ($p<0.01$) by the addition of mesulergine to the primary islet culture (Figure - 47).

Dose-dependent Response of islet DNA Synthesis to Mesulergine

Mesulergine inhibited significantly the DNA synthesis of primary islets in culture induced by 5-HT from 10^{-8}M to 10^{-4}M ($p<0.01$ & $p<0.05$) (Figure - 48).

Dose-dependent Response of EGF Induced Islet DNA Synthesis to Mesulergine

Addition of mesulergine at a concentration from 10^{-8}M to 10^{-4}M dose dependently suppressed ($p<0.01$ & $p<0.001$) the EGF mediated DNA synthesis of cultured islets (Figure - 49).

Dose-dependent Response of TGFβ1 Induced islet DNA Synthesis to Mesulergine

Mesulergine at a concentration of 10^{-6}, 10^{-5} and 10^{-4}M significantly ($p<0.05$, $p<0.01$) decreased TGFβ1 mediated DNA synthesis in primary islet culture (Figure - 50).

DISCUSSION

The mechanisms regulating islet growths under different situations have been studied extensively (Corbett *et al.*, 1997). Most studies have been concerned with intraislet factors that are expressed during islet regeneration or triggering factors thought to be released by the neighboring parenchyma. A large number of growth factors and growth-stimulating peptides are expressed in or have stimulatory effects in the growing islets (Corbett *et al.*, 1997). Biogenic monoamines such as 5-HT and dopamine are present in higher amounts in the islets of young animals (Cegrell, 1968) that also show a larger proliferative capacity. 5-HT has short-term effects on ion channels and other effectors such as adenylyl cyclase but also have growth factor-like effects in developing brain (Lauder, 1993) and mitogenic effects on fibroblasts (Gerhardt & Van Heerikhuizen, 1997). Islet cell neogenesis can be reactivated by applying external stimuli such as partial pancreatectomy (90%) (Bonner-Weir, 1993). Partial pancreatectomy is an established model to study pancreatic regeneration (Pearson *et al.*, 1977).

DNA synthesis in pancreas after partial pancreatectomy

In this study we examined the regeneration of β-cells of the islets of Langerhans of the pancreas in weanling rats using partial pancreatectomy as the stimulus to regenerate. [^{3}H]thymidine incorporation into replicating DNA was used as a biochemical index for quantifying DNA synthesis during pancreatic regeneration after partial pancreatectomy. DNA synthesis was found to be increased 12 hrs after partial pancreatectomy. The maximal rate of [^{3}H]thymidine incorporation was observed at 72 hrs and declined at 7 days after partial pancreatectomy. The peak in the DNA synthesis is concordant with the previous reports (Pearson *et al.*, 1977;

Brockenbrough *et al.*, 1988). Enhanced β-cell function and proliferation maintains the normoglycemic level in rats during pancreatic regeneration (Leahy *et al.*, 1988).

Circulating insulin levels during pancreatic regeneration

Circulating insulin level was found to be increased during pancreatic regeneration. Insulin was reported to increase the cell proliferation of β-cells *in vitro* (Rabinovitch *et al.*, 1982). Previous studies suggest that the increase in the β-cell proliferation is related to the degree to which insulin biosynthesis and/or release is increased (Chick *et al.*, 1975; King & Chick, 1976). Insulin can stimulate β-cell replication directly possibly through a receptor for multiplication stimulating activity or another insulin like growth factor (Rabinovitch *et al.*, 1982). There are also reports about the increase in the insulin secretion after the partial pancreatectomy, besides maintaining the normoglycaemic level, it also helps the remaining β-cell mass to regain its original mass and volume by inducing cell division. The signal for islet cell proliferation is related to a long standing demand for increased insulin secretion (Dubuc, 1976). After major pancreatectomy in dogs, insulin treatment enhances the proliferation of the remnant pancreas and maintains endogenous insulin secretion for a long period, prolonging survival and promoting pancreatic regeneration (Ohashi, 1993).

5-HT content in the brain regions during pancreatic regeneration

Serotonin is involved in regulating cellular functions of central and peripheral nervous systems, endocrine and exocrine organs, as well as vascular and hematopoietic systems (Wilkinson & Dourish, 1991). An increase in 5-HT content was observed during active pancreatic proliferation in brain stem, cerebral cortex and hypothalamus. The increased contents in the cerebral cortex and hypothalamus were reversed to basal level when pancreatic DNA synthesis declined to control level. The

relationship between enhanced monoamine content in the ventromedial hypothalamus (VMH), a characteristic of hyperinsulinemic and insulin-resistant animals and islet dysfunction is already reported (Liang & Cincotta, 1999). The increase in brain 5-HT content may be due to increase in tryptophan uptake through the BBB with other neutral amino acids. Increased insulin in the plasma during pancreatic regeneration tends to release the tryptophan bound to albumin and hence increasing the concentration of free tryptophan in plasma (Trulson & Mackenzie, 1978). Central serotonergic neurons participate in the regulation of sympathetic nerve discharge. Brain serotonergic changes are reported to regulate autonomic nerve function in rats (Kuhn *et al.*, 1980). Thus our result suggests a close relationship between 5-HT level in brain regions and pancreatic islet cell growth. The increased 5-HT content in the brain regions during pancreatic regeneration may inhibit sympathetic nervous system and thus increases insulin secretion from pancreatic β-cells.

5-HT content in the pancreas and plasma during pancreatic regeneration

Pancreas is a rich source of 5-HT (Bird *et al.*, 1980). The 5-HT content in the pancreas decreased at the time of peak DNA synthesis. Low concentration of 5-HT in the pancreatic islets can stimulate insulin synthesis within the pancreatic islets. 5-HT is taken up into the insulin granules and secretes 5-HT/insulin in a pulsatile fashion on stimulation of pancreatic islet β-cells under physiologic conditions (Zhou & Misler, 1996). 5-HT induced a dose dependent hpoglycaemia and an increase in serum insulin levels (Sugimoto *et al*, 1990) due to a decreased uptake of 5-HT at high concentration. This shows that low concentration of 5-HT within the pancreatic islets has a role in the regulation of insulin secretion and islet cell proliferation during pancreatic regeneration

5-HT level in the plasma increased during pancreatic regeneration and it reversed to normal level by 7 days after pancreatectomy. Decreased 5-HT content in the islets may be responsible for the increased serum insulin levels. The increase in serum 5-HT levels during pancreatic regeneration suggests a decreased uptake of 5-HT into pancreas. Higher amounts of 5-HT promote proliferative capacity of the islets of young animals (Cegrell, 1968).

EPI and NE content decreased in plasma and adrenals during pancreatic regeneration

Norepinephrine and epinephrine concentrations decreased in the plasma and adrenals during regeneration of the pancreas. Sympathetic system is inhibitory to insulin secretion. 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 & Williams, 1966). 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). Activation of the splanchnic nerves innervating the adrenals results in the catecholamine release from chromaffin cells into the circulation.

Brain 5-HT_{1A} receptor alterations

Serotonin containing neurons are concentrated in the raphé nuclei of the brainstem and connect to the cerebral cortex, hypothalamus, and major autonomic nuclei, where they appear to exert broad regulatory control. Central serotonergic activity influences autonomic functions like thermogenesis, cardiovascular control, circadian rhythms and pancreatic function (McCall & Clement, 1990; Ramage, 2000; Liang & Cincotta, 1999). Serotonergic neurons act through the autonomic nervous

system or hypothalamic-pituitary axis to affect BP and key metabolic processes (Fuller, 1990).

Several studies have described the role of 5-HT_{1A} receptors in the neuroendocrine regulation (Fuller, 1990; Van de Kar, 1991). The relationship between the serotonergic and the sympathoadrenal system lead in turn to a control of both plasma glucose levels and insulin release (Chaouloff, 1990a). 5-HT_{1A} receptor agonist, 8-OH DPAT has been shown also to act on the endocrine system. 8-OH DPAT through activation of 5-HT_{1A} receptors decreases plasma insulin and increases basal plasma glucose levels in several rat strains, via a central mechanism of action (Chauloff & Jeanrenaud, 1987).

This effect of insulin release is attributed to regulation of catecholamine-epinephrine and norepinephrine, plasma levels because of the stimulation or inhibition of the adrenal medulla. Pre-ganglionic sympathetic nerve fibres pass from the intermediolateral horn cells of the spinal chord through the sympathetic chains, through the splanchnic nerves and synapse in the adrenal medullae. They synapse on cells that are derived from nervous tissue that secrete nor-adrenaline and adrenaline directly into the circulation. Wallin *et al* (1981) have demonstrated that measurements of peripheral sympathetic nerve activity correlate well with measurements of plasma nor-adrenaline. Epinephrine and norepinephrine have long been known to inhibit insulin secretion *in vivo* (Porte Jr D, 1967b); Brunicardi, 1995) and *in vitro* (Brunicardi, 1995; Sorenson *et al.*, 1979).

In our present study we analysed the receptor binding parameters and expression of the 5-HT receptors in sham operated and pancreatectomised rats. Scatchard analysis was performed for determining the B_{max} and K_d of these receptors (Scatchard, 1949). Receptor mRNA status was analysed by RT-PCR technique using specific primers. 5-HT_{1A} receptor binding parameters were analysed using the receptor specific agonist [^{3}H]8-OH DPAT (Nenonene *et al.*, 1994). 5-HT_{1A}

receptor has two affinity sites and the double affinity status of the receptor was confirmed by displacement analysis using 8-OH DPAT.

Cerebral cortex receives an extensive 5-HT input originating from midbrain raphe 5-HT neurons (Tork, 1990). Scatchard analysis of the cerebral cortex showed that the affinity of the high affinity receptor binding decreased in 72 hrs pancreatectomised rats. The low affinity receptor number and affinity decreased during active pancreatic proliferation. $Ki_{(H)}$ increased in 72 hrs pancreatectomised rats along with an increase in the log (EC_{50})-1 indicating a shift in high affinity towards low affinity. $Ki_{(L)}$ and log (EC_{50})-2 showed an increase in 72 hrs pancreatectomised rats indicating a shift in affinity of the low affinity site towards much lower affinity. We observed an increased 5-HT content in the cerebral cortex during pancreatic regeneration. These results indicate that the increased 5-HT is able to down regulate the $5\text{-}HT_{1A}$ receptors in the cerebral cortex. Adrenocortical secretion in response to intraperitoneal and intracerebroventricular administration of the $5\text{-}HT_{1A}$ receptor agonist 8-OH DPAT involves a sympathomedullary activation (Saphier & Welch, 1994). Since our results indicate a decreased $5\text{-}HT_{1A}$ receptor binding, this reduces the sympathetic stimulation and increases insulin secretion from pancreatic islets mediated by a decreased norepinephrine release from adrenal glands. Our RT-PCR studies were also concordant with the receptor studies. Decreased expression of $5\text{-}HT_{1A}$ mRNA is reported in long-term adrenalectomised rats in the dentate gyrus (Liao *et al.*, 1993). The transduction action of the $5\text{-}HT_{1A}$ receptor is usually associated with a decrease in adenylyl cyclase activity. In cultures of hippocampal neurons, $5\text{-}HT_{1A}$ agonists block the forskolin-induced formation of p-CREB, an important transcription factor increased by cAMP (Nishi & Azmitia, 1999). Receptors that result in alterations in the cAMP or Ca^{2+} pathways would be expected to result in altered CREB phosphorylation and altered transcriptional

activity (Hyman & Nestler, 1993). Regulation of these transcription factors through 5-HT_{1A} receptors regulates gene expression in the brain.

The midbrain raphe nuclei of brain stem are the source of wide spread serotonergic innervation throughout the brain (Azmitia & Gannon, 1986). In the case of brain stem, Scatchard analysis revealed an increased K_d of the high affinity 5-HT_{1A} receptor indicating a decreased affinity of the receptor in 72 hrs pancreatectomised rats. The low affinity receptor number decreased in 72 hrs pancreatectomised rats without any change in the affinity. The decrease in receptor number during pancreatic proliferation may be due to increased 5-HT brain levels in the brain stem of these rats, since the 5-HT_{1A} receptor expression is sensitive to autoinhibition (Nishi & Azmitia, 1999). In 72 hrs pancreatectomised rats $Ki_{(H)}$ and log (EC_{50})-1 increased indicating a shift in high affinity towards lower affinity side. Our RT-PCR studies also revealed that during pancreatic cell proliferation 5-HT_{1A} receptors are getting down regulated in the brain stem.

An increased sympathetic activity due to the activation of 5-HT_{1A} receptors will induce increased EPI output from the adrenal medulla that will inhibit insulin secretion (Bauhelal & Mir, 1990, 1993). This was proved by injecting the specific 5-HT_{1A} receptor agonist 8-OH DPAT to normal rats. The rats showed a very rapid increase in blood glucose level that reached its peak within 30 min. A similar observation was reported from a number of laboratories (Laude *et al.*, 1990; Bauhelal *et al.*, 1990).

The results of the present study indicate that pancreatectomy trigger a regulatory effect on the 5-HT_{1A} receptor system in the brain stem and cerebral cortex. 5-HT_{1A} receptors were down regulated both in the cerebral cortex and brain stem. An increase in local release of 5-HT may be responsible for the decrease in [^{3}H]8-OH DPAT receptor binding 72 hrs after pancreatectomy. The decreased 5-HT_{1A} receptor binding reduces the sympathetic stimulation and epinephrine release

from adrenal glands, thereby increasing insulin secretion from pancreatic islets. The adrenaline releasing effect of 8-OH DPAT antagonised by the mixed 5-HT_{1A}-β adrenoreceptor antagonist pindolol has been already reported (Chauloff *et al.*, 1990 b). Opposite effects occur with the use of 5-HT depending on whether or not cellular cAMP is elevated and this in turn, depends on the activity of cellular phosphodiesterase (Fanburg & Lee, 1997). Thus, the 5-HT_{1A} receptor down regulation in the brain stem and cerebral cortex during pancreatic regeneration increases insulin secretion from pancreatic β-cells which is mediated through epinehrine release from adrenal medulla.

The hypothalamus plays a central role in the integration of neurohormonal function (Oommura & Yoshimatsu, 1984). 5-HT is known to influence a number of hypothalamic associated functions such as sleep, thermoregulation and neuroendocrine function. 5-HT exerts a modulatory effect on the hypothalamic pituitary-adrenal (HPA) system (Fuller & Snoddy, 1990). When we studied the 5-HT_{1A} receptor status in the hypothalamus, the affinity of the high affinity receptor decreased in 72 hrs pancreatectomised rats as evidenced by the K_d of the receptor. The density and affinity of the low affinity 5-HT_{1A} receptor decreased during active regeneration, i.e., in 72 hrs after pancreatectomy. Both affinity sites shifted toward their corresponding lower affinity sites as indicated by the increase in the $Ki_{(H)}$, $Ki_{(L)}$, log (EC_{50})-1 and log (EC_{50})-2 in pancreatectomised rats. RT-PCR analysis also revealed a down regulation of this receptor during active islet cell proliferation.

Serotonergic neurons innervate hypothalamic neurons that regulate the secretion of several hormones. 5-HT containing neurons in the midbrain directly innervate corticotropin-releasing hormone (CRH)-containing cells located in paraventricular nucleus of the hypothalamus (Hanley & Van de Kar, 2003). Direct synaptic connections between serotonergic nerve terminals and CRH neurons in the hypothalamic PVN have been demonstrated at the electron microscopic level

(Liposits *et al.*, 1987). The CRH neurons in turn stimulate the secretion of ACTH from the anterior lobe of the pituitary gland, which in turn stimulates corticosterone secretion from the adrenal cortex. Other evidence also points to direct serotonergic innervation of oxytocin-containing neurons in the hypothalamic PVN (Saphier, 1991). Additionally, several lesion studies have provided evidence that the serotonergic stimulation of the secretion of ACTH, corticosterone, prolactin, oxytocin and renin is mediated by neurons in the hypothalamic PVN (Bagdy & Makara, 1994).

5-HT_{1A} subtype is involved in the neural regulation of hypothalamo-pituitary-adrenocortical (HPA) secretion (Saphier & Zhang, 1993). Stimulation of 5-HT_{1A} receptors activates the HPA axis (Gilbert *et al.*, 1988). Activation of 5-HT_{1A} receptors has been shown to induce corticotropin (ACTH) and corticosteroid release in rodents (Gilbert *et al.*, 1988), an effect antagonised by pindolol, a stereoselective 5-$HT_{1A/1B}$ blocker (Lesch, *et al*, 1990). 5-HT_{1A} receptor agonists elevate plasma corticosterone and EPI concentrations. 5-HT and 8-OH DPAT stimulate the release of corticotropin-releasing factor (CRF) (Jones *et al.*, 1976). Our data suggest that the HPA axis can be inhibited by the down regulation of 5-HT_{1A} receptors. In addition to its well known ACTH releasing activity, CRF is also known to stimulate directly sympathetic tone to the adrenal medulla via a central site of action (Fisher, 1989), manifested by increase in efferent adrenal nerve activity, plasma adrenaline concentration and an increase in BP. (Brown & Fisher, 1983; Brown *et al.*, 1985). Since 5-HT_{1A} receptors inhibit HPA axis its down-regulation leads to a decreased CRF release and sympathetic activity which is stimulatory to insulin secretion. 5-HT_{1A} receptor antagonist pindolol blocked elevation of the plasma ACTH concentration induced by 5-HT_{1A} receptor subtype mediated release of CRH from the paraventricular nuclei of the hypothalamus in rats (Pan & Gilbert, 1992). 5-HT_{1A} receptors are linked through G proteins to second messenger enzymes, each receptor can stimulate the release of multiple molecules of oxytocin and CRH. CRH also is

linked *via* G proteins to effector enzymes. Hence, activation of each CRH receptor on corticotrophs in the pituitary will lead to the release of multiple molecules of ACTH, which can further stimulate the release of even more molecules of corticosterone (Charmers & Watson, 1991)

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 ventro-medial hypothalamic nuclei is considered as the sympathetic centre and the stimulation of this area decreases insulin secretion (Helman. *et al.*, 1982). Studies of *in vivo* pancreatic nerve activity after VMH lesions show increased parasympathetic and decreased sympathetic nerve firing rates (Oommura & Yoshimatsu, 1984). Decreased 5-HT_{1A} receptor binding observed in the hypothalamus reduces the sympathetic nerve stimulation thus reducing the inhibitory effect of EPI on insulin secretion

Brain 5-HT_{2C} receptor alterations

The 5-HT_{2C} receptor displays a heterogeneous distribution in the CNS (Pazos & Palacios, 1985). 5-HT_2 receptor stimulation has been shown to trigger numerous biochemical and behavioral effects, including phosphatidyl inositol hydrolysis, platelet aggregation, head twitches, and vasoconstriction (Conn *et al.*, 1987). Stimulation of centrally located 5-HT_2 receptor leads to adrenal epinephrine release that elevates plasma glucose levels and inhibits insulin release (Veronique Baudrie & Chauloff, 1992). Acute administration of the 5-$HT_{2C/2B}$ receptor agonist 1-(3-chlorophenyl) piperazine (mCPP) induced hyperglycaemia in rats and it is mediated by the activation of 5-$HT_{2C/2B}$ receptors. The effects of mCPP are considered to be connected to the activation of the sympathoadrenomedullary system and catecholamine release (Sugimoto *et al.*, 1996). Pretreatment with the 5-HT_{1C}

ceptor antagonist ritanserine dose dependently attenuated EPI and NE responses, suggesting 5-HT_{1C} receptor mediated mechanism (Bagdy *et al.*, 1989)

When we analysed the 5-HT_{2C} receptor status in the cerebral cortex, we found that 5-HT_{2C} receptor number decreased significantly in 72 hrs after pancreatectomy as indicated by a decreased B_{max}. There was no shift in affinity of the receptor in 72 hrs pancreatectomised rats as indicated by the unchanged Ki and log (EC_{50}). RT-PCR analysis also confirmed the receptor data. 5-HT_2 receptor stimulation has neuroendocrinological consequences, as exemplified by the activation of the corticotropic and sympathoexcitation (McCall & Harris, 1988; Bagdy *et al.*, 1989). Our results showed a decreased binding of 5-HT_{2C} receptors in the cerebral cortex, which reduces the sympathoexitatory effect. This decreased expression of 5-HT_{2C} receptors observed in the cerebral cortex decreases the sympathetic effect, which is stimulatory to insulin secretion. Increased plasma levels of insulin and leptin analogous to Type 2 diabetes in mice lacking 5-HT_{2C} suggests that the 5-HT_{2C} receptor has a role in tonic inhibition of neuronal excitability (Tecott *et al.*, 1995)

In the brain stem, Scatchard analysis revealed a decreased B_{max} and increased K_d of the high affinity 5-HT_{2C} receptor indicating a reduction in the receptor density as well as the affinity of the receptor in 72 hrs pancreatectomised rats. The expression of different serotonergic receptor mRNAs appears to change during development and 5- HT receptors can be regulated by a variety of exogenous agents (Roth *et al.*, 1991) Down-regulation of receptor binding sites in choroid plexus cells is seen after agonist treatment (Barker & Sanders-Bush, 1993). A decreased mRNA expression as revealed by RT-PCR analysis confirmed our receptor data. Down-regulation of the receptor by 5-HT is associated with an equivalent decrease in the level of receptor mRNA (Ivins & Molinoff, 1991). Previous studies showed that methysergide, which acts as a partial 5-HT_1 receptor agonist and as a

5-HT_{2C} receptor antagonist, potentiates both insulin and glucagon release (Marco *et al.*, 1976). Thus the down-regulation of the 5-HT_{2C} receptors observed in the brai stem during pancreatic regeneration suggests the stimulatory role of insulin secretio mediated by sympathetic system. This indicates that regulation of receptor activit probably occurs at multiple points in the metabolic cycle of the receptor protein.

5-HT_{2C} receptor number and receptor affinity towards its ligand decreased i hypothalamus of 72 hrs pancreatectomised rats. The analysis of Ki and log (EC_{5} showed an inecreased values in 72 hrs after pancreatectomy indicating a shift i affinity of the receptor towards lower affinity. Our RT-PCR results were als consistent with the receptor data. 5-HT has been shown to control the activity c hypothalamic CRF neurons and pituitary corticotrope cells through activation c 5-$HT_{(2A/2C)}$ receptor subtypes (Contesse *et al.*, 2000). Activation of centrally locate 5-HT_{2C} leading to hypoglycaemia due to increased insulin secretion is alread reported (Sugimoto *et al.*, 1996). Our results showed a decreased 5-HT_{2C} binding i the hypothalamus. This decreases the CRF release which in turn decreases th plasma EPI concentration. CRF mediates EPI responses after several stimu (Brown, 1985). Our data suggest that EPI response might be mediated by CR release. The increase in 5-HT turnover in the rat hypothalamus during insulir induced hypoglycaemia described earlier suggests that 5-HT might be the mediat of CRF release and EPI response (Yehuda & Meyer, 1984).

Thus, central 5-HT_{1A} and 5-HT_{2C} receptors can act as stimulators c pancreatic β-cell proliferation depending upon the NE and EPI release by adren medulla through which its function is mediated. The circulating NE and EPI levels i the experimental groups were also consistent with our receptor data.

5-HT_{1A} and 5-HT_{2C} receptor alterations in pancreatic islets

Neurotransmitter receptors are usually restricted to neuronal cells. However, neurotransmitters have been shown to stimulate or inhibit proliferation of non-neuronal cells by activating receptors coupled to different second messenger pathways (Lauder, 1993). 5-HT has been found to promote cell proliferation in various cell types. In aortic smooth muscle cells, 5-HT induced mitogenesis was comparable with that of human-platelet derived growth factor (Nemeck *et al*, 1986).

Scatchard analysis, displacement analysis and RT-PCR studied 5-HT_{1A} receptor functional status of pancreatic islets. The number of 5-HT_{1A} receptor significantly increased in 72 hrs pancreatectomised rats. The affinity of the receptor decreased in 7 days pancreatectomised rats. $Ki_{(H)}$ and $Ki_{(L)}$ increased in 7 days pancreatectomised rats indicating the shift in affinity of the receptor towards low affinity sites. Our results revealed that pancreatic 5-HT_{1A} receptor status is getting up regulated during pancreatic regeneration. This indicates that up-regulation of 5-HT_{1A} receptor expression is facilitating the islet cell proliferation. In pancreatic cell line, activation of pertussis toxin sensitive $5HT_{1A/1B}$ receptors stimulate proliferation through the activation of PLC and PKC that resulted in the down regulation of cAMP (Ishizuka *et al.*, 1992). Agonists at 5-HT_{1A} receptors inhibit adenylyl cyclase and activate phosphoinositide hydrolysis in HeLa cells (Raymond *et al.*, 1991). Moreover the receptor affinity was found to be decreased by 7 days after partial pancreatectomy, which may be a compensatory mechanism for increased receptor number. These receptor data are well supported by our RT-PCR analysis. The translation of the mRNA results in increased protein synthesis finally leading to either growth or differentiation (Fantl, 1993).

5-HT_{2C} receptor number was increased in the islets of 72 hrs and 7 days pancreatectomised rats. This shows the up regulation of 5-HT_{2C} receptors during active proliferation of islet cells. Our present results are well supported by the fact

that 5-HT_{2C} receptor can function as a protooncogene in NIH-3T3 cells. NIH-3T3 cells that express high levels of 5-HT_{2C} receptor form foci in cell culture (Julius 1989). The 5-HT_2 receptors are coupled to phospho-inositide turnover and diacylglycerol formation, which activates protien kinase C (PKC), an important second messenger for cell division (DeCorcelles *et al.*, 1984). Displacement studies on [^{3}H]5-HT binding to crude membranes from control and regenerating liver tissue using cold ketanserin and spiperone, showed an increased involvement of 5-HT_2 receptors in the regenerating liver during the DNA synthetic phase (Sudha, 1997).

Serotonergic stimulation of insulin synthesis and secretion from pancreatic β-cells *in vitro*

Signal-transduction in the pancreatic β-cell and thereby the insulin secretory process is regulated by a sophisticated interplay between glucose and a plethora of additional factors including other nutrients, neurotransmitters, islet generated factors and systemic growth factors. The coupling of glucose metabolism to electrical activity remains central in all models of β-cell stimulus-secretion coupling. The resting membrane potential of the β-cell is set by the ATP-sensitive potassium (K_{ATP}) channel (Ashcroft & Rorsman, 1990). Incubation of the pancreatic β-cells with stimulatory glucose concentrations leads to the activation of a cascade of reactions, which ends in the exocytosis of stored insulin. This complex of processes starts with the uptake of glucose by the β-cell high-K_m/low affinity glucose transporter GLUT2 and proceeds with the conversion of glucose into glucose-6-phosphate by the β-cell isoform of glucokinase (Randel, 1993; Matschinsky, 1996). Metabolism of glucose in glycolysis and the Krebs cycle results in the generation of ATP. Elevation in the ATP/ADP ratio leads to closure of the K_{ATP}, which in turn results in depolarization of the plasma membrane. The subsequent opening of voltage-gated L-type Ca^{2+} channels leads to an increase in the cytoplasmic free Ca^{2+} concentration, $[Ca^{2+}]_i$ which promotes insulin secretion (Berggren & Larsson, 1994).

Pancreatic islet is considered as a tissue rich in 5-HT (Bird *et al.*, 1980). 5-HT has a direct effect on the insulin secretion from the pancreatic islets (Peschke *et al.*, 1997). Serotonergic receptors play a role in the regulation of blood glucose by facilitating insulin release (Jun Yamada *et al.*, 1990). 5-HT stimulated the output of insulin in the presence of a low concentration of glucose. When the islets were incubated with glucose at a higher concentration there was a lower insulin release in the presence of 5-HT than that obtained with glucose alone at the same concentration (Lechin *et al.*, 1975). High concentrations of NE, DA, and 5-HT in the pancreatic islets can decrease glucose-stimulated insulin secretion (Zern *et al.*, 1980). 5-HT dose dependently inhibited insulin secretion from pancreatic islets in the presence of 20mM glucose. This result indicates that although 5-HT may help in the maintenance of the blood sugar level in normal pancreas by increasing insulin secretion it may also aggravate the hyperglycaemia observed in diabetes mellitus (Adeghate *et al.*, 1999). 5-HT inhibits glucose-induced insulin release by affecting early steps in the β-cell stimulus-secretion coupling (Lindstrom & Sehlin, 1983).

5-HT_{1A} agonist, 8-OH DPAT, at low concentrations (10^{-6}M & 10^{-7}M) stimulated insulin secretion in the presence of 4mM glucose. But at high concentration (10^{-4}M) 8-OH DPAT inhibited the insulin secretion from pancreatic islets. There was a dose dependent inhibition of insulin secretion by 8-OH DPAT in the presence of 20mM glucose. In the isolated perfused pancreas of the rat, 8-OH-DPAT, at 10^{-8}M and 10^{-7}M, concentrations known to activate 5-HT_{1A} receptors *in vitro* (Bouhelal *et al.*, 1990), this induces glucose-stimulated insulin release

The 5-HT_{2C} receptors stimulate phospholipase C, and increases IP_3 and DAG (Conn *et al.*, 1986) which leads to increased intracellular calcium (Watson *et al.*, 1995). IP_3 mediates Ca^{2+} mobilization from intracellular Ca^{2+} stores and plays an important role in insulin secretion from pancreatic β-cells (Laychock, 1990). IP_3 exerts its action through receptors that are ligand-activated, Ca^{2+} selective channels.

IP_3 receptors have been localized to the endoplasmic reticulum, nucleus and insulin granules (Yoo *et al.*, 1990). We examined the effects of 5-HT_{2C} receptor antagonist, mesulergine on insulin secretion. Mesulergine inhibited 5-HT mediated insulin secretion at both 4mM and 20mM glucose concentrations. Our results indicate that mesulergine inhibits 5-HT_{2C} receptor activity and blocked the increase in intracellular calcium and thus inhibited insulin release.

Twenty four hrs islet cell culture was done to study the long-term effect of 5-HT, 5-HT_{1A} and 5-HT_{2C} receptors on insulin synthesis and release from the isolated islets. Long-term insulin secretion studies showed similar changes as in the 1 hour incubations. 5-HT stimulated insulin secretion at lower concentrations and inhibited insulin secretion at higher concentration in the presence of 4mM glucose. There was a dose dependent inhibition of insulin secretion by 5-HT from pancreatic islets in the presence of 20mM glucose. In the long-term studies 8-OH DPAT showed stimulatory effect at lower concentrations and inhibitory effect at higher concentration at 4mM glucose. Similar to 1 hour secretion studies high concentration of 8-OH DPAT abolished the glucose stimulated insulin secretion. Mesulergine inhibited 5-HT mediated insulin secretion at both 4mM and 20mM glucose concentration.

Long-term and 1 hr culture showed stimulatory and inhibitory role of 5-HT, 5-HT_{1A} and 5-HT_{2C} receptors on insulin secretion.

Effect of 5-HT, 8-OH DPAT and mesulergine on islet DNA synthesis

Effect of 5-HT on islet DNA synthesis

Higher concentration of 5-HT (10^{-4}M) when added to primary islet culture did not increase DNA synthesis but was able to increase DNA synthesis at lower concentration. In the absence of growth factors, 5-HT is potent at inducing cell-cycle progression of L cells expressing the 5-HT_{2B} receptor (LM6) (Canan *et al.*, 2000).

Mene *et al* suggetsed activation of a 5-HT_2 receptor in rat mesangial cells to acount for 5-HT induced cell proliferation (Mene *et al.*, 1991). It also stimulated the EGF and TGFβ1 mediated DNA synthesis. EGF and TGF-β are known mitogens for cultured vascular smooth muscle cells (Huang *et al.*, 1992). There are several reports on effects of growth factors in the normal β-cell growth. EGF was shown to stimulate [^{3}H]-thymidine incorporation in islets (Sieradzski *et al.*, 1987). Furthermore, a recent report showed that EGF was an important factor for pancreas precursor cell proliferation *in vitro* (Corentin *et al.*, 2001), and that islet cell migration and differentiation were impaired in the mice lacking EGF receptors (Miettinen *et al.*, 2000). These findings suggest EGF is important in the growth and differentiation of islet cells.

Effect of 8-OH DPAT on islet DNA synthesis

8-OH DPAT is the specific agonist of the 5-HT_{1A} receptor. We used 8-OHDPAT to study the 5-HT_{1A} receptor mediated effect on DNA synthesis of islets kept in primary culture. 8-OH DPAT at a concentration of 10^{-4} M was enough to inhibit the basal DNA synthesis but it induced EGF mediated DNA synthesis in primary islet cultures. In addition to this it enhanced the TGFβ1 mediated DNA synthesis. When we studied the dose dependent effect of 8-OH DPAT in primary islet DNA synthesis, we found a dose dependent inhibition from 10^{-6}M to 10^{-4}M. 8-OH DPAT at a concentration of 10^{-8}M was also found to increase DNA synthesis. This may be due to the activation of the high affinity receptors of 5-HT_{1A} receptor. In addition to increasing the DNA synthesis by itself, it increased the EGF mediated DNA synthesis from 10^{-8}M to 10^{-4}M significantly. DNA synthesis was found to be maximum at 10^{-6}M concentration. 8-OH DPAT also enhanced the TGFβ1 mediated DNA synthesis in a similar trend as that of 8-OH DPAT alone. Thus from our results it is very clear that 5-HT_{1A} receptor mediates stimulation of DNA synthesis *in vitro*.

G-proteins that are coupled to 5-HT_{1A} receptors are PTX sensitive (Raymond *et al.*, 1991). Pertussis toxin inhibited potentiation of EGF effect induced by 8-OH DPAT. In transfected NIH-3T3 cells, transforming and mitogenic effects of 5-HT_{1A} agonists involve a pertussis toxin-sensitive G protein but do not seem to be linked to adenylyl cyclase inhibition (Varrault, *et al.*, 1992). Activation of ERK2 by the 5-HT_{1A} receptor-selective agonist (8-OH-DPAT) was inhibited completely by pertussis toxin (Daniel *et al.*, 1996)

Effect of mesulergine on islet DNA synthesis

We have studied the role of 5-HT_{2C} receptor in mediating the islet DNA synthesis by blocking the 5-HT_{2C} receptor using mesulergine. Addition of mesulergine to the primary islet culture resulted in a decrease in the basal and EGF mediated DNA synthesis and enhanced the TGFβ1 mediated DNA synthesis suppression. It also inhibited the basal DNA synthesis induced by 5-HT from (10^{-8}M to 10^{-4}M) and EGF mediated DNA synthesis of primary islets in culture. Mesulergine enhanced the TGFβ1 mediated DNA synthesis inhibition at a concentration from 10^{-8}M to 10^{-4}M. Thus, mesulergine was able to completely inhibit the EGF mediated DNA synthesis indicating the regulatory role of 5-HT_{2C} receptor in islet cell proliferation. Our results are well supported by the reports that in NIH-3T3 cells 5-HT_{2C} receptor functions as a protooncogene. Moreover the formation of foci is dependent on activation of the 5-HT_{2C} receptor by 5-HT (Julius, 1989).

SUMMARY

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

2. Primary cultures of pancreatic islets were used as the *in vitro* system to study pancreatic islet cell proliferation

3. [^{3}H]thymidine incorporation was used as an index for pancreatic DNA synthesis. DNA synthesis was peaked at 72 hrs after partial pancreatectomy and reversed to control level by 7 days.

4. 5-HT content was analysed using HPLC. It increased in the brain regions during active islet cell proliferation

5. EPI and NE contents were analysed using HPLC. It decreased in the adrenals during active pancreatic islet regeneration. Plasma EPI and NE level also decreased during pancreatic regeneration.

6. 5-HT receptor functional status was analysed by Scatchard and displacement analysis using [^{3}H] ligands. Receptor analysis was confirmed by studying the mRNA status of the corresponding receptor using RT-PCR. 5-HT_{1A} and 5-HT_{2C} receptors were down regulated in brain regions during active islet cell proliferation.

7. Pancreatic islet 5-HT content decreased in 72 hrs pancreatectomised rats. Pancreatic islet 5-HT_{1A} receptor up regulation was observed during islet DNA synthesis .5-HT_{2C} receptor up regulation was also found during pancreatic regeneration.

8. *In vitro* insulin secretion study showed that low concentration of 5-HT and 5-HT_{1A} agonist, 8-OH DPAT, induced glucose stimulated insulin secretion from pancreatic islets. 5-HT_{2C} antagonist, mesulergine, inhibited glucose induced insulin secretion.

9. *In vitro* DNA synthesis studies showed that activation of 5-HT_{1A} receptor b adding 8-OH DPAT, a specific agonist, induced islet DNA synthesis. Also, th addition of mesulergine, a specific antagonist of 5-HT_{2C} receptor resulted in th inhibition of DNA synthesis.

Thus, the regulation of 5-HT_{1A} and 5-HT_{2C} receptors in the brain an pancreatic islets plays an important role in insulin secretion and islet cell proliferatio during pancreatic regeneration

CONCLUSION

Our findings demonstrate that alterations of 5-HT_{1A} and 5-HT_{2C} receptor function and gene expression in the brain stem, cerebral cortex and hypothalamus play an important role in the sympathetic regulation of insulin secretion during pancreatic regeneration. Though many reports are there implicating the functional interaction between brain 5-HT and the sympathoadrenal system, the involvement of specific receptor subtypes in regulating sympathoadrenal system during pancreatic regeneration has not given emphasis. We observed an increased 5-HT content and downregulation of 5-HT_{1A} and 5-HT_{2C} receptors in the cerebral cortex, brain stem and hypothalamus. RT-PCR analysis confirmed the receptor data in the brain regions. The relationship between 5-HT receptors and adrenal catecholamine release is much more homogenous. Thus, downregulation of 5-HT_{1A} or 5-HT_{2C} receptor leads to decreased adrenomedullary catecholamine release. Plasma NE and EPI levels of different experimental groups were in accordance with the functioning of the 5-HT_{1A} and 5-HT_{2C} receptors. These relationship between the serotonergic and the sympathoadrenal system lead in turn to a control of insulin release. In addition, receptor binding studies and RT-PCR analysis revealed that during pancreatic regeneration 5-HT_{1A} and 5-HT_{2C} receptors were up regulated in pancreatic islets. This suggests a stimulatory role for 5-HT_{1A} and 5-HT_{2C} receptors in islet cell proliferation i.e., the up regulation of this receptor facilitates proliferation. Insulin secretion study showed that 5-HT_{1A} receptor agonist, 8-OH DPAT was stimulatory to insulin secretion at lower concentration and inhibitory at higher concentration. Mesulergine blocked the insulin secretary potential at all concentrations. *In vitro* DNA synthesis studies revealed that 5-HT_{1A} receptor agonist, 8-OH DPAT inhibited DNA synthesis at higher concentration and stimulated DNA synthesis at lower concentration. 5-HT_{2C} receptor antagonist, mesulergine inhibited the pancreatic islet DNA synthesis.

Also, 8-OH DPAT and mesulergine enhanced the mitogenic effect mediated by EGF and TGFβ1. Thus, we conclude that brain and pancreatic 5-HT_{1A} and 5-HT_{2C} receptor gene expression modulates pancreatic endocrine function and islet cell proliferation during pancreatic regeneration. This will have immense clinical significance in the therapeutic applications of diabetes.

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Table-1

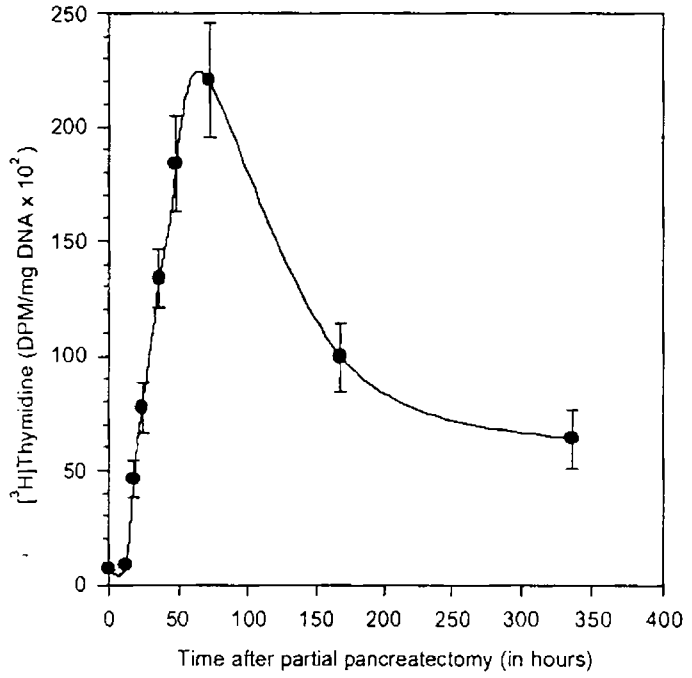
Body weight (gm) and blood glucose level (mg/dL) of experimental rats

Animal status	Body weight (gm)	Glucose level (mg/dL)
Sham	89 ± 2	99.6 ± 6.6
72 hrs pancreatectomy	84 ± 3	85.3 ± 6.1
7 days pancreatectomy	94 ± 3	93.4 ± 6.2

Values are mean ± SEM of 4-6 separate experiments

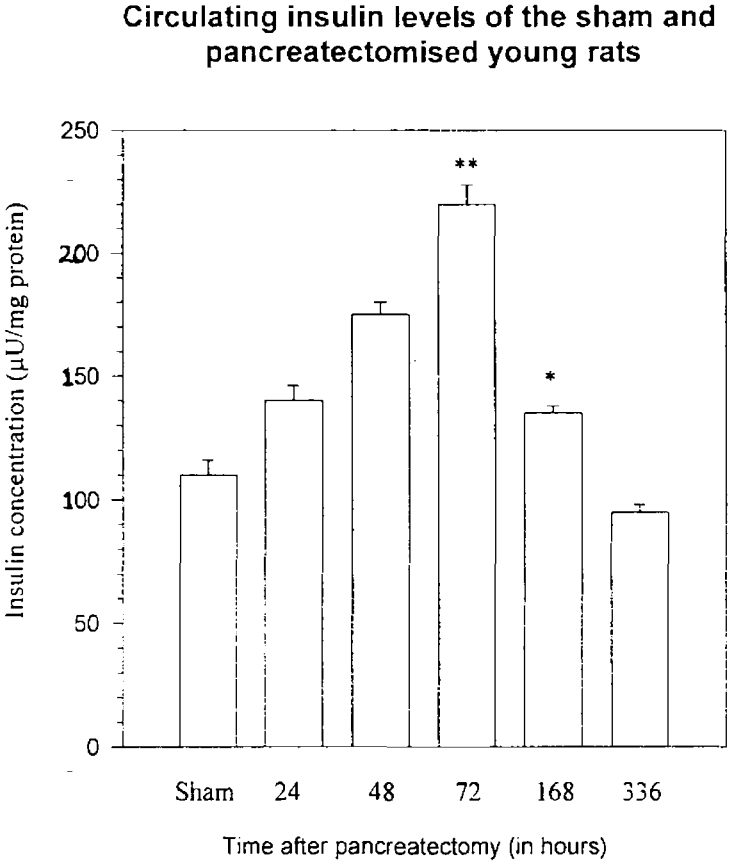
Figure-1

DNA synthesis in the regenerating pancreas of experimental rats



Values are mean ± SEM of 4-6 separate experiments

Figure-2



Values are mean ± SEM of 4-6 separate experiments

*P<0.05, **P<0.01 when compared with sham.

Table-2

5-HT content (nmoles/g wet weight of tissue) in the cerebral cortex, brain stem and hypothalamus of experimental rats

Animal status	Cerebral cortex	Brain stem	Hypothalamus
Sham	0.25 ± 0.07	0.28 ± 0.04	0.31 ± 0.04
72 hrs pancreatectomy	0.62 ± 0.09**	1.12 ± 0.03*	1.08 ± 0.14**
7 days pancreatectomy	0.19 ± 0.06††	0.80 ± 0.07*	0.47 ± 0.05††

Values are mean ± SEM of 4-6 separate experiments

**P<0.01, *P<0.05 when compared with sham

††P<0.01 when compared with 72 hrs.

Table-3

5-HT and 5-HIAA content (nmoles/g wet weight of tissue) in the pancreas of experimental rats

Animal status	5-HT	5-HIAA	5-HIAA/5-HT
Sham	0.69 ± 0.09	0.20 ± 0.01	0.30 ± 0.05
72 hrs pancreatectomy	0.23 ± 0.04*	0.48 ± 0.07*	2.1 ± 0.08***
7 days pancreatectomy	0.67 ± 0.04†	0.25 ± 0.01†	0.67 ± 0.04*†††

Values are mean ± SEM of 4-6 separate experiments

*P<0.05, ***P<0.001 when compared with sham.

†p<0.05, †††P<0.001 when compared with 72 hrs.

Table-4

5-HT, Norepinephrine and Epinephrine level (nmoles/ml of plasma) in the plasma of experimental rats

Animal status	5-HT	Norepinephrine	Epinephrine
Sham	1.3 ± 0.10	4.80 ± 0.48	5.75 ± 0.11
72 hrs pancreatectomy	2.2 ± 0.20**	2.86 ± 0.01*	1.50 ± 0.32***
7 days pancreatectomy	0.95 ± 0.10††	4.21 ± 0.91†	4.95 ± 0.17†††

Values are mean ± SEM of 4-6 separate experiments

*P<0.05, **P<0.01, ***P<0.001 when compared with sham

†P<0.05, ††P<0.01, †††P<0.001 when compared with 72 hrs

Table-5

Norepinephrine and Epinephrine content (nmoles/g wet weight of tissue) in the adrenals of experimental rats

Animal status	Norepinephrine	Epinephrine
Sham	475 ± 29	3255 ± 210
72 hrs pancreatectomy	186 ± 23***	1478 ± 72***
7 days pancreatectomy	421 ± 31†††	2984 ± 107†††

Values are mean ± SEM of 4-6 separate experiments

***P<0.001 when compared with sham, †††P<0.001 when compared with 72hrs

Figure-3

Scatchard analysis of high affinity [^{3}H]8-OH DPAT receptor binding against 5-HT in the cerebral cortex of rats

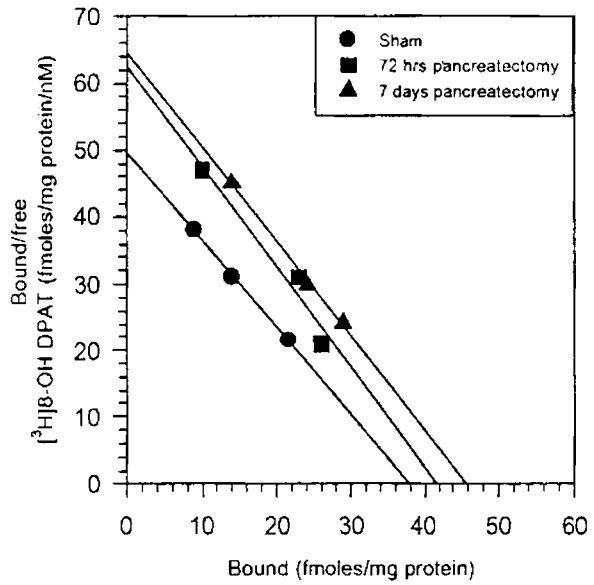


Table-6
[^{3}H]8-OH DPAT high affinity receptor binding parameters in the cerebral cortex of rats

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	39.5 ± 2.5	0.72 ± 0.05
72 hrs pancreatectomy	45.0 ± 5.0	1.03 ± 0.05**††
7 days pancreatectomy	45.0 ± 5.0	0.73 ± 0.05

Values are mean ± S.E.M. of 4-6 separate experiments
**p<0.01 when compared with sham
††p<0.01 when compared with 7 days pancreatectomy

Figure-4

Scatchard analysis of low affinity [^{3}H] 8-OH DPAT receptor binding against 5HT in the cerebral cortex of rats

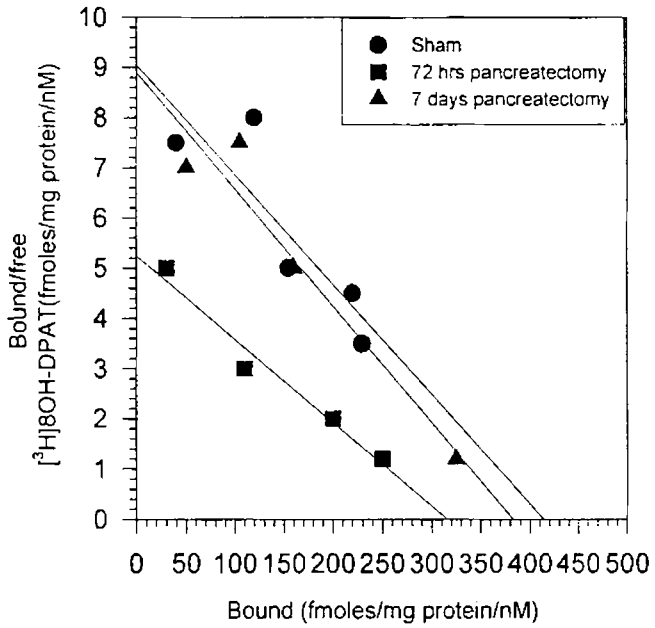


Table-7
[^{3}H]8-OH DPAT low affinity receptor binding parameters in the cerebral cortex of rats

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	395 ± 15	43.9 ± 2.6
72 hrs pancreatectomy	315 ± 15**††	63.0 ± 1.4**††
7 days pancreatectomy	405 ± 13	42.7 ± 1.7

Values are mean ± S.E.M. of 4-6 separate experiments
**p<0.01 when compared with sham
††p<0.01 when compared with 7 days pancreatectomy.

Binding parameters of [^{3}H]8-OH DPAT against 5-HT in the cerebral cortex of experimental rats

Experimental Group	Best-fit model	log (EC_{50})-1	log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Sham	Two-site	-10.10	-7.05	3.1×10^{-11}	8.5×10^{-8}	-0.29
72 hrs pancreatectomy	Two-site	-8.00	-4.60	9.5×10^{-9}	2.2×10^{-5}	-0.38
7 days pancreatectomy	Two-site	-11.14	-6.70	7.0×10^{-11}	1.7×10^{-7}	-0.26

Values are mean of 4-6 separate experiments

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

Figure - 5

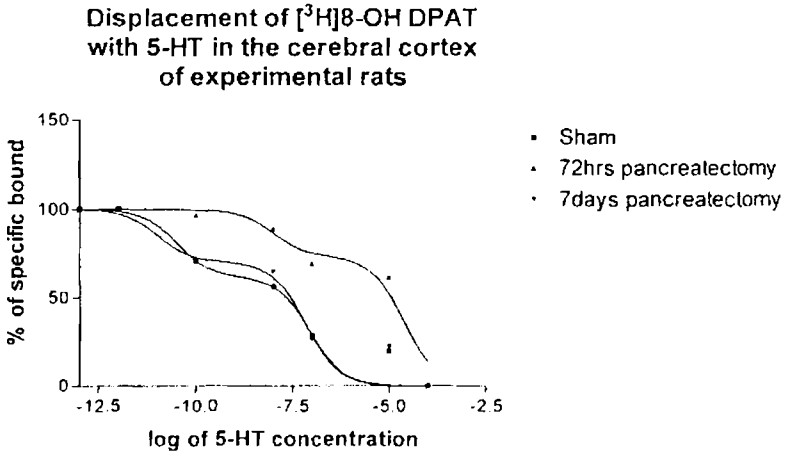


Figure-6
RT-PCR amplification product of 5-HT_{1A} receptor mRNA from the cerebral cortex of experimental rats

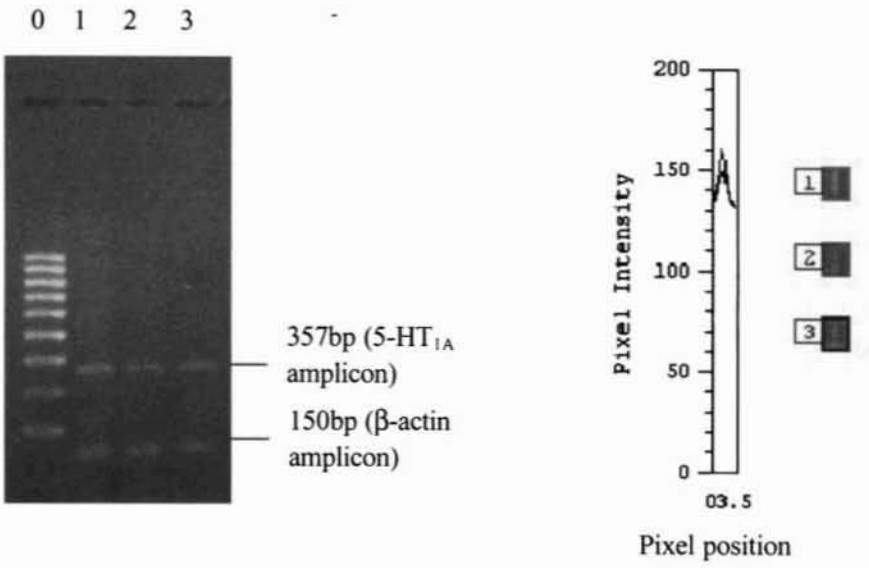


Table-9
Band properties of 5-HT_{1A} receptor mRNA RT-PCR amplicon

Lane No	Raw volume	Area	Peak
1	16198	160.1	109
2	15527	147.9	103
3	15584	149.1	106

0 - 100bp ladder
1 - Sham
2 - 72 hrs pancreatectomy
3 - 7 days pancreatectomy

Figure-7

Scatchard analysis of high affinity [^{3}H]8-OH DPAT receptor binding against 5-HT in the brain stem of rats

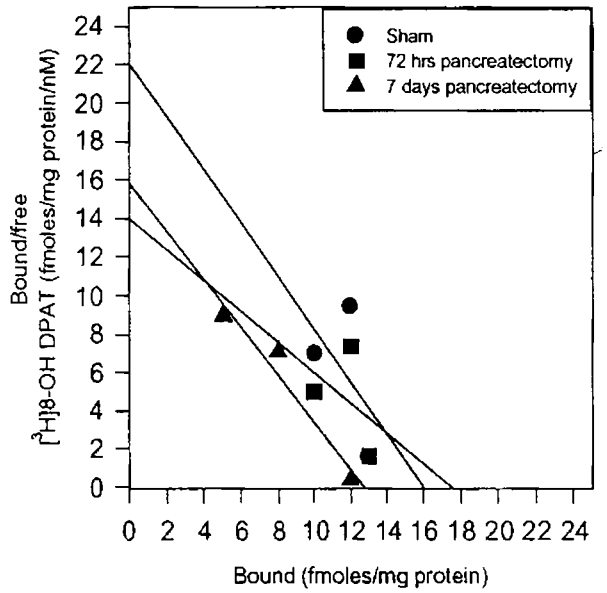


Table-10
[^{3}H]8-OH DPAT high affinity receptor binding parameters in the brain stem of rats

Experimental Group	B_{max} (fmoles/mg protein/nM)	K_d (nM)
Sham	16.0 ± 1.5	0.69 ± 0.04
72 hrs pancreatectomy	17.5 ± 2.5	1.06 ± 0.12**††
7 days pancreatectomy	14.0 ± 2.0	0.77 ± 0.06

Values are mean ± S.E.M. of 4-6 separate experiments
**P<0.01 when compared with sham
††P<0.01 when compared with 7 days pancreatectomy

Figue-8

Scatchard analysis of low affinity [^{3}H]8-OH DPAT receptor binding against 5-HT in the brain stem of rats

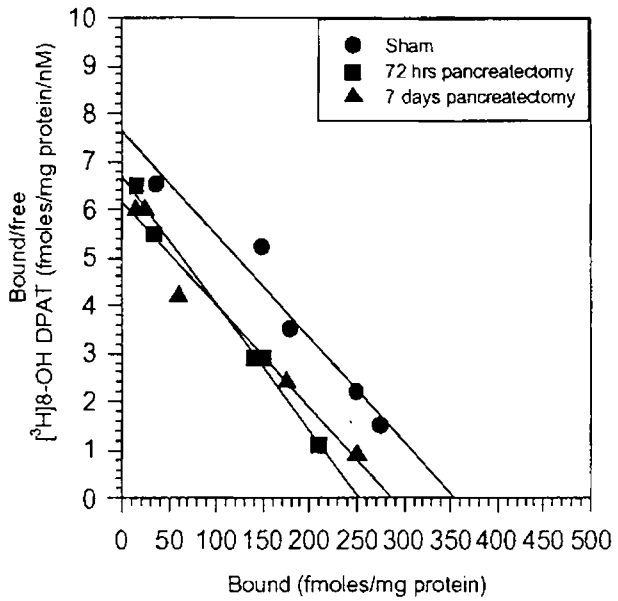


Table-11
[^{3}H]8-OH DPAT low affinity receptor binding parameters in the brain stem of rats

Experimental Group	B_{max} (fmoles/mg protein/nM)	K_d (nM)
Sham	350.0 ± 18.0	35.6 ± 9.2
72 hrs pancreatectomy	258.5 ± 15.0**†	30.6 ± 5.4
7 days pancreatectomy	295.0 ± 10.0*	41.9 ± 6.1

Values are mean ± S.E.M. of 4-6 separate experiments
**P<0.01, *P<0.05 when compared with sham
†P<0.05 when compared with 7 days pancreatectomy

Table - 12

Binding parameters of [^{3}H]8-OH DPAT against 5-HT in the brain stem of experimental rats

Experimental Group	Best-fit model	log (EC_{50})-1	log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Sham	Two-site	-9.2	-5.43	5.5×10^{-10}	3.7×10^{-6}	-0.32
72 hrs pancreatectomy	Two-site	-8.7	-5.03	1.8×10^{-9}	9.0×10^{-6}	-0.33
7 days pancreatectomy	Two-site	-9.2	-5.20	6.5×10^{-10}	6.8×10^{-6}	-0.30

Values are mean of 4-6 separate experiments

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

Figure - 9

Displacement of [^{3}H]8-OH DPAT with 5-HT in the brain stem of experimental rats

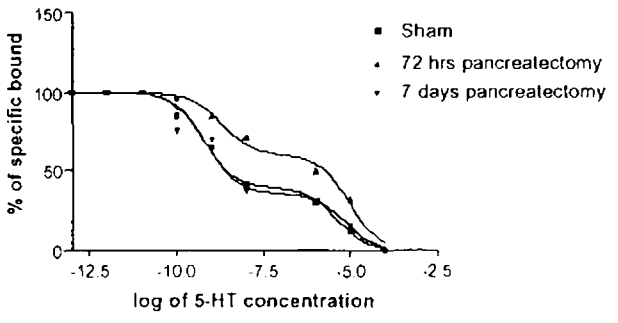


Figure-10
RT-PCR amplification product of 5-HT_{1A} receptor mRNA from the brain stem of experimental rats

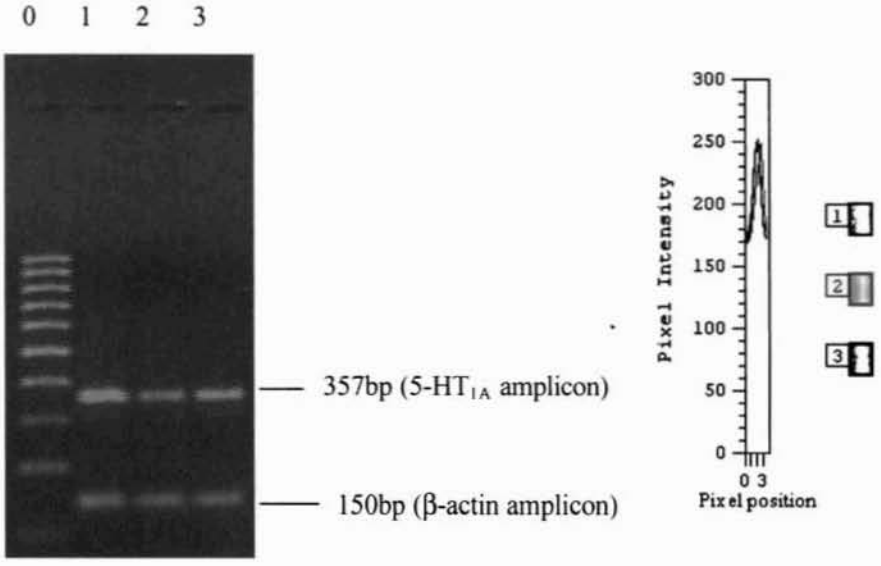


Table-13
Band properties of 5-HT_{1A} receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	47850	220	242.0
2	36004	180	229.7
3	40348	200	246.5

0 - 100bp ladder
1 - Sham
2 - 72 hrs pancreatectomy
3 - 7 days pancreatectomy

Figure-11

Scatchard analysis of high affinity [^{3}H]8-OH DPAT receptor binding against 5-HT in the hypothalamus of rats.

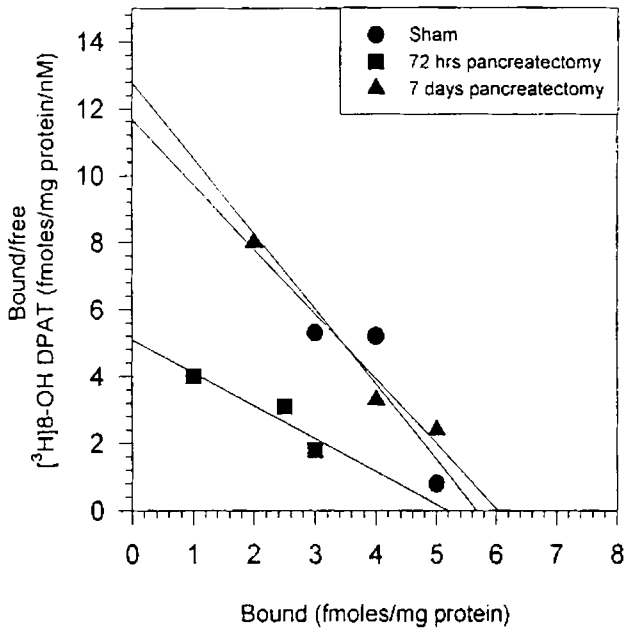


Table-14
[^{3}H]8-OH DPAT high affinity receptor binding parameters in the hypothalamus of rats

Experimental Group	B_{max} (fmoles/mg protein/nM)	K_d (nM)
Sham	7.0 ± 1.0	0.29 ± 0.03
72 hrs pancreatectomy	7.3 ± 2.3	1.05 ± 0.05**††
7 days pancreatectomy	6.2 ± 1.4	0.43 ± 0.03

Values are mean ± S.E.M. of 4-6 separate experiments
**P<0.01 when compared with sham
††P<0.01 when compared with 7 days pancreatectomy.

Figure-12

Scatchard analysis of low affinity [^{3}H]8-OH DPAT receptor binding against 5-HT in the hypothalamus of rats

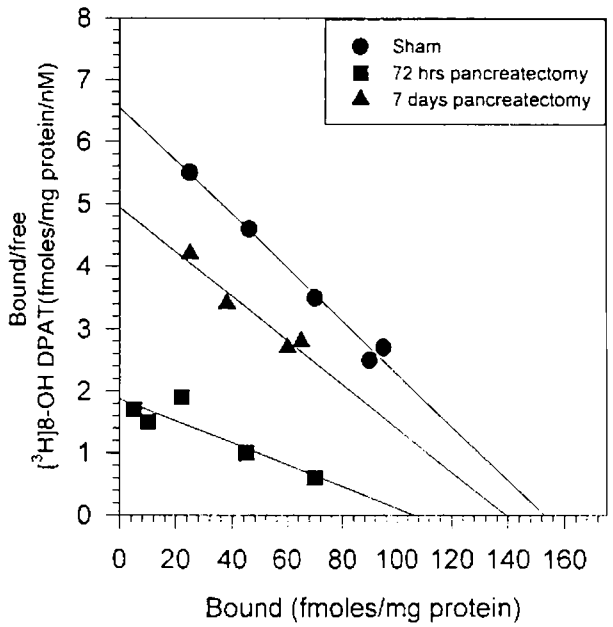


Table - 15
[^{3}H]8-OH DPAT low affinity receptor binding parameters in the hypothalamus of rats

Experimental Group	B_{max} (fmoles/mg protein/nM)	K_d(nM)
Sham	152.0 ± 7.0	30.1 ± 2.1
72 hrs pancreatectomy	110.5 ± 4.5**†	71.3 ± 5.8*†
7 days pancreatectomy	128.5 ± 5.5	51.9 ± 6.4*

Values are mean ± S.E.M. of 4-6 separate experiments
**P<0.01 when compared with sham, *P<0.05 when compared with sham
†P<0.05 when compared with 7 days pancreatectomy.

Table - 16

Binding parameters of [^{3}H]8-OH DPAT against 5-HT in the hypothalamus of experimental rats

Experimental Group	Best-fit model	log (EC_{50})-1	log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Sham	Two-site	-10.21	-5.21	6.2×10^{-11}	6.01×10^{-6}	-0.28
72 hrs pancreatectomy	Two-site	-8.25	-4.6	5.4×10^{-9}	2.5×10^{-5}	-0.28
7 days pancreatectomy	Two-site	-10.32	-5.1	4.6×10^{-11}	7.8×10^{-6}	-0.26

Values are mean of 4-6 separate experiments

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

Figure - 13

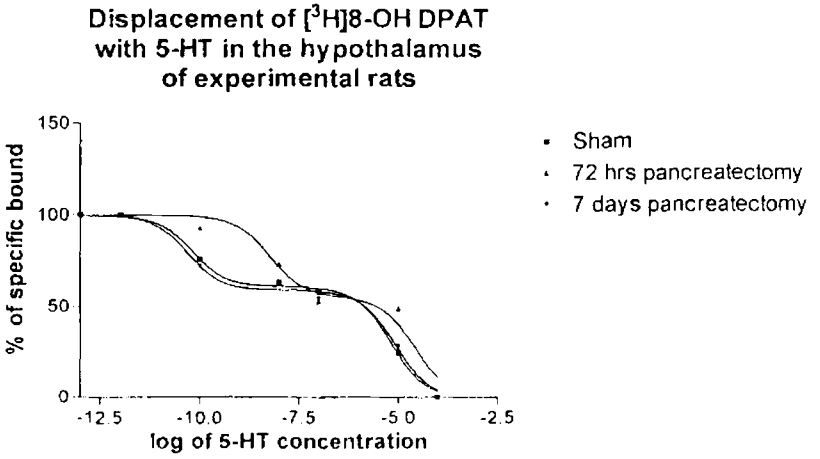


Figure-14
RT-PCR amplification product of 5-HT_{1A} receptor mRNA from the hypothalamus of experimental rats

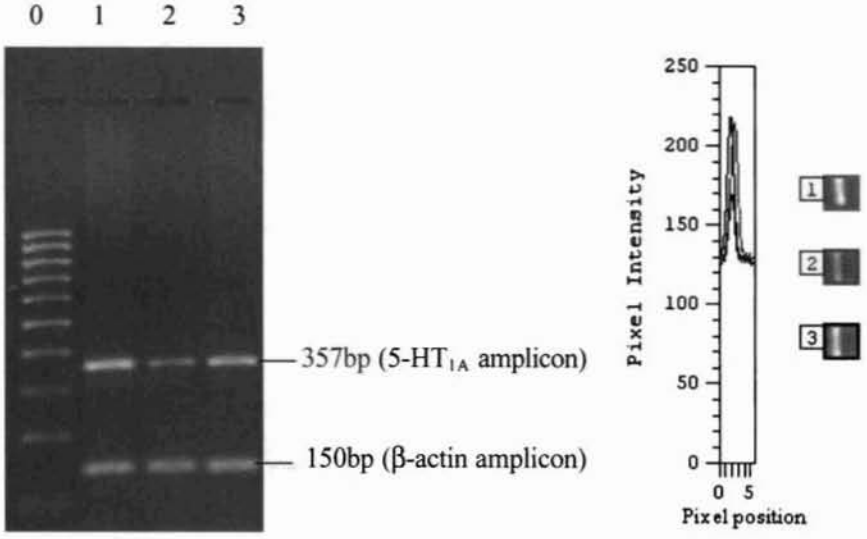


Table - 17
Band properties of 5-HT_{1A} receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	39,961	240	214.0
2	26,400	180	168.4
3	31,274	180	217.5

0 – 100bp ladder
1 - Sham
2 - 72 hrs pancreatectomy
3 - 7 days pancreatectomy

Figure-15

Scatchard analysis of [^{3}H]Mesulergine binding against 5-HT in the cerebral cortex of rats

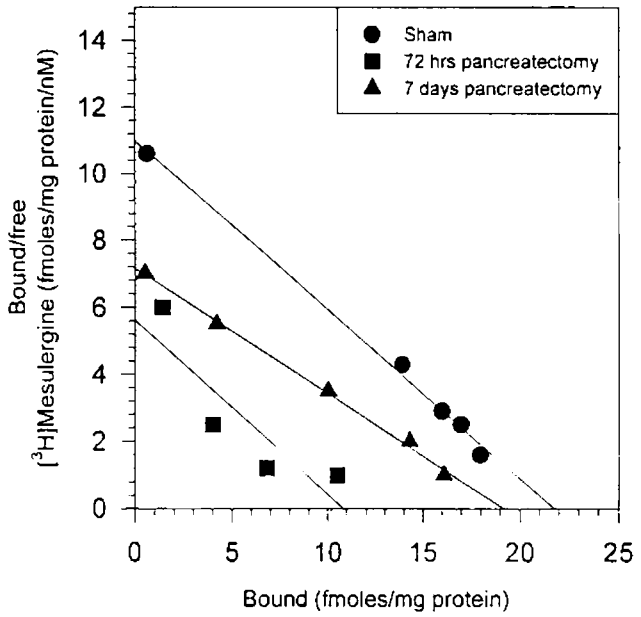


Table-18

[^{3}H]Mesulergine binding parameters in the cerebral cortex of rats

Experimental Group	B_{max} (fmoles/mg protein/nM)	K_d (nM)
Sham	21.6 ± 1.9	2.8 ± 0.4
72 hrs pancreatectomy	10.9 ± 2.5*†	2.9 ± 0.5
7 days pancreatectomy	20.2 ± 1.9	2.2 ± 0.3

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

*P<0.05 when compared with sham

†P<0.05 when compared with 7 days pancreatectomy.

Table - 19

Binding parameters of [^{3}H]mesulergine in the cerebral cortex of experimental rats

Experimental Group	Best-fit model	Log (EC_{50})	Ki	Hill slope
Sham	One-site	-8.90	1.25×10^{-9}	-0.96
72 hrs pancreatectomy	One-site	-8.30	4.6×10^{-9}	-0.97
7 days pancreatectomy	One-site	-9.01	9.5×10^{-10}	-0.92

Values are mean of 4-6 experiments

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

Figure - 16

Displacement of [^{3}H]mesulergine with 5-HT in the cerebral cortex of experimental rats

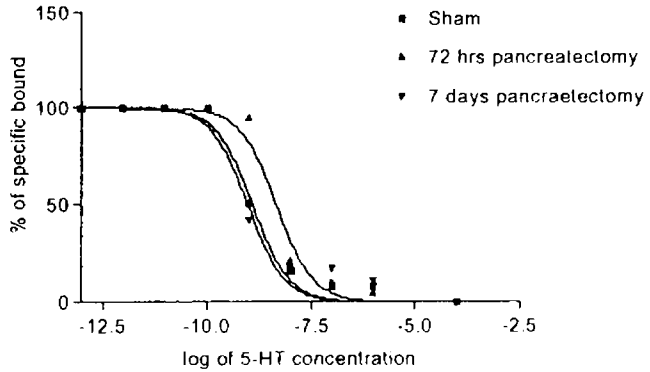


Figure-17
RT-PCR amplification product of 5-HT_{2C} receptor mRNA from the cerebral cortex of experimental rats

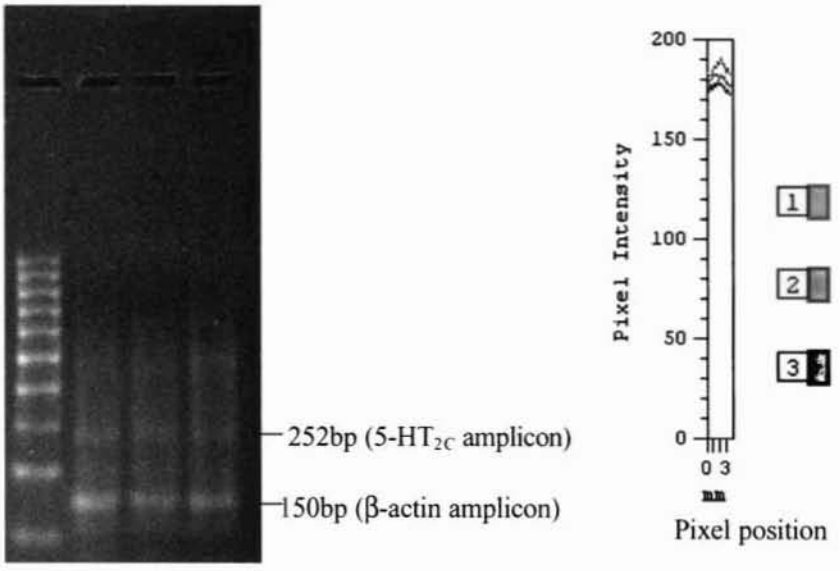


Table-20
Band properties of 5-HT_{2C} receptor mRNA RT-PCR amplicon

Lane No	Raw volume	Area	Peak
1	6277	195	48
2	5982	187	42
3	6073	192	46

0 - 100bp ladder
1 - Sham
2 - 72 hrs pancreatectomy
3 - 7 days pancreatectomy

Figure -18

Scatchard analysis of [^{3}H]Mesulergine binding against 5-HT in the brain stem of rats

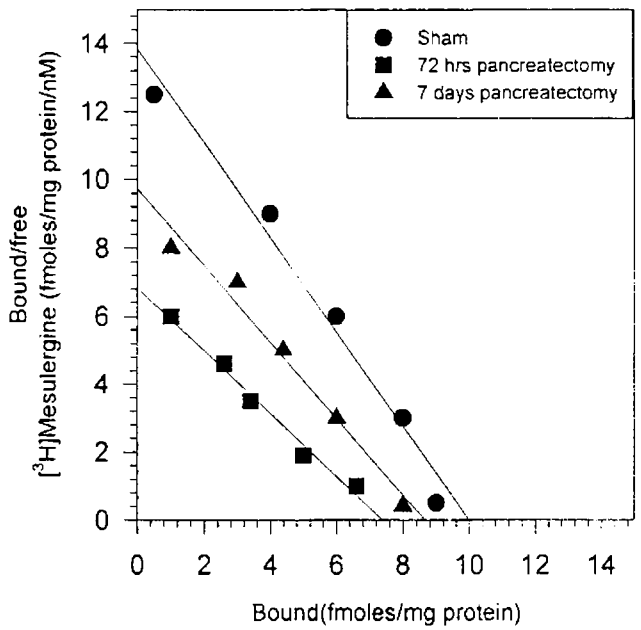


Table-21
[^{3}H]Mesulergine binding parameters in the brain stem of rats

Experimental Group	B_{max} (fmoles/mg protein/nM)	K_d (nM)
Sham	10.2 ± 1.2	0.65 ± 0.04
72 hrs pancreatectomy	6.5 ± 1.4**	1.03 ± 0.07**
7 days pancreatectomy	8.1 ± 0.8	0.95 ± 0.04

Values are mean ± S.E.M. of 4-6 separate experiments
**P<0.01 when compared with sham
**P<0.01 when compared with 7 days pancreatectomy.

Table - 22

Binding parameters of [^{3}H]mesulergine in the brain stem of rats

Experimental Group	Best-fit model	Log (EC_{50})	Ki	Hill slope
Sham	One-site	-8.86	5.6×10^{-10}	-1.03
72 hrs pancreatectomy	One-site	-7.97	4.4×10^{-9}	-0.98
7 days pancreatectomy	One-site	-8.70	8.3×10^{-10}	-1.01

Values are mean of 4-6 experiments

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

Figure - 19

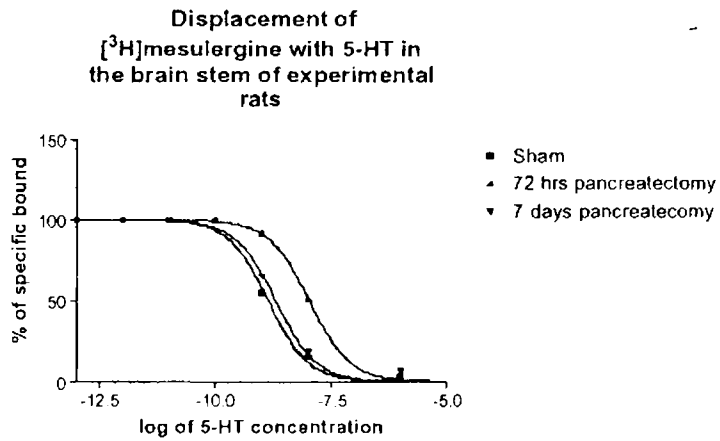


Figure-20
RT-PCR amplification product of 5-HT_{2C} receptor mRNA from the brain stem of experimental rats

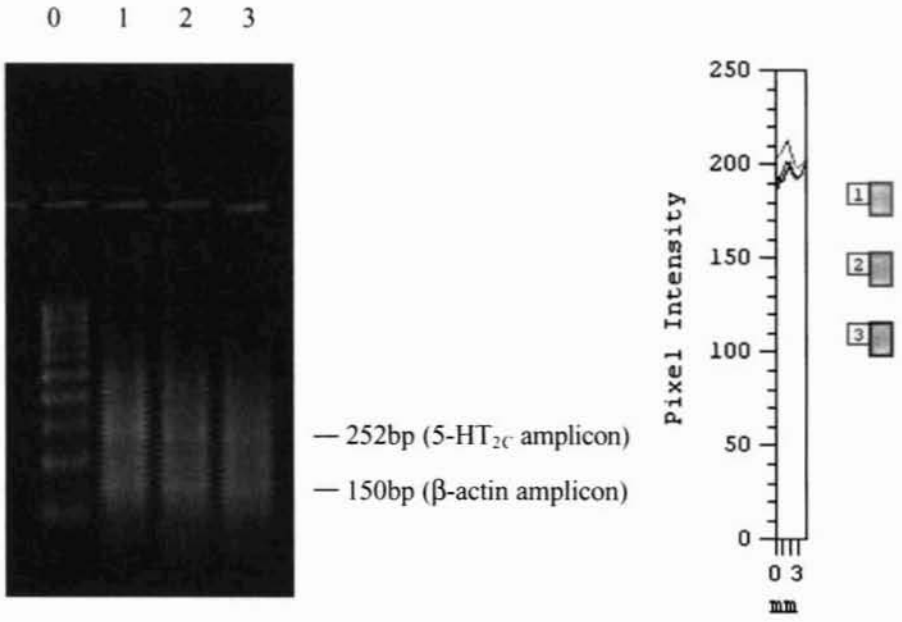


Table-23
Band properties of 5-HT_{2C} receptor mRNA RT-PCR amplicon

Lane No	Raw volume	Area	Peak
1	31708	154	211.6
2	26104	132	200.7
3	30081	154	198.2

0 - 100bp ladder
1 - Sham
2 - 72 hrs pancreatectomy
3 - 7 days pancreatectomy

Figure-21

Scatchard analysis of [^{3}H]Mesulergine binding against 5-HT in the hypothalamus of rats

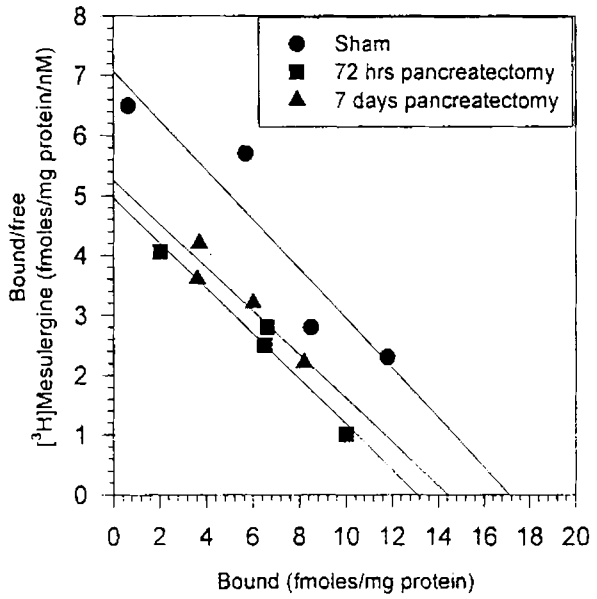


Table-24
[^{3}H]Mesulergine binding parameters in the hypothalamus of rats

Experimental Group	B_{max} (fmoles/mg protein/nM)	K_d (nM)
Sham	17.8 ± 1.6	2.29 ± 0.07
72 hrs pancreatectomy	12.8 ± 1.8**†	3.10 ± 0.10**†
7 days pancreatectomy	16.5 ± 1.1	2.80 ± 0.09

Values are mean ± S.E.M. of 4-6 separate experiments
**P<0.01 when compared with sham
†P<0.05 when compared with 7 days pancreatectomy.

Table - 25

Binding parameters of [^{3}H]mesulergine in the hypothalamus of experimental rats

Experimental Group	Best-fit model	Log (EC_{50})	Ki	Hill slope
Sham	One-site	-8.9	1.2×10^{-9}	-1.02
72 hrs pancreatectomy	One-site	-7.4	3.5×10^{-8}	-1.07
7 days pancreatectomy	One-site	-8.7	1.6×10^{-9}	-0.93

Values are mean of 4-6 experiments

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

Figure - 22

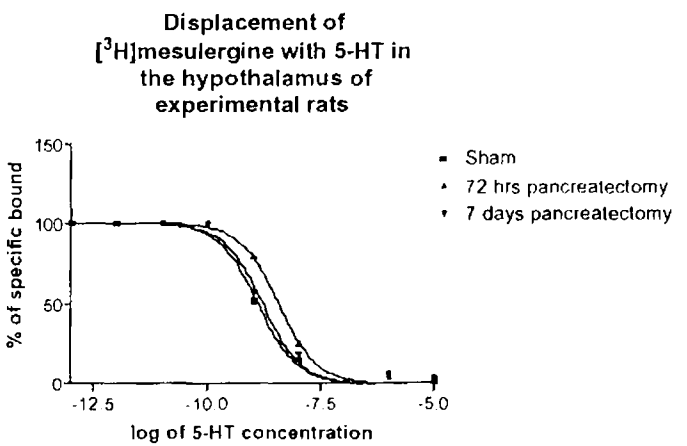


Figure - 23
RT-PCR amplification product of 5-HT_{2C} receptor mRNA from the hypothalamus of experimental rats

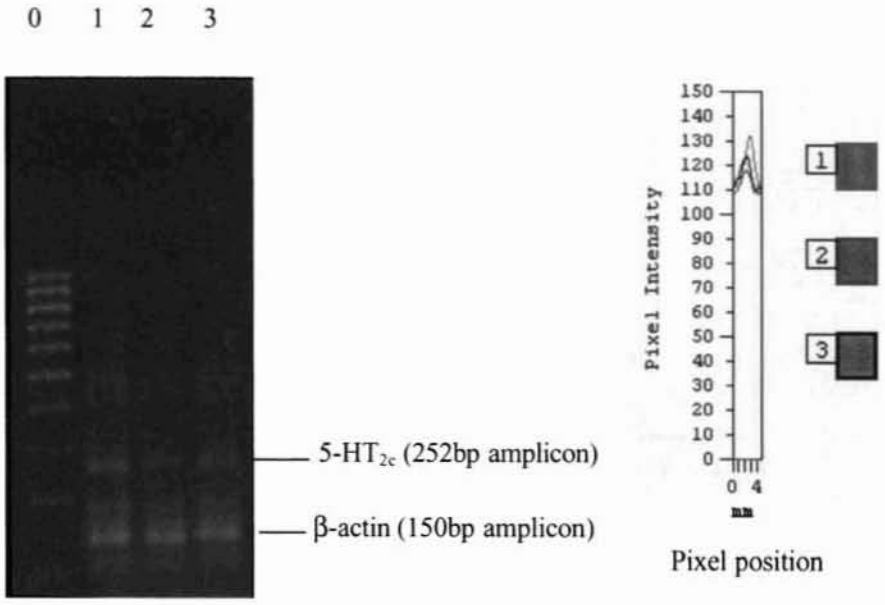


Table-26
Band properties of 5-HT_{2C} receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	27,101	220	206
2	22,590	198	175
3	25,748	206	198

0 - 100bp ladder
1 - Sham
2 - 72 hrs pancreatectomy
3 - 7 days pancreatectomy

Figure-24

Scatchard analysis of [^{3}H]8-OH DPAT receptor binding against 5-HT in the pancreatic islets of rats

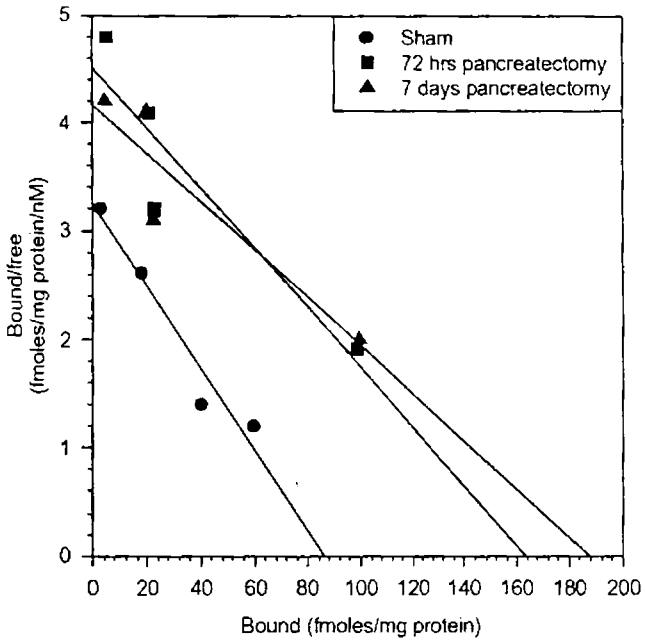


Table - 27
[^{3}H]8-OH DPAT receptor binding parameters in the pancreatic islets of rats

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	84.3 ± 4.6	30.6 ± 2.1
72 hrs pancreatectomy	171.3 ± 5.8**	35.7 ± 5.3
7 days pancreatectomy	180.7 ± 7.0**	45.0 ± 3.2*†

Values are mean ± S.E.M. of 4-6 separate experiments
**P<0.01, *P<0.05 when compared with sham
†P<0.05 when compared with 72 hrs pancreatectomy

Table-28

Binding parameters of [^{3}H]8-OH DPAT binding against 5-HT in the pancreatic islets of experimental rats

Experimental Group	Best-fit model	Log (EC_{50})	Ki	Hill slope
Sham	One-site	-8.6	2.15×10^{-9}	-0.97
72 hrs pancreatectomy	One-site	-8.4	3.80×10^{-9}	-0.74
7 days pancreatectomy	One-site	-7.9	1.12×10^{-8}	-1.02

Values are mean of 4-6 experiments

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

Figure - 25

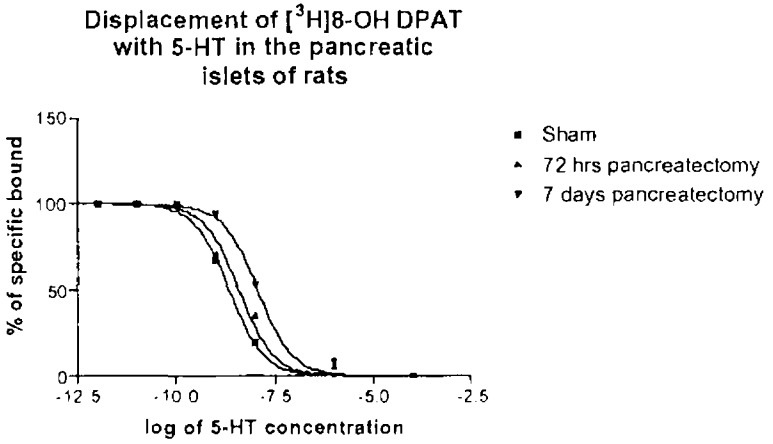


Figure- 26
RT-PCR amplification product of 5-HT_{1A} receptor mRNA from the pancreatic islets of experimental rats

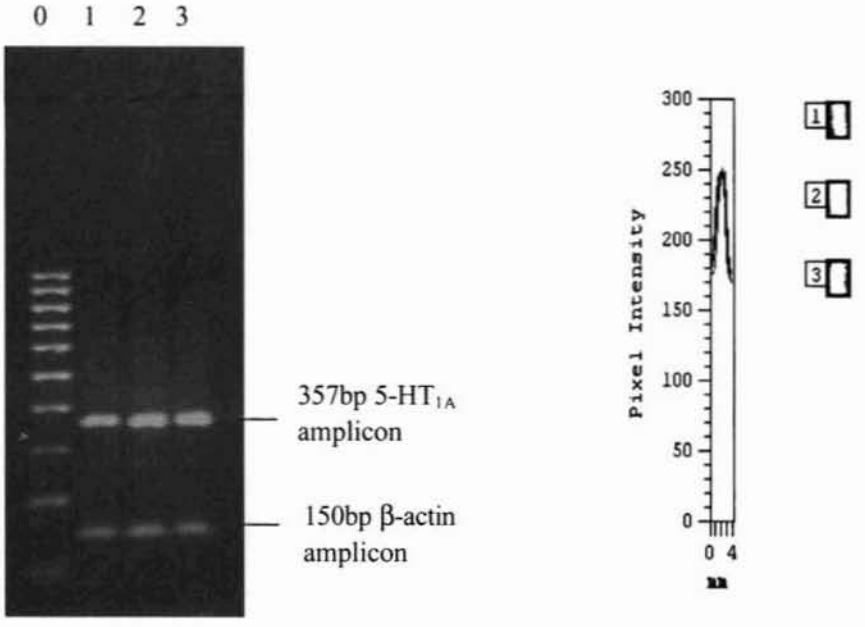


Table - 29
Band properties of 5-HT_{1A} receptor mRNA RT-PCR amplicon

Lane No	Raw Volume	Area	Peak
1	46813	233	220
2	48991	240	239
3	48105	241	240

0 - 100bp ladder
1 - Sham
2 - 72 hrs pancreatectomy
3 - 7 days pancreatectomy

Figure - 27

Scatchard analysis of [^{3}H]Mesulergine binding against 5-HT in the pancreatic islets of rats

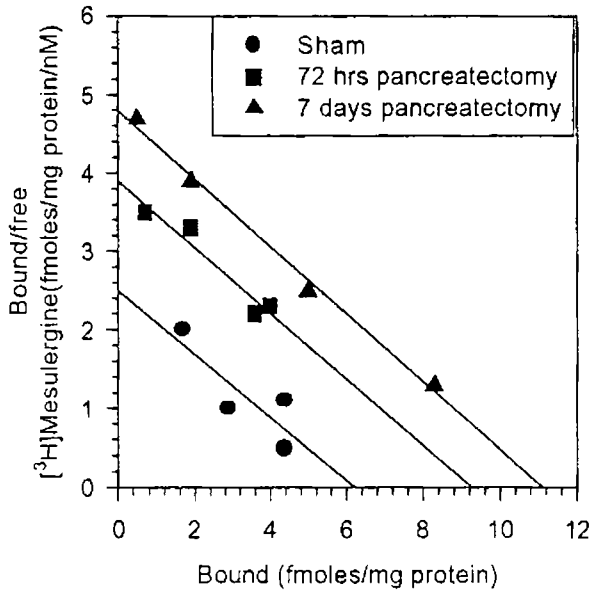


Table - 30
[^{3}H]Mesulergine binding parameters in the pancreatic islets of rats

Experimental Group	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	5.5 ± 0.2	2.3 ± 0.1
72 hrs pancreatectomy	9.4 ± 0.3**	2.6 ± 0.3
7 days pancreatectomy	10.9 ± 0.3**	2.7 ± 0.1

Values are mean ± S.E.M. of 4-6 separate experiments
**P<0.01 when compared with sham

Table - 31

Binding parameters of [^{3}H]mesulergine in the pancreatic islets of experimental rats

Experimental Group	Best-fit model	Log (EC_{50})	Ki	Hill slope
Sham	One-site	-8.7	1.3×10^{-9}	-1.08
72 hrs pancreatectomy	One-site	-8.4	2.4×10^{-9}	-1.09
7 days pancreatectomy	One-site	-8.7	1.1×10^{-9}	-0.94

Values are mean of 4-6 experiments

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

Figure - 28

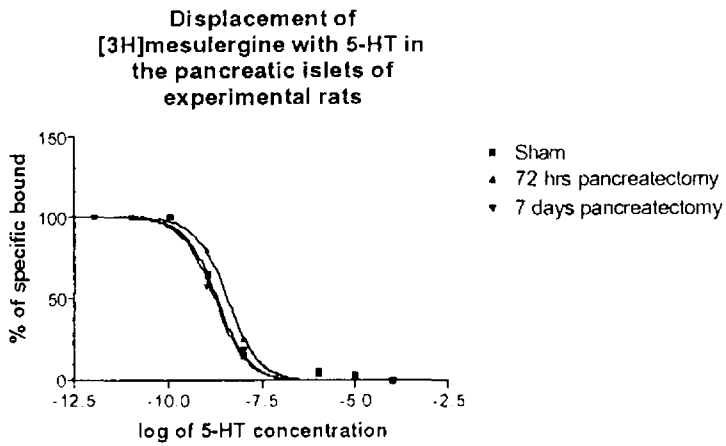
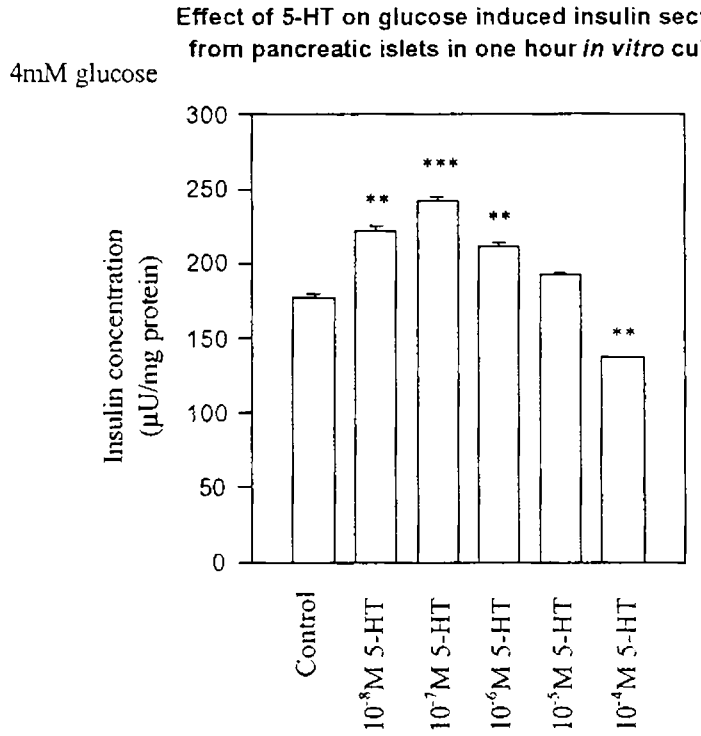


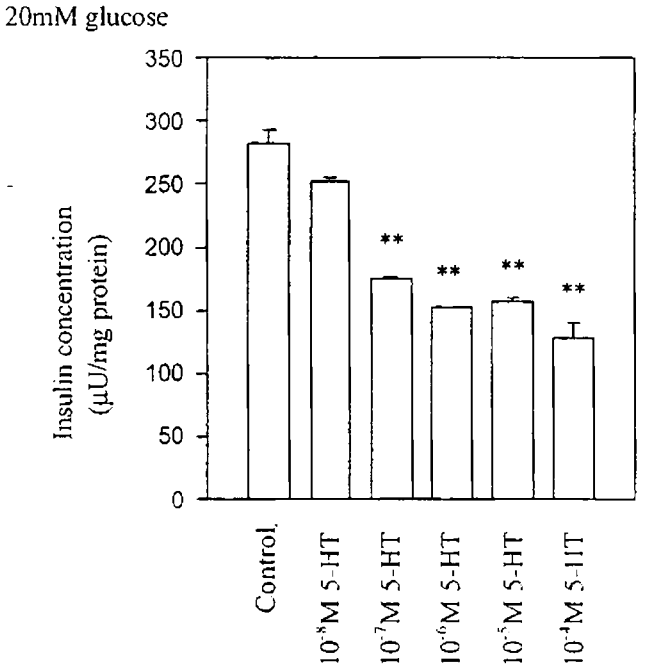
Figure- 29



**P<0.01 when compared with control (medium only)
***P<0.001 when compared with control (medium only)
Islets were incubated in KRB buffer with different concentrations of 5-HT and 4mM glucose for one hour

Figure- 30

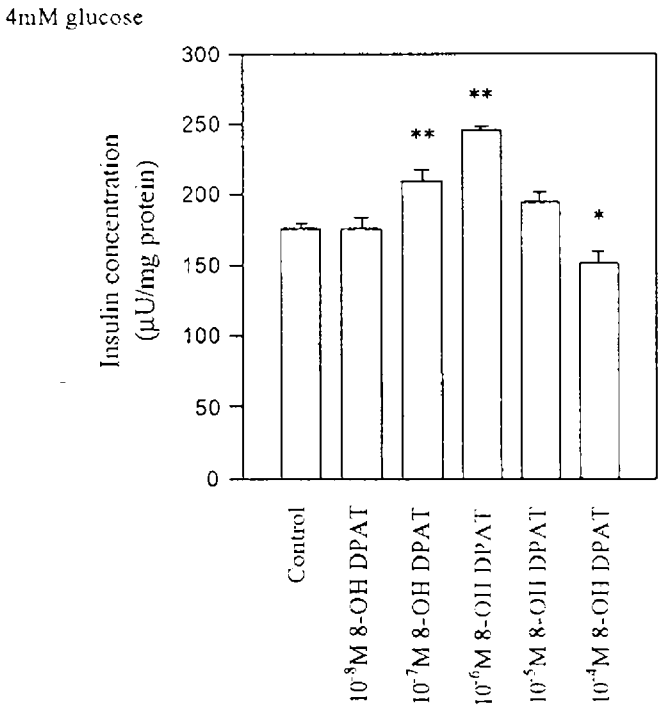
Effect of 5-HT on glucose induced insulin secretion from pancreatic islets in one hour *in vitro* culture



**P<0.01 when compared with control (medium only)
Islets were incubated in KRB buffer with different concentrations of 5-HT and 20mM glucose for one hour

Figure- 31

Effect of 8-OH DPAT on glucose induced insulin secretion from pancreatic islets in one hour *in vitro* culture



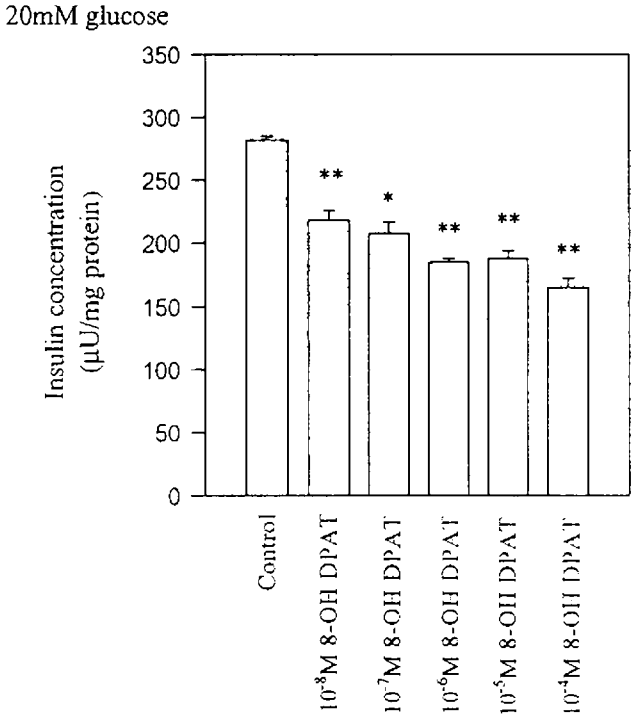
*P<0.05 when compared with control (medium only)

**P<0.01 when compared with control (medium only)

Islets were incubated in KRB buffer with different concentrations of 8-OH DPAT and 4mM glucose for one hour

Figure- 32

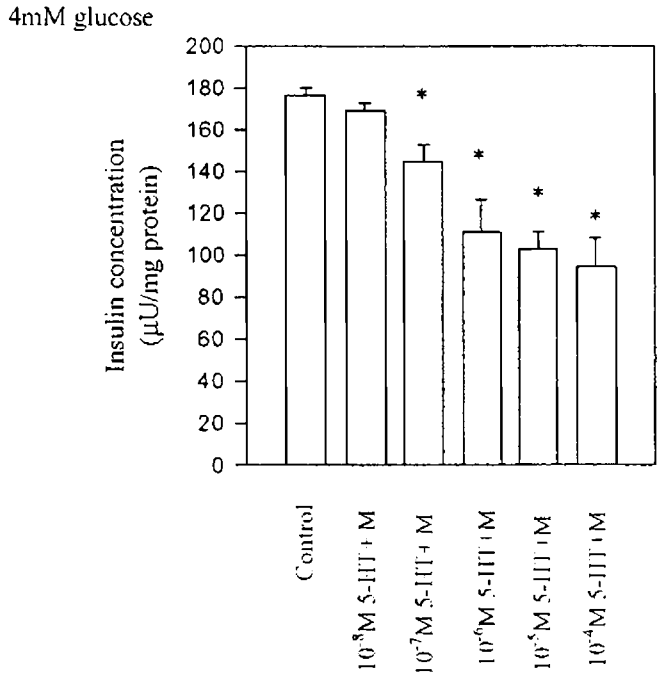
Effect of 8-OH DPAT on glucose induced insulin secretion from pancreatic islets in one hour *in vitro* culture



*P<0.05 when compared with control (medium only)
**P<0.01 when compared with control (medium only)
Islets were incubated in KRB buffer with different concentrations of 8-OH DPAT and 20mM glucose for one hour

Figure- 33

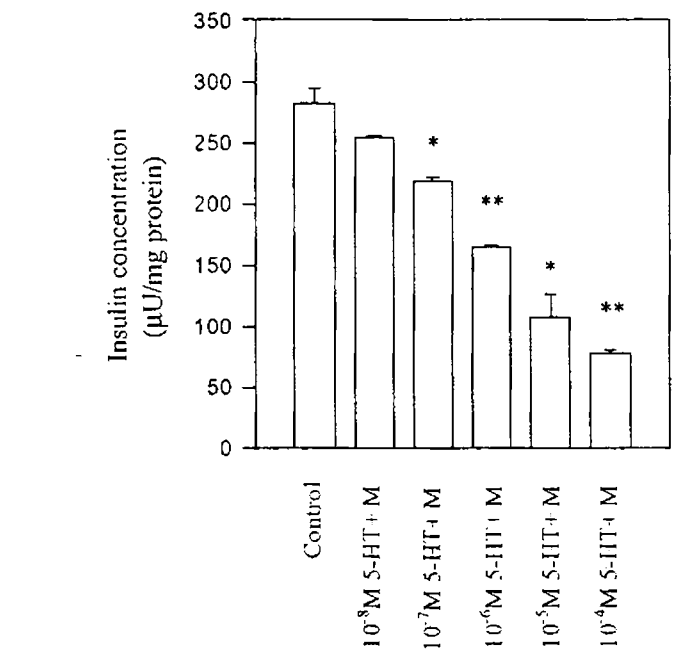
Effect of mesulergine on insulin secretion from pancreatic islets in one hour *in vitro* culture



*P<0.05 when compared with control (medium only)
Islets were incubated in KRB buffer with different concentrations of 5-HT, mesulergine (M) (10^{-4}M) and 4mM glucose for one hour

Figure- 34

Effect of mesulergine on glucose induced insulin secretion from pancreatic islets in one hour *in vitro* culture

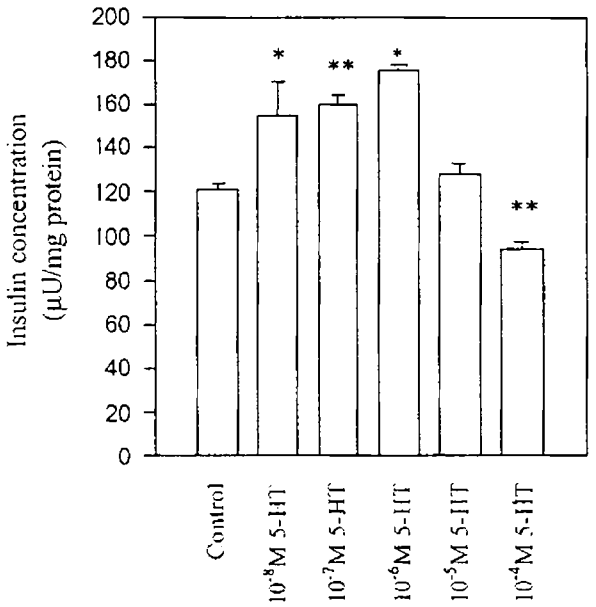


*P<0.05 when compared with control (medium only)
**P<0.01 when compared with control (medium only)
Islets were incubated in KRB buffer with different concentrations of 5-HT, mesulergine (M) 10^{-4}M) and 20mM glucose for one hour

Figure - 35

Effect of 5-HT on glucose induced insulin secretion from pancreatic islets in 24 hours *in vitro* culture

4mM glucose



*P<0.05 when compared with control (medium only)

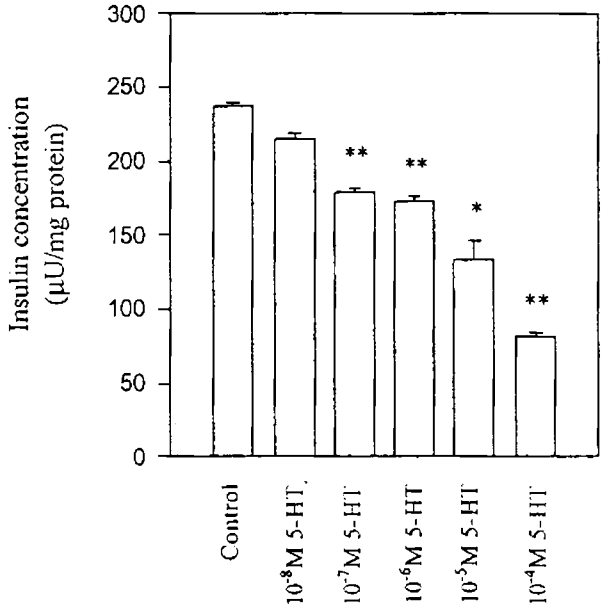
**P<0.01 when compared with control (medium only)

Islets were incubated in RPMI - 1640 medium with different concentrations of 5-HT and 4mM glucose for 24 hours. Later one hour incubation was carried out in KRB instead of RPMI - 1640

Figure - 36

Effect of 5-HT on glucose induced insulin secretion from pancreatic islets in 24 hours *in vitro* culture

20mM glucose



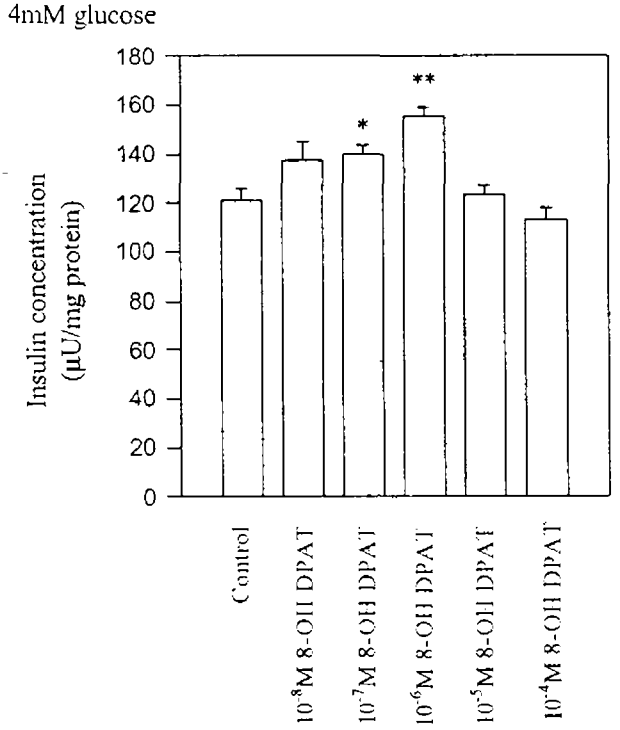
*P<0.05 when compared with control (medium only)

**P<0.01 when compared with control (medium only)

Islets were incubated in RPMI - 1640 medium with different concentrations of 5-HT and 20mM glucose for 24 hours. Later one hour incubation was carried out in KRB instead of RPMI - 1640

Figure - 37

Effect of 8-OH DPAT on glucose induced insulin secretion from pancreatic islets in 24 hours *in vitro* culture

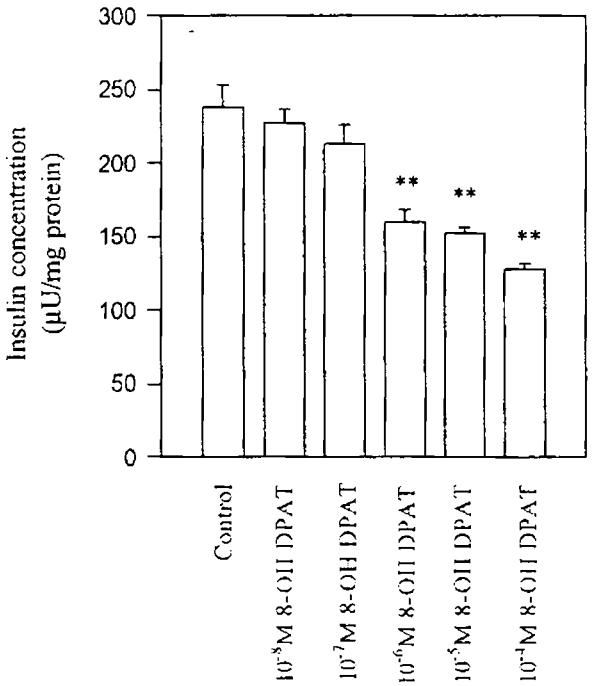


*P<0.05 when compared with control (medium only)
**P<0.01 when compared with control (medium only)
Islets were incubated in RPMI - 1640 medium with different concentrations of 8-OH DPAT and 4mM glucose for 24 hours. Later one hour incubation was carried out in KRB instead of RPMI - 1640

Figure - 38

Effect of 8-OH DPAT on glucose induced insulin secretion from pancreatic islets in 24 hours *in vitro* culture

20mM glucose



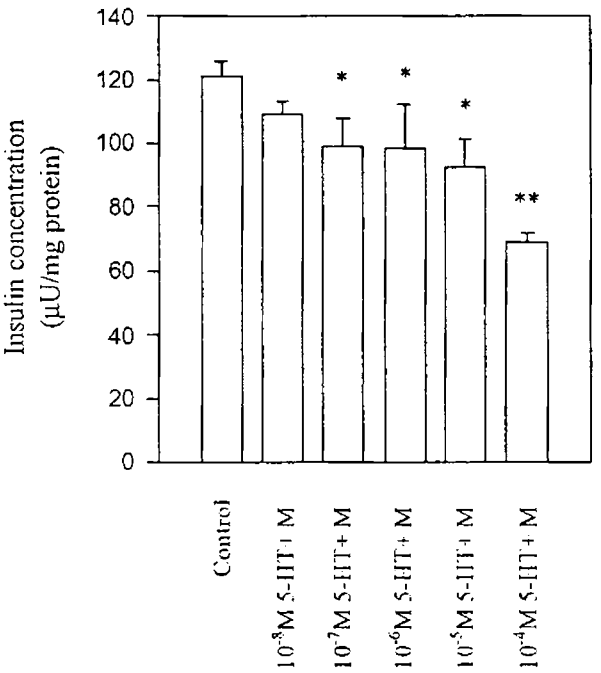
**P<0.01 when compared with control (medium only)

Islets were incubated in RPMI - 1640 medium with different concentrations of 8-OH DPAT and 20mM glucose for 24 hours. Later one hour incubation was carried out in KRB instead of RPMI - 1640

Figure - 39

Effect of mesulergine on glucose induced insulin secretion from pancreatic islets in 24 hours *in vitro* culture

4mM glucose



*P<0.05 when compared with control (medium only)

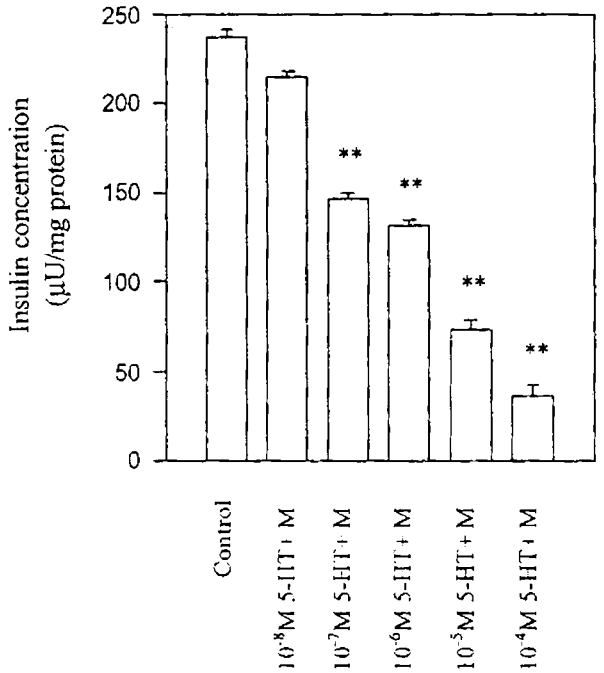
**P<0.01 when compared with control (medium only)

Islets were incubated in RPMI - 1640 medium with different concentrations of 5-HT, mesulergine (M) (10^{-4}M) and 4mM glucose for 24 hours. Later one hour incubation was carried out in KRB instead of RPMI - 1640

Figure - 40

Effect of mesulergine on glucose induced insulin secretion from pancreatic islets in 24 hours *in vitro* culture

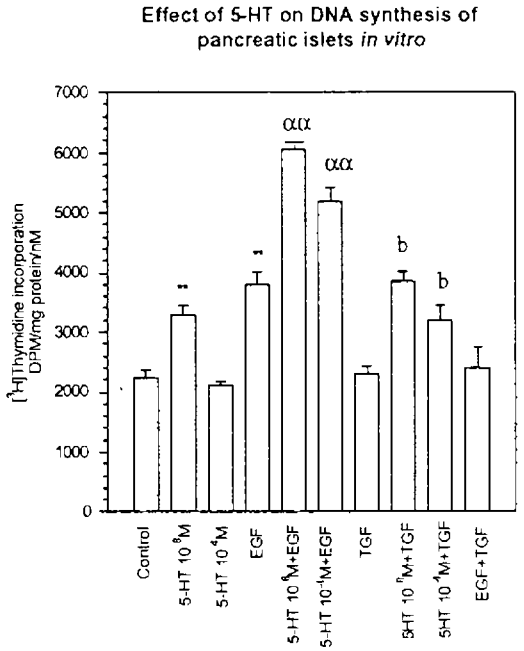
4mM glucose



**P<0.01 when compared with control (medium only)

Islets were incubated in RPMI - 1640 medium with different concentrations of 5-HT, mesulergine (10^{-4}M) and 20mM glucose for 24 hours. Later one hour incubation was carried out in KRB instead of RPMI - 1640

Figure - 41



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

**p<0.01 when compared with control

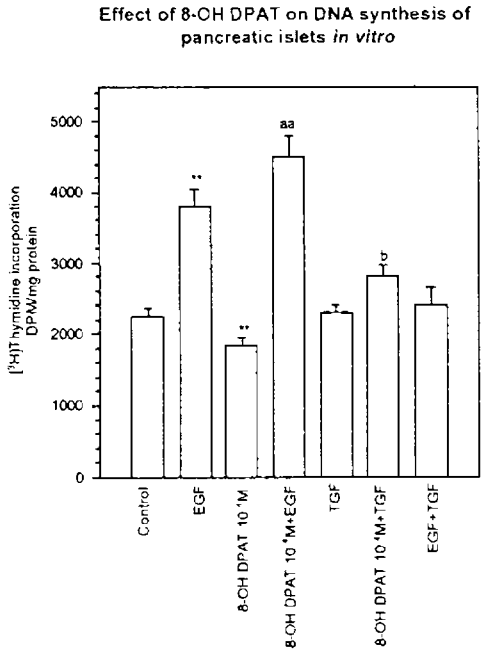
αα p<0.01 when compared with EGF, b p<0.05 when compared with TGF

EGF - Epidermal growth factor (10ng/ml)

TGF - Transforming growth factor β1(1ng/ml)

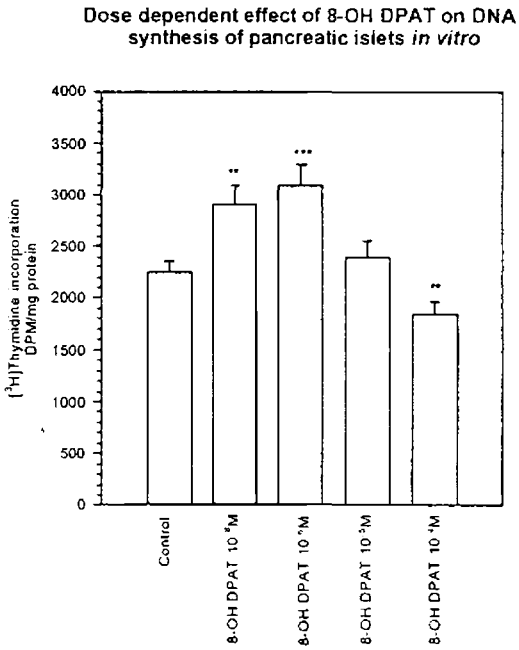
Islets were incubated in RPMI - 1640 medium with EGF, TGF, 4mM glucose, 5-HT (10^{-4}M and 10^{-8}M) and 2.5μCi [^{3}H]thymidine

Figure - 42



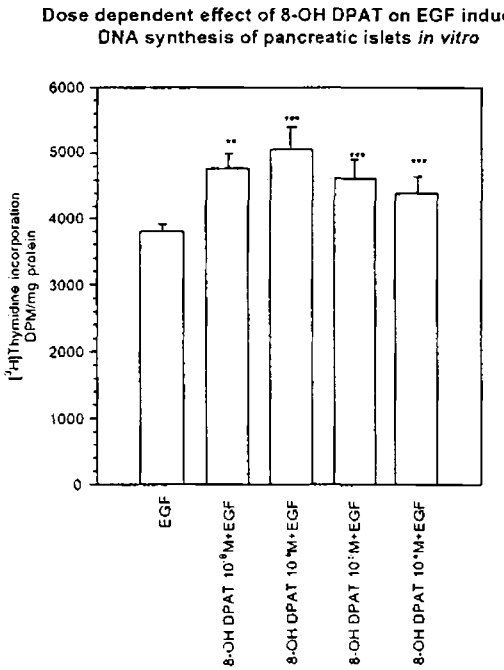
Values are mean ± S.E.M. of 4-6 separate experiments
**p<0.01 when compared with control, aa p<0.01 when compared with EGF,
b p<0.05 when compared with TGF,
EGF - Epidermal growth factor (10ng/ml)
TGF - Transforming growth factor β1 (1ng/ml)
Islets were incubated in RPMI - 1640 medium with EGF, TGF, 10^{-4}M 8-OH DPAT, 4mM glucose and 2.5μCi [^{3}H]thymidine.

Figure - 43



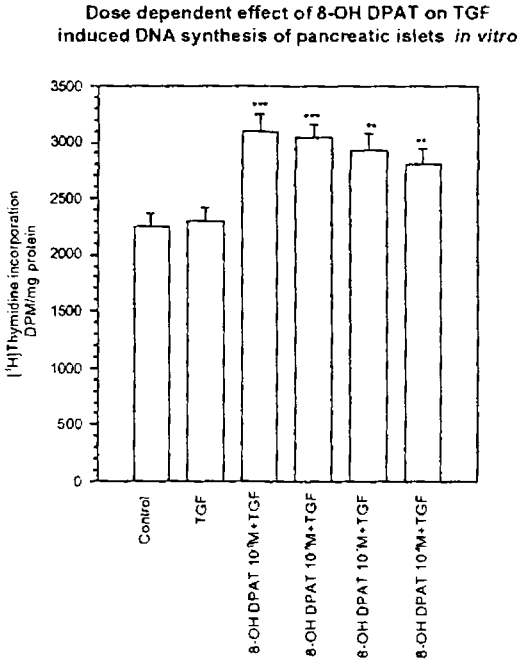
*P<0.01, **p<0.001 when compared with control
Islets were incubated in RPMI - 1640 medium with 8-OH DPAT (10^{-8} - 10^{-4}M), 4mM glucose and 2.5μCi [^{3}H]thymidine.

Figure - 44



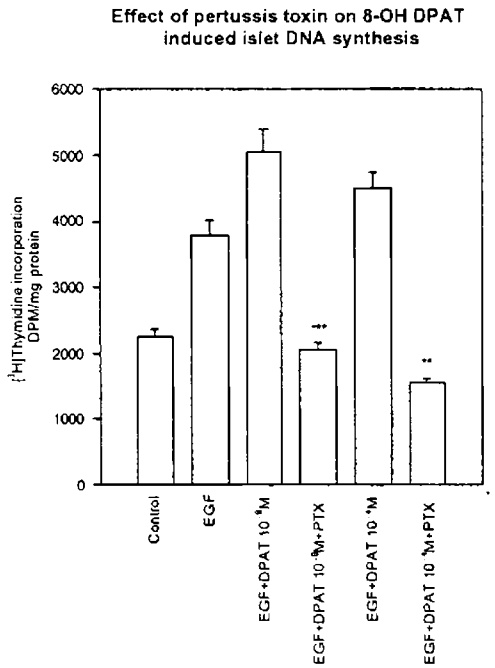
**P<0.01, ***p<0.001 when compared with EGF
EGF - Epidermal growth factor (10ng/ml)
Islets were incubated in RPMI - 1640 medium with EGF and 8-OH DPAT (10^{-8}M - 10^{-4}M), 4mM glucose and 2.5μCi [^{3}H]thymidine

Figure - 45



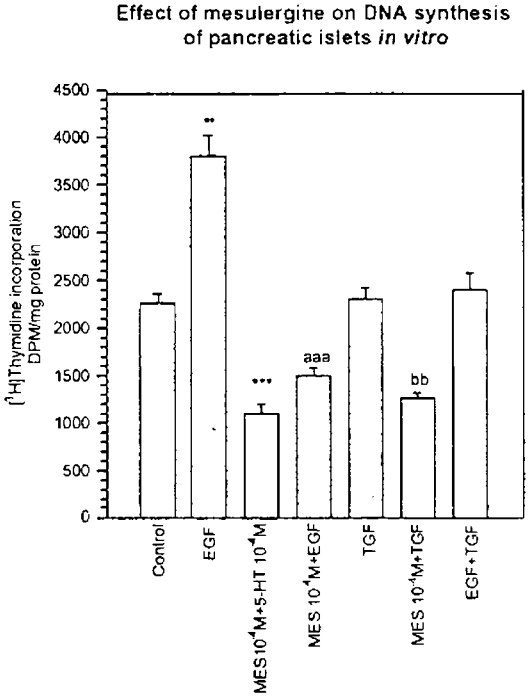
*P<0.01, **p<0.001 when compared with TGF
TGF - Transforming growth factor β1 (1ng/ml)
Islets were incubated in RPMI - 1640 medium with TGF, 8-OH DPAT (10^{-8}M - 10^{-4}M), 4mM glucose and 2.5μCi [^{3}H]thymidine

Figure - 46



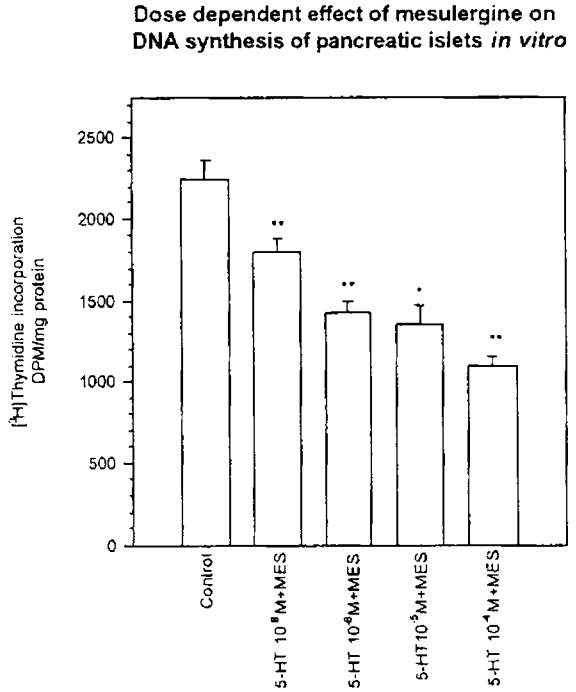
**P<0.01, ***p<0.001 when compared with EGF + DPAT group
EGF - Epidermal growth factor (10ng/ml)
PTX - Pertussis toxin (50ng/ml)
Islets were incubated in RPMI - 1640 medium with EGF, DPAT (8-OH DPAT 10^{-8}M & 10^{-4}M), 4mM glucose and 2.5µCi [^{3}H]thymidine

Figure - 47



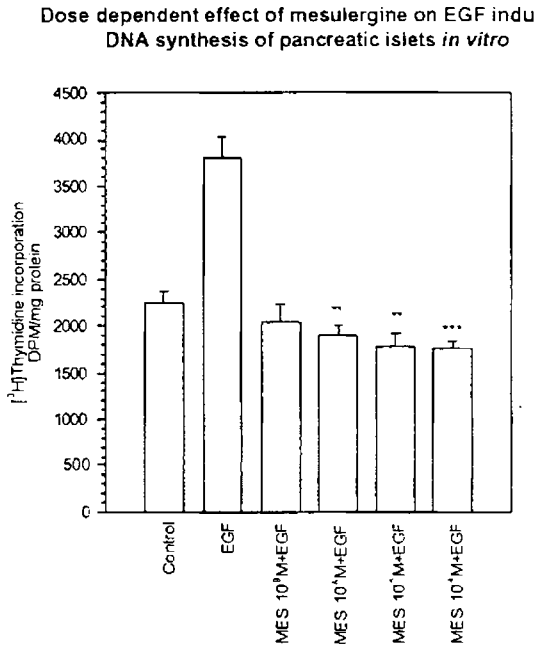
*P<0.01, **p<0.001 when compared with control
aaa p<0.001 when compared with EGF
bb p<0.01 when compared with TGF
EGF - Epidermal growth factor (10ng/ml)
TGF - Transforming growth factor β1 (1ng/ml)
Islets were incubated in RPMI - 1640 medium with 5-HT (10^{-4}M), MES (10^{-4}M), EGF, TGF, 4mM glucose and 2.5μCi [^{3}H]thymidine

Figure - 48



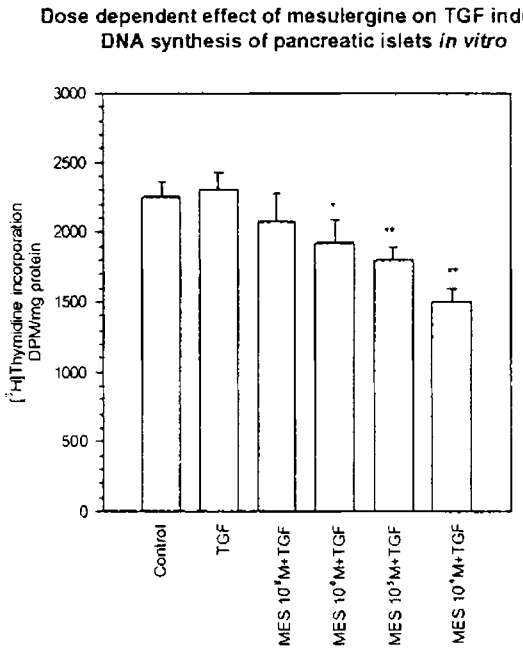
*P<0.05, **p<0.01 when compared with control
Islets were incubated in RPMI - 1640 medium with 5-HT (10^{-8}M - 10^{-4}M), mesulergine (10^{-4}M), 4mM glucose and 2.5µCi [^{3}H]thymidine.

Figure - 49



*P<0.01, **p<0.001 when compared with EGF
EGF - Epidermal growth factor (10ng/ml)
Islets were incubated in RPMI - 1640 medium with EGF, mesulergine (10^{-8}M - 10^{-4}M), 4mM glucose and 2.5µCi [^{3}H]thymidine

Figure - 50



*P<0.05, **p<0.01 when compared with TGF
TGF - Transforming growth factor β1 (1ng/ml)
Islets were incubated in RPMI - 1640 medium with TGF, MES (10^{-8}M - 10^{-4}M), 4mM glucose and 2.5μCi [^{3}H]thymidine

G9019

LIST OF PUBLICATIONS

PAPERS PRESENTED IN SCIENTIFIC MEETINGS

C.S.Paulose and **Mohanan V.V.** "Decreased 5-HT_{1A} receptor gene expression in the hypothalamus and increased insulin secretion from pancreas during pancreatic regeneration". XXII Symposium on Reproductive Biology and Comparative Endocrinology , January 2004, Chennai, India.

Mohanan V.V and C.S.Paulose. "Down regulation of $5HT_{1A}$ receptors in the brain and increased insulin secretion from pancreas during pancreatic regeneration". International Medical Sciences Academy (IMSA) Annual Conference, September 2003, Cochin, India.

P.N. Eswar Shankar., **Mohanan V.V.** and C.S.Paulose. "Dopaminergic neuromodulation in streptozotocin induced diabetes and pancreatic islet function" (ABSTRACT). Indo-US symposium on "Brain Reasearch". January 2002, New Delhi, India.

Mohanan V.V, Renuka T.R, Pyroja S, Karunakar Narayan and C.S Paulose. "Antidiabetic effect of Diabaid and the role of malate dehydrogenase activity in streptozotocin induced diabetic rats". National Symposium on Medical, Plant and Industrial Biotechnology December 2000, Cochin, India.

PAPERS COMMUNICATED

Mohanan.V.V and C.S.Paulose. "Decreased $5HT_{2C}$ receptors in the cerebral cortex during pancreatic regeneration in rats" Life Sciences (Communicated}

Mohanan.V.V. and C.S.Paulose . "$5HT_{1A}$ and $5HT_{2C}$ receptor down regulation in the hypothalamus during pancreatic regeneration in rats" Biochimica et Biophysica Acta.

Mohanan.V.V. and C.S.Paulose. "Differential regulation of 5-HT_{1A} and 5-HT_{2C} receptors in the brain stem of pancreatectomised rats" Brain Reasearch.

Mohanan.V.V. and C.S.Paulose. Up regulation of 5-HT_{1A} receptors in the pancreatic islets during pancreatic regeneration in weanling rats. Current Science.