GABA$_A$ AND GABA$_B$ RECEPTOR GENE EXPRESSION AND FUNCTIONAL REGULATION DURING PANCREATIC REGENERATION AND INSULIN SECRETION IN RATS

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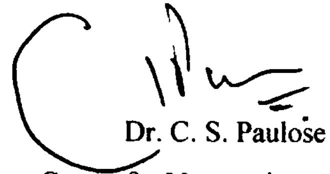
JULY 2005

CERTIFICATE

This is to certify that the thesis entitled **"GABA$_A$ AND GABA$_B$ RECEPTOR GENE EXPRESSION AND FUNCTIONAL REGULATION DURING PANCREATIC REGENERATION AND INSULIN SECRETION IN RATS"** is a bonafide record of the research work carried out by Mr. Balarama Kaimal. S, under my guidance and supervision in the Department of Biotechnology, Cochin University of Science and Technology and that no part thereof has been presented for the award of any other degree.



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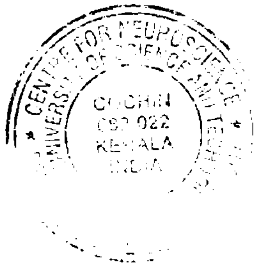

DECLARATION

I hereby declare that this thesis entitled **"$GABA_A$ AND $GABA_B$ RECEPTOR GENE EXPRESSION AND FUNCTIONAL REGULATION DURING 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 and Head, Department of Biotechnology and no part thereof has been presented for the award of any other degree, diploma, associateship or other similar titles or recognition.

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(Balarama Kaimal S)

DEDICATED TO

The People of Mother India and her Democratic Values, which Aid the Research Scenario of this Great Nation

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 monophosphate
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	Deoxycytosine triphosphate
DEPC	Diethyl pyrocarbonate
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 1
OBJECTIVES OF THE PRESENT STUDY 7

LITERATURE REVIEW 8
PANCREAS 8
PARTIAL PANCREATECTOMY AND PANCREATIC REGENERATION 10

FACTORS AFFECTING INSULIN SECRETION FROM PANCREATIC β-CELLS 11
GLUCOSE 11
AMINO ACIDS 12
FATTY ACIDS 12
GLUCAGON 12
SUBSTRATES DERIVED FROM NUTRIENTS 13
SOMATOSTATIN 13
PANCREASTATIN 14
AMYLIN 14
ADRENOMEDULLIN 15
GALANIN 15
MACROPHAGE MIGRATION INHIBITORY FACTOR (MIF) 15
OTHER AGENTS 16

ROLE OF NEUROTRANSMITTERS IN INSULIN REGULATION 16
EPINEPHRINE AND NOREPINEPHRINE 16
ACETYLCHOLINE 17
DOPAMINE 17
SEROTONIN 18
γ-AMINOBUTYRIC ACID 19

PANCREATIC REGENERATION AND β-CELL GROWTH 19
ISLET CELLS IN REGENERATION 20
MECHANISM OF β-CELL GROWTH 21
FACTORS REGULATING β-CELL GROWTH 25

NEUROTRANSMITTERS AS GROWTH SIGNALS 27
NOREPINEPHRINE 27
GAMMA AMINO BUTYRIC ACID 28
ACETYLCHOLINE 29
SEROTONIN 29

GABA 30
$GABA_A$ RECEPTORS 30
$GABA_B$ RECEPTORS 31
GABA IN PANCREAS 32

MATERIALS AND METHODS 33
BIOCHEMICALS AND THEIR SOURCES 33
BIOCHEMICALS: (SIGMA CHEMICAL CO., USA) 33
RADIOCHEMICALS 33
MOLECULAR BIOLOGY CHEMICALS 33
ANIMALS 33
PARTIAL PANCREATECTOMY AND SACRIFICE 34
TISSUE PREPARATION 34
ESTIMATION OF BLOOD GLUCOSE 34

IN VIVO DNA SYNTHESIS STUDIES IN PANCREAS 35

ESTIMATION OF CIRCULATING INSULIN BY
RADIOIMMUNOASSAY 36
PRINCIPLE OF THE ASSAY 36
ASSAY PROTOCOL 36

GABA RECEPTOR STUDIES USING [^{3}H] RADIOLIGANDS 37
GABA RECEPTOR BINDING ASSAYS 37
$GABA_A$ RECEPTOR BINDING ASSAYS 37
$GABA_B$ RECEPTOR BINDING STUDIES 38
RECEPTOR BINDING PARAMETERS ANALYSIS 38
QUANTIFICATION OF GABA USING [^{3}H]RADIOLIGANDS 39
PROTEIN DETERMINATION 39
ISOLATION OF PANCREATIC ISLETS 39

INSULIN SECRETION STUDIES WITH GABA, AGONISTS AND
ANTAGONIST OF $GABA_A$ AND $GABA_B$ RECEPTORS *IN VITRO* 40
INSULIN SECRETION STUDY - 1 HOUR 40
INSULIN SECRETION STUDY - 24 HOURS 41

PANCREATIC DNA SYNTHESIS STUDIES *IN VITRO* 41

STATISTICS 42
DISPLACEMENT CURVE ANALYSIS 42

REVERSE TRANSCRIPTION POLYMERASE CHAIN
REACTION (RT-PCR) 42
ISOLATION OF mRNA 42

THERMOCYCLING PROFILE FOR REAL TIME-PCR 44
ANALYSIS OF REAL TIME PCR PRODUCT 45

RESULTS 46
BODY WEIGHTS AND BLOOD GLUCOSE LEVELS 46
DNA SYNTHESIS IN THE REGENERATING PANCREAS 46
CIRCULATING INSULIN LEVEL 46
GABA CONTENT IN THE BRAIN REGIONS (BRAIN STEM, CEREBELLUM AND HYPOTHALAMUS) OF EXPERIMENTAL RATS 46
GABA CONTENT IN THE PANCREAS OF EXPERIMENTAL RATS 47
RECEPTOR ALTERATIONS IN THE BRAIN REGIONS OF EXPERIMENTAL RATS 47

$GABA_A$ RECEPTOR ANALYSIS 47

BRAIN STEM 47
[^{3}H]GABA BINDING PARAMETERS 47
DISPLACEMENT ANALYSIS OF [^{3}H]GABA 47
[^{3}H]BICUCULLINE BINDING PARAMETERS 48
DISPLACEMENT ANALYSIS OF [^{3}H]BICUCULLINE 48
REAL TIME-PCR ANALYSIS OF $GABA_A$ RECEPTOR 48

HYPOTHALAMUS 49
[^{3}H]GABA BINDING PARAMETERS 49
DISPLACEMENT ANALYSIS OF [^{3}H] GABA 49
[^{3}H]BICUCULLINE BINDING PARAMETERS 49
DISPLACEMENT ANALYSIS OF [^{3}H] BICUCULLINE 50
REAL TIME-PCR ANALYSIS OF $GABA_A$ RECEPTOR 50

CEREBELLUM 51
[^{3}H]GABA BINDING PARAMETERS 51
DISPLACEMENT ANALYSIS OF [^{3}H] GABA 51
[^{3}H]BICUCULLINE BINDING PARAMETERS 51
DISPLACEMENT ANALYSIS OF [^{3}H] BICUCULLINE 51
REAL TIME-PCR ANALYSIS OF $GABA_A$ RECEPTOR 52

PANCREAS 52
[^{3}H]GABA BINDING PARAMETERS 52
DISPLACEMENT ANALYSIS OF [^{3}H] GABA 53

[^{3}H]BICUCULLINE BINDING PARAMETERS 53
DISPLACEMENT ANALYSIS OF [^{3}H] BICUCULLINE 53
REAL TIME-PCR ANALYSIS OF $GABA_A$ RECEPTOR 53

$GABA_B$ RECEPTOR ANALYSIS 54

BRAIN STEM 54
[^{3}H]BACLOFEN BINDING PARAMETERS 54
DISPLACEMENT ANALYSIS OF [^{3}H]BACLOFEN 54

HYPOTHALAMUS 55
[^{3}H]BACLOFEN BINDING PARAMETERS 55
DISPLACEMENT ANALYSIS OF [^{3}H]BACLOFEN 55

CEREBELLUM 55
[^{3}H]BACLOFEN BINDING PARAMETERS 55
DISPLACEMENT ANALYSIS OF [^{3}H]BACLOFEN 56

PANCREAS 56
[^{3}H]BACLOFEN BINDING PARAMETERS 56
DISPLACEMENT ANALYSIS OF [^{3}H]BACLOFEN 56

INSULIN SECRETION STUDIES IN PANCREATIC ISLETS 57
ONE HOUR *IN VITRO* INSULIN SECRETION 57

EFFECT OF GABA ON GLUCOSE INDUCED
INSULIN SECRETION *IN VITRO* 57
EFFECT OF GABA IN THE PRESENCE OF $GABA_A$
ANTAGONIST BICUCULLINE ON GLUCOSE INDUCED
INSULIN SECRETION *IN VITRO* 57
EFFECT OF MUSCIMOL ON GLUCOSE INDUCED
INSULIN SECRETION *IN VITRO* 57
EFFECT OF BACLOFEN ON GLUCOSE INDUCED
INSULIN SECRETION *IN VITRO* 58

24 HRS *IN VITRO* CULTURE 58

EFFECT OF GABA ON GLUCOSE INDUCED INSULIN
SECRETION IN 24 HRS ISLET CULTURES 58
EFFECT OF GABA IN THE PRESENCE OF $GABA_A$
ANTAGONIST BICUCULLINE ON GLUCOSE INDUCED
INSULIN SECRETION *IN VITRO* 58

EFFECT OF MUSCIMOL ON GLUCOSE INDUCED INSULIN SECRETION *IN VITRO* 59
EFFECT OF BACLOFEN ON GLUCOSE INDUCED INSULIN SECRETION *IN VITRO* 59

IN VITRO DNA SYNTHESIS STUDIES IN PANCREATIC ISLETS 59

EFFECT OF GABA ON ISLET DNA SYNTHESIS 59
EFFECT OF MUSCIMOL ON ISLET DNA SYNTHESIS 60
EFFECT OF BACLOFEN ON ISLET DNA SYNTHESIS 60
DOSE-DEPENDENT RESPONSE OF ISLET DNA SYNTHESIS TO MUSCIMOL 61
DOSE-DEPENDENT RESPONSE OF EGF INDUCED ISLET DNA SYNTHESIS TO MUSCIMOL 61

DOSE-DEPENDENT RESPONSE OF ISLET DNA SYNTHESIS TO BACLOFEN 61
DOSE-DEPENDENT RESPONSE OF EGF INDUCED ISLET DNA SYNTHESIS TO BACLOFEN 61

EFFECT OF PERTUSSIS TOXIN ON BACLOFEN MEDIATED DNA SYNTHESIS 61

DISCUSSION 62
DNA SYNTHESIS IN PANCREAS AFTER PARTIAL PANCREATECTOMY 62
GABA CONTENT IN BRAIN REGIONS DURING PANCREATIC REGENERATION 63
GABA CONTENT IN PANCREAS DURING PANCREATIC REGENERATION 64

BRAIN $GABA_A$ AND $GABA_B$ RECEPTOR ALTERATIONS IN THE RATS DURING PANCREATIC REGENERATION 65
$GABA_A$ AND $GABA_B$ RECEPTOR ALTERATIONS IN THE BRAIN STEM 66
$GABA_A$ AND $GABA_B$ RECEPTOR ALTERATIONS IN HYPOTHALAMUS 68
$GABA_A$ AND $GABA_B$ RECEPTOR ALTERATIONS IN CEREBELLUM 69

PANCREATIC $GABA_A$ AND $GABA_B$ RECEPTOR ALTERATIONS IN THE RATS DURING PANCREATIC REGENERATION 69

GABAERGIC INHIBITION OF INSULIN SYNTHESIS AND SECRETION FROM PANCREATIC B-CELLS *IN VITRO* 70

EFFECT OF GABA, MUSCIMOL AND BACLOFEN ON PANCREATIC DNA SYNTHESIS 73

SUMMARY 77

CONCLUSION 79

REFERENCES 81

LIST OF PUBLICATIONS 107

TABLES AND FIGURES

INTRODUCTION

The brain neurotransmitters' receptor activity and hormonal pathways control many physiological functions in the body. γ- aminobutyric acid, also known as GABA was discovered over 40 years ago as a key inhibitory neurotransmitter in the brain (Bazemore *et al.*, 1957, Krnjevic & Phillis, 1963). Since then, evidence has accumulated that this amino acid may function as a neurotransmitter not only in the central nervous system but also in the peripheral nervous system, including the myenteric plexus (Amenta, 1986, Hills & Taniyama, 1987), major pelvic ganglia (Akasu *et al.*, 1999), and sympathetic ganglia, encompassing the rat superior cervical ganglion (Bertilsson *et al.*, 1976, Kasa *et al.*, 1988, Wolff *et al.*, 1986) and abdominal prevertebral ganglia (Parkman & Stapelfeldt, 1993). In the mammalian central nervous system (CNS), GABA is the most important inhibitory neurotransmitter occurring in 30-40% of all synapses. Three types of GABA receptors have been identified: $GABA_A$ and $GABA_C$ receptors are ligand-gated Cl^- channels, while $GABA_B$ receptors are G protein coupled (Chebib & Johnston, 1999). $GABA_A$ receptors are ligand-gated Cl^- channels that consist of a heteromeric mixture of protein subunits forming a pentameric structure, and $GABA_B$ receptors couple to Ca^{2+} and K^+ channels via G proteins and second messengers (Johnston, 1996). In the central nervous system, application of GABA reduces excitability by a combination of $GABA_A$ and $GABA_B$ receptor activation, leading to membrane repolarization, reduced Ca^{2+} influx, and suppression of neurotransmitter release.

Fast inhibitory neurotransmission in the mammalian central nervous system is mediated mainly by the $GABA_A$ receptor, a ligand-gated chloride channel. The receptor complex presumably is composed of five protein subunits, each consisting of an extracellular N-terminal domain with a putative cysteine loop, four largely conserved transmembrane segments (TM), and a variable intracellular region between TM3 and TM4. This topology is characteristic for members of the superfamily of

ligand-gated ion channel receptors (Schofield, 1987). Several $GABA_A$ receptor subunits (α 1–6, β 1–3, γ 1–3, δ, ε, θ, π, and ρ 1–3) have been cloned from mammalian brain (Korpi, 2002; Mehta *et al.*, 1999). Thus, the genetic diversity of multiple $GABA_A$ receptor subunits permits the assembly of a vast number of receptor heteromeric isoforms. Apparently, the subunit composition determines the pharmacological profile of the resulting receptor subtypes (Barnard *et al.*, 1998). Mechanisms that modulate the stability and function of postsynaptic $GABA_A$ receptor subtypes and that are implicated in functional plasticity of inhibitory transmission in the brain are of special interest (Luscher & Keller, 2004).

$GABA_C$ receptors appear to be relatively simple ligand-gated Cl^- channels with a distinctive pharmacology, in that they are not blocked by bicuculline and not modulated by barbiturates, benzodiazepines or neuroactive steroids. Compared with $GABA_A$ receptors, $GABA_C$ receptors are activated at lower concentrations of GABA and are less liable to desensitization. In addition, their channels open for a longer time. The pharmacology of these novel subtypes of GABA receptors are yet to be explored and may yield important therapeutic agents (Johnston, 1996).

GABA has been implicated in cell growth during differentiation in the cultures in at least certain neuron types (Spoerri, 1982). GABA was reported to be present in the pancreas in comparable concentrations with those in the central nervous system during the early seventies (Briel *et al.*, 1975; Okada *et al.*, 1976). Prolonged binding to peripheral benzodiazepine receptors is hypothesized to cause human β-cells functional damage and apoptosis (Marselli *et al.*, 2004). Cytokines produced by immune system cells infiltrating pancreatic islets are candidate mediators of islet β-cells destruction in autoimmune insulin-dependent diabetes mellitus. Peripheral benzodiazepine receptors constitute the aspecific mitochondrial permeability transition pore, and that it has been suggested to be involved in cytokine-induced cell death (Trincavelli *et al.*, 2002). In the CNS, GABA affects

neuronal activity through both the ligand-gated $GABA_A$ receptor channel and the G protein-coupled $GABA_B$ receptor. In the mature nervous system, both receptor subtypes decrease neural excitability, whereas in most neurons during development, the $GABA_A$ receptor increases neural excitability and raises cytosolic Ca^{2+} levels. $GABA_B$ receptor activation depresses $GABA_A$ receptor-mediated Ca^{2+} rises by both reducing the synaptic release of GABA and decreasing the postsynaptic Ca^{2+} responsiveness. Collectively, $GABA_B$ receptors play an important inhibitory role regulating Ca^{2+} rises elicited by $GABA_A$ receptor activation. Changes in cytosolic Ca^{2+} during early neural development would, in turn, profoundly affect a wide array of physiological processes, such as gene expression, neurite outgrowth, transmitter release and synaptogenesis (Obrietan & van der Pol, 1998).

The endocrine part of the pancreas plays a central role in blood-glucose regulation. GABA released from β-cells is considered as an inhibitor of insulin secretion in pancreatic islets and that the effect is principally due to direct suppression of exocytosis in which $GABA_B$ receptors are said to play a role when activated (Braun *et al.*, 2004). GABA has been proposed to function as a paracrine signaling molecule in islets of Langerhans and the Glucose inhibition of glucagon secretion from rat alpha-cells is mediated by GABA released from neighboring β-cells (Wendt *et al.*, 2004). GAD_{65} and the second isoform of glutamate decarboxylase, GAD_{67}, catalyze the formation of the inhibitory neurotransmitter GABA from glutamate. Both GAD and GABA are present in pancreatic islets at concentrations similar to those encountered in classical GABAergic regions of the brain (Taniguchi *et al.*, 1977). In pancreatic islets, both GAD and GABA selectively localize to β-cells (Michalik & Erecinska, 1992). GABA is associated with a vesicular compartment distinctly different from insulin secretory granules (Sorenson *et al.*, 1991). Type I diabetes mellitus is caused by the selective autoimmune destruction of insulin-producing ß-cells in pancreatic islets of Langerhans. One of the

most important autoantigens in type I diabetes is the 65-kD isoform of glutamate decarboxylase (GAD_{65}). GAD_{65}-reactive cytotoxic T-lymphocytes can mediate the autoimmune destruction of the β-cells (Yoon *et al.*, 1999). Peripheral benzodiazepine receptors are present in purified human pancreatic islets suggesting their role in the mechanisms of insulin release (Giusti *et al.*, 1997). PK 11195 [1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline-carboxami de], a potent and selective ligand for peripheral benzodiazepine binding sites inhibits insulin release from rat pancreatic islets (Pujalte *et al.*, 2000). $GABA_B$ receptors play a role in the regulation of the endocrine pancreas with mechanisms probably involving direct activation or inhibition of voltage dependent Ca^{2+}-channels, cAMP generation and G-protein-mediated modulation of K_{ATP} channels (Brice *et al.*, 2002).

In a study of conversion of glutamate to GABA (Fernandez-Pascual *et al.*, 2004) L-glutamine is metabolized preferentially to GABA and L-aspartate. They accumulate in islets preventing its complete oxidation in the Krebs cycle, which accounts for its failure to stimulate insulin secretion.

There is complex nature of GABAergic neurons and β-cells GABA in regulation of islet function. The mammalian pancreas, like the gut wall, has an intrinsic nervous system consisting of ganglia, interconnecting intrinsic nerve fibers, and extrinsic parasympathetic and sympathetic nerves (Berthoud *et al.*, 1991, 2001). Pancreatic ganglion neurons contain $GABA_A$ receptors. Exogenously added GABA acts through $GABA_A$ receptors to cause depolarization, inhibiting excitatory postsynaptic field potentials (fEPSPs). Ganglionic glial cells store and can release endogenous GABA. The presence of GABA in glial cells and the absence of GABA immunoreactivity in ganglion neurons and nerve fibers and endings suggest that GABA in pancreatic ganglia functions as a paracrine messenger molecule rather than as a neurotransmitter substance (Sha *et al.*, 2001). New studies provide evidence demonstrating the presence of GABAergic nerve cell bodies at the periphery of islets

with numerous GABA-containing processes extending into the islet mantle. This close association between GABAergic neurons and islet α and δ-cells strongly suggests that GABA inhibition of somatostatin and glucagon secretion is mediated by these neurons (Sorenson *et al.*, 1991). In mammalian peripheral sympathetic ganglia GABA acts presynaptically to facilitate cholinergic transmission and postsynaptically to depolarize membrane potential. Endogenous GABA released from ganglionic glial cells acts on pancreatic ganglion neurons through $GABA_A$ receptors (Sha *et al.*, 2001). The mammalian pancreas, like the gut wall, has an intrinsic nervous system consisting of ganglia, interconnecting intrinsic nerve fibers, and extrinsic parasympathetic and sympathetic nerves.

The natural source for new pancreatic β-cells is an important issue both for understanding the pathogenesis of diabetes, and for possibly curing diabetes by increasing the number of β-cells. Transplantation of pancreatic islets can now be applied successfully to treat diabetes, but its widespread use is hampered by a shortage of donor organs. Since insulin-producing β-cells cannot be expanded significantly in vitro, efforts are under way to identify stem or progenitor cells that potentially could be grown and differentiated into β-cells *in vitro*. Such cells could provide an ample supply of transplantable tissue. Current research in this field focuses mainly on pluripotential embryonic stem cells and on pancreas-specific adult progenitor cells. β-cell replication is the only source for new β-cells without contributions from stem cells or other non- β-cells. The pancreatic gland has an enormous potential for growth and regeneration, mainly in rodents. Animal models of pancreatic regeneration can be easily established in weanling rats. There are no reports that the human pancreas shows proliferative properties after partial pancreatectomy, but research in this field has been scarce.

Partial pancreatectomy is an established model to study the pancreatic regeneration. In the present study, we have investigated the changes in the brain and

pancreas- GABA, GABA receptor subtypes and their gene expression during pancreatic regeneration. Also, the effect of GABA, its receptor agonists and antagonists in presence of growth factors on DNA synthesis and insulin secretion were studied *in vitro*.

OBJECTIVES OF THE PRESENT STUDY

1. To induce regeneration of pancreatic tissue 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 GABA content in various rat brain regions – brain stem, cerebellum and hypothalamus during pancreatic regeneration.
4. To study the changes in GABA content in the pancreas of experimental rats during pancreatic regeneration.
5. To study the GABA, $GABA_A$ and $GABA_B$ receptor alterations during pancreatic regeneration in brain stem, hypothalamus and cerebellum of sham and experimental rats.
6. To study the GABA, $GABA_A$ and $GABA_B$ receptor alterations during pancreatic regeneration in pancreas of different experimental rat groups.
7. To study the alterations in the GABA receptor subtypes gene expression during pancreatic regeneration in brain stem, hypothalamus, cerebellum and pancreas of sham and experimental rats.
8. To study the effect of GABA, $GABA_A$ receptor agonist muscimol and $GABA_B$ receptor agonist baclofen on insulin secretion in isolated rat pancreatic islets.
9. To study the effect of GABA, $GABA_A$ receptor agonist muscimol and $GABA_B$ receptor agonist- baclofen on DNA synthesis in rats *in vitro.*

LITERATURE REVIEW

Diabetes mellitus is one of the diseases familiar since the ancient times, 'Ayurveda' the 3000-5000 year old traditional system of Indian herbal medicine describes it as *'Meha' or 'Madhumeha* meaning 'Honey urine' (Shashtri & Chaturvedi, 1977) and one among *Ashtamaharogaa:* (the eight major diseases as described in *Ashtangahrihaya,* the Ayervedic text written in Sanskrit). In diabetic state the body does not produce or properly use insulin. Type 1 diabetes results from the body's failure to produce insulin, which is allowing glucose to enter and fuel the cells. It is caused by autoimmune destruction of pancreatic islet β-cells. The presence of healthy β-cells mass in the pancreas is an important factor in maintaining the body homeostasis.

Pancreas

Pancreas is a complex organ consisting of both endocrine and exocrine cells. Approximately 5 percent of the total pancreatic mass is comprised of endocrine cells. These endocrine cells are clustered in groups within the pancreas, which look like little islands of cells when examined under a microscope. This appearance led to these groups of pancreatic endocrine cells being called "pancreatic islets". Within pancreatic islets are cells, which make specific pancreatic endocrine hormones, of which there are only a few, the most famous of course being insulin. Pancreatic islets are scattered throughout the pancreas. Like all endocrine glands, they secrete their hormones into the bloodstream and not into tubes or ducts like the digestive pancreas. Because of this need to secrete their hormones into the blood stream, pancreatic islets are surrounded by small blood vessels. 65-80% of the islets are insulin- secreting β- cells.

The destruction of β-cell mass will lead to impaired insulin secretion and thereby hyperglycemia. Management of diabetes is burdensome both to the individual and society, costing over 100 billion US dollars annually. Transplantation of the pancreatic β-cells to the patient body is suggested as one of the treatment methods. Shortage of pancreatic tissue, together with a lifetime requirement of immunosuppressive drugs to prevent rejection and recurrent disease, remain as major hurdles yet to be overcome prior to widespread applicability. In this context newer techniques such as use of stem cells and regeneration of the remaining healthy β- islet cells have been proposed more interesting alternatives in diabetic therapy. Development of stem cells into potential pancreatic β-cells and the regeneration of existing islets by down-regulation of autoimmunity were recommended for future research to cure this ailment (Ramiya *et al.*, 2004). Indeed, islet-regeneration research will soon match the level of interest.

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

Light and electron microscopic studies have demonstrated that there are three different types of nerve endings in the pancreas: sympathetic, parasympathetic and peptidergic nerves (Miller, 1981). The neurotransmitters found in the first two nerve

terminals are catecholamines and acetylcholine. The peptidergic nerve terminals contain various peptides as neurotransmitters. The nerve fibres enter the pancreas in association with the vascular supply and they are distributed to blood vessels, acinar tissue and islets. Adrenergic fibres innervate vessels, acini and islets. Cholinergic fibres are found mainly in islets. Peptidergic nerves are found in both exocrine and endocrine tissue (Ahren *et al.*, 1986).

Partial pancreatectomy and pancreatic regeneration

The natural source for new pancreatic β-cells is an important issue both for understanding the pathogenesis of diabetes, and for possibly curing diabetes by increasing the number of β-cells. Transplantation of pancreatic islets can now be applied successfully to treat diabetes, but its widespread use is hampered by a shortage of donor organs. Since insulin-producing β-cells cannot be expanded significantly *in vitro*, efforts are under way to identify stem or progenitor cells that potentially could be grown and differentiated into β-cells *in vitro*. Such cells could provide an ample supply of transplantable tissue. Current research in this field focuses mainly on pluripotential embryonic stem cells and on pancreas-specific adult progenitor cells. β-cell replication is the only source for new β-cells without contributions from stem cells or other non- β-cells. The pancreatic gland has an enormous potential for growth and regeneration, mainly in rodents. Animal models of pancreatic regeneration can be easily established in weanling rats.

The pancreatic gland shows a tendency for growth and regeneration, mainly in rodents. The mammalian pancreas has a strong regenerative potential, but the origin of organ restoration is not clear, and it is not known to what degree such a process reflects pancreatic development (Jensen *et al.*, 2005). The human pancreas however does not show proliferative properties after partial pancreatectomy, but research in this field has been scarce (Morisset, 2003).

Streptozotocin (STZ)-induced diabetic mice can be cured by injection of the regenerating pancreatic extract (RPE) of the partially pancreatectomized Wistar-Kyoto rats (Shin *et al.*, 2005). Pancreatitis-associated protein (PAP) and regenerating protein 1_{α} ($Reg1_{A}$) are up-regulated during the pancreas regeneration. Transplantation of pancreas has beneficial effects on impaired islet, inducing regeneration in the spontaneously diabetic Torii rat (25-week-old) (Miao *et al.*, 2005). Pancreatic regeneration following chemically induced pancreatitis in the mouse occurs predominantly through acinar cell dedifferentiation, whereby a genetic program resembling embryonic pancreatic precursors is reinstated (Jensen *et al.*, 2005).

FACTORS AFFECTING INSULIN SECRETION FROM PANCREATIC β-CELLS

Glucose

Insulin is secreted primarily in response to elevated blood glucose concentrations. The mechanism of glucose induced insulin release is not completely understood. Phosphorylation of glucose to glucose-6-phosphate serves as the rate-limiting step in glucose oxidation (Schuit, 1996). Glucokinase acts as a glucose sensor during this process. The entry of glucose into β-cells is followed by an acceleration of metabolism that generates one or several signals that close ATP-sensitive K^{+} channels in the plasma membrane. The resulting decrease in K^{+} conductance leads to depolarisation of the membrane with subsequent opening of voltage dependent Ca^{2+} channels. The rise in the cytoplasmic free Ca^{2+} eventually leads to the exocytosis of insulin containing granules (Dunne, 1991). 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 islet enlargement, an important issue in the progression towards overt diabetes (Wookey & Cooper, 2001).

Adrenomedullin

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

Galanin

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

Macrophage migration inhibitory factor (MIF)

MIF, originally identified as cytokines, is 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 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). EPI and NE also inhibit insulin -stimulated glycogenesis through inactivation of glycogen

synthase and activation of phosphorylase with consequent accumulation of glucose-6-phosphate. In addition, it has been reported that epinephrine enhances glycolysis through an increased activation of phospho-fructokinase. In humans, adrenaline stimulates lipolysis, ketogenesis, thermogenesis and glycolysis and raises plasma glucose concentrations by stimulating both glycogenolysis and gluconeogenesis. Adrenaline is, however, known to play a secondary role in the physiology of glucose counter-regulation. Indeed, it has been shown to play a critical role in one pathophysiological state, the altered glucose counter-regulation in patients with established insulin-dependent diabetes mellitus (Cryer, 1993). The inhibitory effect of EPI upon insulin secretion induced by glucose was reported by Coore & Randle (1964). As judged by Malaisse *et al.*, (1967), the inhibitory effect of EPI on glucose-induced insulin secretion is mediated through the activation of α-adrenoreceptors. 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).

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.

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 content of 5-HIAA and accelerated the cerebral 5-HT turnover (Juszkiewicz, 1985). The 5-HIAA content 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). Studies suggest that the brain 5-HT through 5-HT_{1A} receptor has a functional role in the pancreatic regeneration through the sympathetic regulation (Mohanan *et al.,* 2005).

γ-Aminobutyric acid

Gamma aminobutyric acid 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 (GAD), 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.

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 (Bonner-Weir *et al.*, 1993; Waguri *et al.*, 1997). 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.*, 1995). 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 (Finegood *et al.*, 1995; Bonner-Weir *et al.*, 2000). 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, 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 islets (Bonner-Weir, 2000). 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

β-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 (iii) replication capacity of existing β-cells (Swenne, 1992). The relative contribution of replication, neogenesis or increased β-cell size to the increased β-cell mass is not very clear at this time. The ability of the pancreas to regenerate and the effects of trophic hormones on regeneration of the pancreas after partial pancreatectomy are not completely understood. There is strong evidence to the existence of neogenesis as a plausible mechanism for changes in β-cell mass based on studies in rat models (Swenne, 1982; Swenne & Eriksson, 1982). In contrast, changes in size of individual β-cells is not very well documented, even though, glucose, which is the prime stimulator of β-cell replication, increases β-cell size and apparently leads to increased

insulin synthesis (Hakan Borg *et al.*, 1981). Several studies pioneered by Hellerstrom and Bonner-Weir have lead to an improved understanding of mechanisms associated with β-cell proliferation (Hellerstrom, 1984; Bonner-Weir 1994). Swenne performed the initial cell cycle characterization of β-cells and paved the way for further investigations into the replication capacity of β-cells. Standard thymidine incorporation assays and more recently using antibody-based bromodeoxyuridine assays have determined islet cell replication.

Upon receiving stimulatory influences from either cytokines or growth factors, mammalian cells undergo a regulated cell cycle progression. Every phase of the cell cycle is under regulatory influences of different cell cycle proteins. Changes in cell cycle progression modulate the rate of proliferation and growth. Moreover, 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 by 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 proteins regulating the cell cycle machinery (Grana *et al.*, 1995; Sherr, 1996). The cell cycle is typically divided into the following phases, G_0 (reversible quiescence), G_1 (first gap phase), S (DNA synthesis), G_2 (second gap phase) and M (mitosis).

Pancreatic β-cells, similar to other cell types, pass through several distinct phases of the cell cycle. Studies elucidated the replication capacity of β-cells (Swenne, 1982; Hellerstrom, 1984). 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. 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 G_0 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 G_0 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 G_0 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 G_0-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 molecular mechanisms that 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 B_1 that 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). β-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. 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 over

expression of growth factor receptors such as the platelet-derived growth factor receptor (PDGF-R) and fibroblast growth factor receptor (FGF-R). Growth factor mediated signal transduction pathways lead to changes in expression of cell cycle proteins, eventually, resulting in the increased proliferation effects. Identification of cell cycle modulators of β-cell proliferation will provide insights into the replication potential of fetal, young and adult islet cells.

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 α, β and δ- 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-existing embryonic ductal cells, which is referred to as neogenesis, or by replication of β-cells. While neogenesis is the primary mode of increase in β-cell mass during 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 of factors influencing β-cell replication. Among the various factors, glucose is a prime regulator of β-cell replication and is known to stimulate replication in both fetal and adult rodent islets (Hellerstrom, 1984). In addition, glucose leads to an increased β-cell proliferative compartment (Swenne, 1982). Insulin and IGF-1 stimulate islet β-cell replication in neonatal rodent pancreatic cells in culture providing evidence that

insulin itself can regulate the replication capacity of β-cells in an autocrine fashion (Rabinovitch *et al.,* 1982).

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

Growth hormone elicits many of its actions by inducing local production of IGFs in target cells. Studies aimed at investigating a similar paracrine pathway operative in islet cells have yielded confusing results. GH, but not glucose, stimulated the release of IGF-1 from fetal and adult rat islets leading to mitogenesis, which could be partially negated by addition of monoclonal antibodies to IGF-1 (Swenne *et al.*, 1987). The presence of high-affinity IGF-1 receptors on β-cells and the finding that exogenous IGF-1 stimulates β-cell replication (Van Schravendijk *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). They 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, 1991_a), 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, 1991_b). IL1-β suppresses islet cell proliferation in adult mice and rats (Southern *et al.*, 1990). However, the role of IL1-β in fetal islet cell proliferation is slightly complex with the first 24 hrs of stimulation leading to a suppression of β-cell proliferation followed by a potent mitogenic stimulus after 3 days of cytokine exposure. Sjoholm *et al.*, (1991_c) identified pancreastatin and diazepam-binding inhibitor (acyl-CoA binding protein) as inhibitors of β-cell replication. Both pancreastatin and diazepam-binding inhibitor are produced by islet cells (Chen *et al.*, 1988) and inhibit insulin secretion and may function as inhibitors of β-cell replication *in vivo*.

NEUROTRANSMITTERS AS GROWTH SIGNALS

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 G_i, or positively coupled to phospholipase C (G_q) or to pertussis toxin–sensitive pathways (G_o, G_i) (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 (Michalopoulos & 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^{2+} 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 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.*, 2002_a). 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). During the regeneration of liver, $GABA_A$ receptor acts as an inhibitory signal for hepatic cell proliferation (Biju *et al.*, 2001_b).

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 parameters 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 cell proliferation (Watanabe *et al.*, 2001). Serotonin is supposed to act as a potent hepatocyte comitogen and induce DNA synthesis in primary cultures of rat hepatocytes, which is suggested to mediate through the serotonin S_2 receptors of hepatocytes (Balasubrahmanian & Paulose, 1998). During the regeneration

of pancreas 5-HT1A and 5-HT2C receptors get downregulated in brain stem and cerebral cortex and brain stem, which the changes are mediated through the sympathetic system (Mohanan *et al.*, $2005_{a, b}$).

GABA

GABA is an inhibitory neurotransmitter present in large quantities the inhibitory neurons of the central nervous system (Csillag, 1991). GABA is formed by the decarboxylation of L-glutamic acid by glutamate decarboxylase (GAD). All interneurons are GABAergic neurons and they comprise a great number in the body. In the central nervous system, GABA acts at two distinct types of receptors, ligand-gated ionotropic $GABA_A$ receptors and $GABA_C$ receptors and G-protein linked metabotropic $GABA_B$ receptors, thus mediating both fast and slow inhibition of excitability at central synapses (Vicini, 1999; Marshall *et al.*, 1999). $GABA_A$ receptor is a ligand-gated chloride ion channel playing an important part in polarizing the cell membrane and reducing neuronal excitability in the neuron. $GABA_A$ and $GABA_B$ receptors are abundant but $GABA_C$ receptors are very much localized in areas such as retina etc.

$GABA_A$ receptors

In developing neurons by contrast, GABA, acting at the $GABA_A$ receptor, can be excitatory. As an excitatory transmitter in the developing brain, GABA can depolarize the membrane potential and increase the frequency of action potentials (Chen *et al.*, 1996; Owens *et al.*, 1996). Functional $GABA_A$ receptors are expressed at the earliest development time studied, embryonic day 15th in rats (Chen *et al.*, 1995, van den Pol *et al.*, 1995). GABA immunoreactivity is found in axonal growth cones (van den Pol, 1997).

$GABA_B$ receptors

$GABA_B$ receptors are expressed both presynaptically (Howe *et al.,* 1987; Dutar & Nicoll 1988; Yoon & Rothman 1991) and postsynaptically (Dutar & Nicoll 1989; Solis & Nicoll 1992). Presynaptic $GABA_B$ receptors have been shown to decrease neurotransmitter release by increasing K^- conductance or decreasing Ca^{2+} conductance or through a mechanism independent of changes in membrane conductance (Misgeld *et al.,* 1995). Additionally, $GABA_B$ receptors have been shown to play a role as autoreceptors, providing a negative feedback control for synaptic GABA secretion (Anderson & Mitchell 1985; Pittaluga et al., 1987). Multiple neurochemical studies at a variety of CNS preparations in which transmitter release has been monitored suggest that subtypes of $GABA_B$ receptors are present on various nerve terminals. Molecular biological cloning of $GABA_B$ receptors has provided additional support to the concept of multiple $GABA_B$ receptors. The initial cloning of a $GABA_B$ receptor that demonstrated the presence of two splice variants ($GABA_BR1a$ and $GABA_BR1b$). This as recently been expanded by the demonstration of a second and different $GABA_B$ clone: $GABA_BR2$ (Kaupman *et al.*, 1997). A major function of $GABA_B$ receptors is to modulate transmitter release (Bowery *et al.*, 1980; Alford & Grillner 1991; Davies *et al.*, 1991; Thompson *et al.*, 1993; Kombian *et al.*, 1996; Mouginot & Gähwiler 1996).

During this early stage of development, activation of the $GABA_A$ receptor usually leads to depolarization and the resultant opening of plasmalemmal Ca^{2+} channels that raise intracellular Ca^{2+} (Obrietan & van den Pol, 1995). In contrast, activation of the $GABA_B$ receptor tends to reduce the GABA-mediated elevations in Ca^{2+}, at both presynaptic and postsynaptic sites of action. Thus GABA would generate two opposing actions, one at the $GABA_A$ receptor that initially depolarizes the cell, raising Ca^{2+}, and a slightly later effect at the $GABA_B$ receptor that would reduce the Ca^{2+} rise (Karl Obrietan *et al.,* 1998). Baclofen, the $GABA_B$ agonist,

dose-dependently induced EGF mediated DNA synthesis in primary hepatocyte cultures and it significantly reduced the TGF $_{\beta1}$ suppression of EGF induced DNA synthesis (Biju *et al.*, 2002_a).

GABA in pancreas

In addition to its presence in the central nervous system, GABA and GAD have been demonstrated in the pancreatic β-cells of normal rat (Garry *et al.*, 1986). GABA is present in large number in the islet cells in the pancreas. The concentration of GABA in the endocrine pancreas is comparable to that measured in the in the central nervous system (Rorsman *et al.*, 1989). In addition, the GABA metabolizing enzyme, GABA-transaminase (GABA-T), was localized in the pancreatic β-cells (Vincent *et al.*, 1983). It is known that the β-cells can produce and release GABA in response to glucose (Okada *et al.*, 1976; Gilon *et al.*, 1991; Satin *et al.*, 1998). It is possible that GABA and Glutamate mediate a paracrine signaling pathway whereby α and β-cells communicate within the islets (Rorsman *et al.*, 1989; Satin *et al.*, 1998; Skerry *et al.*, 2001; Gill *et al.*, 2001).

In the present study we examined the GABA receptor functional regulation and gene expression in an animal model of regulated cell proliferation *in vivo* to elucidate their role in pancreatic cell proliferation. *In vitro* studies were conducted to confirm the involvement of $GABA_A$ and $GABA_B$ receptors in the regulation of pancreatic β-cell proliferation using specific ligands in primary cultures.

MATERIALS AND METHODS

Biochemicals and Their Sources

Biochemicals: (Sigma Chemical Co., USA)

γ-aminobutyric acid (GABA), HEPES (N—[2-hydroxymethyl] piperazine-N'- [2-ethanesulfonic acid), 2-propyl thiol, Tris buffer, foetal calf serum (heat inacticvated), collagenase type XI, Muscimol, Baclofen, Bicuculline methoiodide, Epidermal growth factor, (EGF) and Insulin

Radiochemicals

4-amino-*n*-[2,3-^{3}H]butyric acid (Specific activity- 84.0 Ci/mmol) was purchased from Amersham Bioscience, USA, Baclofen, (-)-[butyl-4-3H(N)] (Specific activity- 42.9 Ci/mmol), Bicuculline methyl chloride, (-)-[methyl-^{3}H] (Specific activity- 82.9 Ci/mmol), were purchased from NEN Life Sciences Products, Inc., Boston USA, [^{3}H]Thymidine (Specific activity 18 Ci/mmol) was purchased from Amersham Bioscience, USA

Molecular biology chemicals

Random hexamer and human placental RNAse inhibitor were purchased from Bangalore Genei, India. Tri-reagent kit was purchased from Sigma Chemical Co., USA. dNTPs were purchased from Bangalore Genei. Real Time-PCR SyBr Green mix was purchased from Takara, Japan. PCR primers used in this study were synthesised by Genemed, San Francisco, USA.

Animals

Weanling male Wistar rats weighing 80-100g were obtained from Kerala Agricultural University, Mannuthy, Thrissur and Amrita Institute of Medical Sciences and Research Centre, Cochin and used for all experiments. All animals

were housed under conditions of controlled temperature and light with free access to food and water.

Partial Pancreatectomy and Sacrifice

Wistar weanling rats, 4-5 weeks old, were anaesthetised under aseptic conditions, the body wall was cut opened and 60-70% of the total pancreas, near to the spleen and duodenum, was removed (Pearson, 1977). The removal of most of the pancreas was done by gentle abrasion with cotton applications, leaving the major blood vessels supplying other organs intact (Zangen, 1997). The sham was done in an identical procedure except that the pancreatic tissue was only lightly rubbed between fingertips using cotton for a minute instead of being removed. Body weight and blood glucose levels were checked daily. The rats were maintained for different time intervals -72 hours and 7 days. The rats sacrificed by decapitation between 7 and 9am for all the experiments.

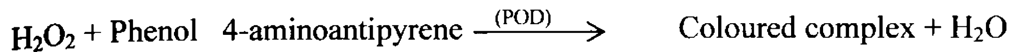
Tissue preparation

The brain regions- cerebral cortex, brain stem and hypothalamus were dissected out quickly over ice according to the procedure of Glowinski and Iversen (1966). The tissues were stored at $^{-}70^{0}$ C until assay. Pancreas was also dissected out and stored.

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:

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



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

The hydrogen peroxide formed 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^{0}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 was 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) 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_o on the Y-axis and insulin concentration/ml on the X-axis of a log-logit graph. %B/B_o was calculated as:

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

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

GABA Receptor Studies Using [^{3}H] Radioligands

GABA Receptor Binding Assays

[^{3}H]GABA binding to the GABA receptor was assayed in Triton X-100 treated synaptic membranes (Kurioka *et al.*, 1981). Crude synaptic membranes were prepared using sodium-free 10mM tris buffer (pH 7.4). Each assay tube contained a protein concentration of 0.3-0.4 mg. In saturation binding experiments, 1-10 nM of [^{3}H]GABA incubated with and without excess of unlabelled GABA (100μM) and in competition binding experiments the incubation mixture contained 2nM of [^{3}H]GABA with and without muscimol at a concentration range of 10^{-9}M to 10^{-4}M. The incubation was continued for 20 min at 0-4°C and terminated by centrifugation at 35,000 x*g* for 20 min. [^{3}H]GABA in the pellet was determined by liquid scintillation spectrometry. Specific binding was determined by subtracting non-specific binding from the total binding.

GABA$_A$ Receptor Binding Assays

[^{3}H]bicuculline binding to the GABA receptor was assayed in Triton X-100 treated synaptic membranes (Kurioka *et al.*, 1981). Crude synaptic membranes were prepared using sodium-free 10mM tris buffer (pH 7.4). Each assay tube contained a protein concentration of 0.3-0.4 mg. In saturation binding experiments, 5nM to 75nM concentrations of [^{3}H]bicuculline incubated with and without excess of unlabelled bicuculline (100μM) and in competition binding experiments the incubation mixture contained 2nM of [^{3}H]bicuculline with and without bicuculline at a concentration range of 10^{-9}M to 10^{-4}M. The incubation was continued for 20 min

at 0-4 ^{o}C and terminated by centrifugation at 35,000xg for 20 min. [^{3}H]bicuculline in the pellet was determined by liquid scintillation spectrometry. Specific binding was determined by subtracting non-specific binding from the total binding.

$GABA_B$ Receptor Binding Studies

[^{3}H]baclofen binding to GABA receptor in the synaptic membrane preparations were assayed as previously described (Hill *et al.*, 1984). Crude synaptic membrane preparation was suspended in 50mM Tris-HCl buffer (pH 7.4) containing 2mM $CaCl_2$ and 0.3-0.4 mg protein. In saturation binding experiments, 10-100nM of [^{3}H]baclofen was incubated with and without excess of unlabelled baclofen (100μM) and in competition binding experiments the incubation mixture contained 2nM of [^{3}H]GABA with and without baclofen at a concentration range of 10^{-9}M to 10^{-4}M. The incubations were carried out at 20 ^{o}C for 20 min. The binding reactions were terminated by centrifugation at 14000xg for 10 min. [^{3}H]baclofen and [^{3}H]GABA in the pellet was determined by liquid scintillation spectrometry. Specific binding was determined by subtracting non-specific binding from total binding.

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 specific binding of the radioligand on X-axis and bound/free on Y-axis using Sigma plot computer software. This is called a Scatchard plot. The B_{max} is a measure of the total number of receptors present in the tissue and the K_d represents affinity of the

receptors for the radioligand. The K_d is inversely related to receptor affinity or the "strength" of binding. Competitive binding data were analyzed using non-linear regression curve-fitting procedure (GraphPad PRISM™, San Diego, USA). The concentration of competitor that competes for half the specific binding was defined as EC_{50}. It is same as IC_{50}. The affinity of the receptor for the competing drug is designated as K_i and is defined as the concentration of the competing ligand that will bind to half the binding sites at equilibrium in the absence of radioligand or other competitors (Cheng & Prusoff., 1973)

Quantification of GABA Using [^{3}H]Radioligands

GABA content in the brain regions and pancreas of the sham and experimental rat groups was quantified by displacement method (Kurioka *et al.*, 1981) where the incubation mixture contained 1nM [^{3}H]GABA with and without GABA at a concentration range of 10^{-9}M to 10^{-4}M. The unknown concentrations were determined from the standard displacement curve using appropriate dilutions and calculated for µmoles/ gm wt. of the tissue.

Protein determination

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

Isolation of Pancreatic Islets

Pancreatic islets were isolated from male weanling Wistar rats by standard collagenase digestion procedures using aseptic techniques (Howell, 1968). The islets were isolated in HEPES-buffered sodium free Hanks Balanced Salt Solution (HBSS)

(Pipeleers, 1985) with the following composition: 137mM Choline chloride, 5.4mM KCl, 1.8mM $CaCl_2$, 0.8mM $MgSO_4$, 1mM KH_2PO_4, 14.3mM $KHCO_3$ and 10mM HEPES. Autoclaved triple distilled water was used in the preparation of the buffer.

The pancreas 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.

Insulin Secretion Studies With GABA, Agonists and Antagonist of $GABA_A$ and $GABA_B$ Receptors *in vitro*

Pancreatic islets were isolated by collagenase digestion and islets were incubated in RPMI-1640 medium for 16 hours in 5% CO_2 at 37^0C for fibroblast attachment. Islets were harvested after 16 hours and used for secretion studies.

Insulin secretion study - 1 hour

Pancreatic islets were harvested after removing the fibroblasts and resuspended in Krebs Ringer Bicarbonater buffer (KRB), pH 7.3. The isolated islets were incubated for 1hour at 37^0C with 10^{-8}-10^{-4} M concentrations of GABA, bicucilline, muscimol and baclofen and two different concentrations of glucose i.e., (i) 4mM glucose and (ii) 20mM glucose, which represent the normal and diabetic

conditions respectively. To study the effect of different GABA receptor subtypes, islets were incubated with combinations of agonists and subtype specific antagonist. After incubation cells were centrifuged at 1,500xg for 10 minutes at $4^{0}C$ and the supernatant were transferred to fresh tubes for insulin assay by radioimmunoassay.

Insulin secretion study - 24 hours

The islets were harvested after removing the fibroblasts and cultured for 24hours in RPMI-1640 medium. Insulin secretion study was carried out by preincubating the cells in 4mM and 20mM glucose concentrations with different concentrations of GABA, bicuculline, muscimol and baclofen. The cells were then harvested and washed with fresh KRB and then incubated for another 1 hour in the presence of same concentrations of glucose, GABA, bicuculline, muscimol and baclofen. At the end of incubation period the medium was collected and insulin content was measured by radioimmunoassay using kit from BARC, Mumbai.

PANCREATIC DNA SYNTHESIS STUDIES *IN VITRO*

Pancreatic islets were prepared by the collagenase digestion method as mentioned earlier. The isolated islets were then suspended in RPMI 1640 medium containing 10% FCS, and incubated for 16 hours at $37^{0}C$ and 5% CO_2 to remove the fibroblasts. The cells were recultured for three days after fibroblast removal to remove all other non-endocrine tissue. The medium will be rich in β-cells after the incubation. Groups of 100 islets were transferred at the end of culture period to 1ml fresh medium containing 5% FCS, antibiotics, different concentrations of glucose (4 and 20mM), GABA, bicuculline, muscimol, baclofen and EGF (10ng) were added and cultured free floating for an additional 24 hours in the presence of 1μCi of $[^3H]$thymidine (Sjoholm, 1991). 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 liquid 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).

Displacement Curve Analysis

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

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Isolation of mRNA

25-50 mg tissue was homogenized in 0.5 ml Tri Reagent. The homogenate was centrifuged at 12,000xg for 10 minutes at 4^0C. The clear supernatant was transferred to a fresh tube and it was allowed to stand at room temperature for 5 minutes. 100µl of chloroform was added to it, shaken vigorously for 15 seconds and

allowed to stand at room temperature for 15 minutes. The tube was centrifuged at 12,000x*g* for 15 minutes at 4^0C. Three distinct phases appear after centrifugation. The bottom red organic phase contained protein, interphase contained DNA and a colourless upper aqueous phase contained RNA. The upper aqueous phase was transferred to a fresh tube and 250µl of isopropanol was added and the tubes allowed to stand at room temperature for 10 minutes. The tubes were centrifuged at 12,000x*g* for 10 min at 4^0C. RNA precipitate forms a pellet on the sides and bottom of the tube. The supernatant was removed and the RNA pellet was washed with 500µl of 75% ethanol, vortexed and centrifuged at 12,000x*g* for 5 min at 4^0C. The pellet was semi dried and dissolved in minimum volume of DEPC-treated water. 2 µl of RNA was made up to 1 ml and absorbance was measured at 260nm and 280nm. For pure RNA preparation the ratio of absorbance at 260/280 was ≥ 1.7. The concentration of RNA was calculated as one absorbance $_{260}$ = 42µg.

RT PCR Primers

5' ACA AGA AGC CAG AGA ACA AGC CAG 3'	α_2 GABA
5' GAG GTC TAC TGG TAA GCT CTA CCA 3'	
5'TGA GAT GGC CAC ATC AGA AGC AGT 3'	β_2 GABA
5' TCA TGG GAG GCT GGA GTT TAG TTC 3'	
5' CAG AGA CAG GAA GCT GAA AAG CAA 3'	γ1 GABA
5' CGA AGT GAT TAT ATT GGA CTA AGC 3'	
5'-TGT GAG CAA CCG GAA ACC AAG CAA-3'	γ2 GABA
5'-CGT GTG ATT CAG CGA ATA AGA CCC-3'	

***RT-PCR of GABA$_A$ receptor* subunits**

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.

Thermocycling profile for Real Time-PCR

For obtaining higher stringency conditions RT-PCR profile was adopted. PCR was carried out in a 25µl volume reaction mixture in the specially designed Real Time PCR tubes provided by Takara, Japan, containing 2µl cDNA, 12.5µl reaction mixture and 1µl of primer and 9.5µl DEPC water. The reaction mixtureis SYBR Premix EX Taq of which the unit definition is one unit is the amount of the enzyme that will incorporate 10nmol of dNTP into acid insoluble products in 30 minutes at 74°C with activated salmon sperm DNA as the template-primer. The ingredients of the reaction mixture are TAPS (pH 9.3 at 25°C, KCl, $MgCl_2$, 2-mercaptoethanol, each dATP, dGTP, dTTP, [a^{-32}]-dCTP and activated salmon sperm DNA).

Following is the thermocycling profile used for GABA$_A$ receptor α_2, β_2, β_3 and γ_1 and γ_2 subunits.

95°C -- 30 seconds	Initial denaturation	
95°C -- 10 seconds	Denaturation	
65°C -- 30 seconds	Annealing	45 cycles
72°C -- 30 seconds	Extension	

Analysis of Real Time PCR product

The Crossing Threshold (Ct) represents the comparative expression of the mRNA of the gene of interest from the samples used. The Ct values are taken from the graph directly in the software of the Real Time PCR provided along with (Cephied SmartCycler Software v2.0) and selected for each experimental group separate. The lowest Ct value represents the higher expression of the mRNA isolated from the samples of the sham and experimental groups.

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

[^{3}H]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 (Figure 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).

GABA Content in the Brain Regions (Brain Stem, Cerebellum and Hypothalamus) of Experimental Rats

In the brainstem, cerebellum and hypothalamus the GABA content was decreased significantly ($p<0.001$) at 72 hrs after partial pancreatectomy when compared with sham. The decreased contents were reversed to near normal by 7days after partial pancreatectomy in the brainstem, cerebellum and hypothalamus (Table - 2).

GABA Content in the Pancreas of Experimental Rats

There was a significant decrease ($p<0.001$) in the pancreatic GABA cont during active cell proliferation when compared with sham in the pancreatic islets the experimental rats. The decreased content was reversed to near normal at 7d after partial pancreatectomy (Table-2).

Receptor Alterations in the Brain Regions of Experimental Rats

$GABA_A$ Receptor Analysis

Brain Stem

[³H]GABA Binding Parameters

Scatchard analysis in the brain stem of rats showed a significant decre ($p<0.001$) in the B_{max} of [^{3}H]GABA binding to the membrane preparation of P 72 (72 hours pancretectomised rats) when compared with sham. The K_d value of P hrs also decreased significantly ($p<0.01$) when compared with sham. The P 7 d days pancretectomised rats) group showed a signioficant reversal of B_{max} and ($p<0.05$) towards sham from the P72 hrs group (Fig.3 & Table-3).

Displacement Analysis of [³H] GABA

The competition curve for GABA against [^{3}H]GABA fitted for two-si model in all the groups with Hill slope value away from Unity. The $Ki_{(H)}$ increa in 72 hrs pancreatectomised rats along with an increase in the log (EC_{50})-1 indicat a shift in high affinity towards low affinity. $Ki_{(L)}$ also showed an increase in 72 pancreatectomised rats with an increase in log (EC_{50})-2 denoting a shift in the l affinity site towards much lower affinity (Fig.-4 & Table -4).

[^{3}H]Bicuculline Binding Parameters

Scatchard analysis in the brain stem of rats showed a significant decrease ($p<0.001$) in the B_{max} of [^{3}H]bicuculline binding to the membrane preparation of P 72hrs when compared with sham. The K_d value of P 72hrs also decreased significantly ($p<0.01$) when compared with sham. The P 7d showed a significant reversal of B_{max} ($p<0.001$) with a significant increase in K_d ($p<0.05$) towards sham (Fig.-5 & Table -5).

Displacement Analysis of [^{3}H]Bicuculline

The competition curve for bicuculline against [^{3}H]bicuculline 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 (Fig.-6 & Table -6).

Real Time-PCR analysis of $GABA_A$ receptor

α_2 subunit of $GABA_A$ receptor mRNA showed an increase in Ct value showing decreased expression in 72 hrs pancreatectomised rats when compared with sham. The Ct value of the P 7d decreased showing an increased expression in mRNA synthesis (Fig.– 7, and & Table - 7). β_2 subunit of $GABA_A$ receptor mRNA showed an increase in Ct value showing decreased in expression 72 hrs pancreatectomised rats when compared with sham. The Ct value of the P 7d decreased showing an increased expression in mRNA synthesis (Fig.– 8 & Table - 8). γ_1 subunit of $GABA_A$ receptor mRNA expression did not show any change in 72 hrs pancreatectomised rats when compared with sham (Fig.- 9 &Table - 9). γ_2 subunit of

$GABA_A$ receptor mRNA showed an increase in Ct value showing decreased expression in 72 hrs pancreatectomised rats when compared with sham. The Ct value of the P 7d decreased showing an increased expression in mRNA synthesis (Fig.- 10 &Table - 10).

Hypothalamus

[^{3}H]GABA Binding Parameters

Scatchard analysis in the hypothalamus of rats showed a significant decrease ($p<0.001$) in the B_{max} of [^{3}H]GABA binding to the membrane preparation of P 72hrs when compared with sham. The k_d value of P72 h also decreased significantly ($p<0.05$) when compared with sham. The P 7d showed a significant reversal of B_{max} and K_d towards sham ($p<0.05$) (Fig.- 11 &Table - 1).

Displacement Analysis of [^{3}H] GABA

The competition curve for GABA against [^{3}H]GABA 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 (Fig.-12 & Table -12).

[^{3}H]Bicuculline Binding Parameters

Scatchard analysis in the hypothalamus of rats showed a significant decrease ($p<0.001$) in the B_{max} of [^{3}H]bicuculline binding to the membrane preparation of P 72hrs when compared with sham. The K_d value of P72 h also decreased significantly ($p<0.01$) when compared with sham. The P 7d showed a significant reversal of B_{max} and K_d towards sham ($p<0.01$) (Fig.- 13 &Table - 13)

Displacement Analysis of [^{3}H]bicuculline

The competition curve for bicuculline against [^{3}H]bicuculline 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 (Fig.- 14 &Table - 14).

Real Time-PCR analysis of $GABA_A$ receptor

α_2 subunit of $GABA_A$ receptor mRNA showed an increase in Ct value showing decrease in expression 72 hrs pancreatectomised rats when compared with sham. The Ct value of the P 7d decreased showing an increased expression in mRNA synthesis (Fig.- 15 & Table - 15). β_2 subunit of $GABA_A$ receptor mRNA showed an increase in Ct value showing decreased expression in 72 hrs pancreatectomised rats when comperes with sham. The Ct value of the P 7d decreased showing an increased expression in mRNA synthesis (Fig.- 16 & Table - 16). γ_1 subunit of $GABA_A$ receptor mRNA expression did not alter in 72 hrs pancreatectomised rats (Fig.- 17 & Table - 17) when compared with sham. γ_2 subunit of $GABA_A$ receptor mRNA showed an increase in Ct value showing decreased in expression 72 hrs pancreatectomised rats when compered with sham. The Ct value of the P 7d decreased showing an increased expression in mRNA synthesis (Fig.- 18 &Table - 18).

Cerebellum

[^{3}H]GABA Binding Parameters

Scatchard analysis in the cerebellum of rats showed a significant decrease (p<0.001) in the B_{max} of [^{3}H]GABA binding to the membrane preparation of P 72hrs when compared with sham. The K_d value of P72 h also decreased (p<0.05) when compared with sham. The P 7d showed a reversal of B_{max} (p<0.01) and K_d (p<0.05) towards sham (Fig.- 19 & Table - 19).

Displacement Analysis of [^{3}H]GABA

The competition curve for GABA against [^{3}H]GABA 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 (Fig.- 20 & Table - 20).

[^{3}H]Bicuculline Binding Parameters

Scatchard analysis in the cerebellum of rats a significant decrease (p<0.001) in the B_{max} of [^{3}H]bicuculline binding to the membrane preparation of P 72hrs when compared with sham. The K_d value of P72 hrs also decreased (p<0.05) when compared with sham. The P 7d showed a significant reversal of B_{max} (p<0.001) towards sham but without any change in K_d (Fig.- 21 & Table - 21).

Displacement Analysis of [^{3}H]Bicuculline

The competition curve for bicuculline against [^{3}H]bicuculline 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

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 (Fig.- 22 & Table – 22).

Real Time-PCR analysis of $GABA_A$ receptor

α_2 subunit of $GABA_A$ receptor mRNA showed an increase in Ct value showing decreased in expression 72 hrs pancreatectomised rats. The Ct value of the P 7d decreased showing an increased expression in mRNA synthesis (Fig.- 23 & Table – 23). β_2 subunit of $GABA_A$ receptor mRNA showed an increase in Ct valueshowing decreased in expression 72 hrs pancreatectomised rats. The Ct value of the P 7d decreased showing an increased expression in mRNA synthesis (Fig. - 24 & Table - 24). γ_1 subunit of $GABA_A$ receptor mRNA showed only a slight increase in Ct value showing decreased in expression 72 hrs pancreatectomised rats. The Ct value of the P 7d decreased showing an increased expression in mRNA synthesis (Fig.- 25 & Table - 25), γ_2 subunit of $GABA_A$ receptor mRNA did not show any change in Ct value (Fig.- 26 & Table - 26).

Pancreas

[³H]GABA Binding Parameters

Scatchard analysis in the pancreas of rats showed a significant decrease ($p<0.001$) in the B_{max} of [^{3}H]GABA binding to the membrane preparation of P 72hrs when compared with sham. The K_d value of P 72hrs also significantly decreased ($p<0.05$) when compared with sham. The P 7 d group showed a significant reversal ($p<0.001$) in B_{max} towards sham, without any significant change in K_d (Fig.- 27 & Table - 27).

Displacement Analysis of [^{3}H] GABA

The competition curve for GABA against [^{3}H]GABA 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 (Fig.- 28 & Table - 28).

[^{3}H]Bicuculline Binding Parameters

Scatchard analysis in the pancreas of rats showed a significant decrease ($p<0.001$) in the B_{max} of [^{3}H]bicuculline binding to the membrane preparation of P 72hrs when compared with sham. The K_d value of P72 hrs also decreased significantly ($p<0.001$) when compared with sham. The P 7d showed a significant reversal of B_{max} and K_d towards sham ($p<0.01$) (Fig.- 29 & Table - 29).

Displacement Analysis of [^{3}H] Bicuculline

The competition curve for bicuculline against [^{3}H]bicuculline 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 (Fig.- 30 & Table - 30).

Real Time-PCR analysis of $GABA_A$ receptor

α_2 subunit of $GABA_A$ receptor mRNA showed an increase in Ct value showing decrease in expression in 72 hrs pancreatectomised rats when compared

with sham. The Ct value of the P 7d decreased showing an increased expression in mRNA synthesis (Fig.- 31 & Table - 31). β_2 subunit of $GABA_A$ receptor mRNA showed an increase in Ct value showing decrease in expression 72 hrs pancreatectomised rats when compared with sham. The Ct value of the P 7d decreased showing an increased expression in mRNA synthesis (Fig.- 32 & Table - 32). γ_1 subunit of $GABA_A$ receptor mRNA expression did not change in 72 hrs pancreatectomised rats when compared with sham (Fig.- 33 & Table - 33). γ_2 subunit of $GABA_A$ receptor mRNA expression also did not alter, since there was not much change in the Ct value in 72 hrs pancreatectomised rats (Fig.- 34 & Table - 34).

$GABA_B$ Receptor Analysis

Brain Stem

[³H]Baclofen Binding Parameters

Scatchard analysis in the brainstem of rats showed a significant increase ($p<0.001$) in the B_{max} of [^{3}H]baclofen binding to the membrane preparation of P 72hrs when compared with sham. The K_d value of P72 hrs also increased ($p<0.01$) when compared with sham. The P 7 d group showed a reversal of B_{max} ($p<0.001$) and K_d ($p<0.05$) towards sham (Fig.- 35 & Table - 35).

Displacement Analysis of [³H]baclofen

The competition curve for baclofen against [^{3}H] baclofen fitted for two-sited model in all the groups with Hill slope value away from Unity. The $Ki_{(H)}$ decresaed in 72 hrs pancreatectomised rats along with an increase in the log (EC_{50})-1 indicating a shift in affinity towards high 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 (Fig.- 36 & Table - 36).

Hypothalamus

[^{3}H]Baclofen Binding Parameters

Scatchard analysis in the hypothalamus of rats showed a significant increase ($p<0.001$) in the B_{max} of [^{3}H]baclofen binding to the membrane preparation of P 72hrs when compared with sham. The K_d value of P 72hrs also increased ($p<0.01$) when compared with sham. The P 7d showed a significant reversal of B_{max} ($p<0.001$) and K_d ($p<0.05$) towards sham (Fig.- 37 & Table - 37).

Displacement Analysis of [^{3}H]Baclofen

The competition curve for baclofen against [^{3}H] baclofen fitted for two-sited model in all the groups with Hill slope value away from Unity. The $Ki_{(H)}$ decresaed in 72 hrs pancreatectomised rats along with an increase in the log (EC_{50})-1 indicating a shift in affinity towards high 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 (Fig.- 38 & Table - 38).

Cerebellum

[^{3}H]Baclofen Binding Parameters

Scatchard analysis in the cerebellum of rats showed a significant increase ($p<0.001$) in the B_{max} of [^{3}H]baclofen binding to the membrane preparation of P 72hrs when compared with sham. The K_d value of P 72hrs also increased significantly ($p<0.05$) when compared with sham. The P 7d showed a significant reversal of B_{max} ($p<0.001$) towards sham without any significant change in K_d (Fig.- 39 & Table - 39).

Displacement Analysis of [^{3}H]baclofen

The competition curve for baclofen against [^{3}H] baclofen fitted for two-sited model in all the groups with Hill slope value away from Unity. The $Ki_{(H)}$ decresaed in 72 hrs pancreatectomised rats along with an increase in the log (EC_{50})-1 indicating a shift in affinity towards high 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 (Fig.- 40 & Table - 40).

Pancreas

[^{3}H]Baclofen Binding Parameters

Scatchard analysis in the pancreas of rats showed a significant increase ($p<0.001$) in the B_{max} of [^{3}H]baclofen binding to the membrane preparation of P 72hrs when compared with sham. The K_d value of P 72hrs also increased ($p<0.05$) when compared with sham. The P 7d showed a reversal of B_{max} ($p<0.001$) and k_d ($p<0.01$) towards sham (Fig.- 41 & Table - 41).

Displacement Analysis of [^{3}H]Baclofen

The competition curve for baclofen against [^{3}H] baclofen fitted for two-sited model in all the groups with Hill slope value away from Unity. The $Ki_{(H)}$ decresaed in 72 hrs pancreatectomised rats along with an increase in the log (EC_{50})-1 indicating a shift in affinity towards high 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 (Fig.- 42 & Table - 42).

INSULIN SECRETION STUDIES IN PANCREATIC ISLETS

One hour *in vitro* insulin secretion

Effect of GABA on Glucose Induced Insulin Secretion in vitro

The isolated islets were incubated for one hour with 10^{-9} to 10^{-4}M concentrations of GABA and with two different concentrations of glucose, 4mM and 20mM. There was no significant change insulin secretion in presence of 10^{-9}M GABA, but at other concentrations (10^{-9} to 10^{-4}M) insulin secretion was significantly decreased ($P<0.01$ and $p<0.001$) in the presence of 4mM glucose (Fig. - 43). GABA dose dependently inhibited ($p<0.01$ and $p<0.001$) insulin secretion from 10^{-7} to 10^{-4}M concentration in the presence of 20mM glucose (Fig. - 44).

Effect of GABA in the Presence of $GABA_A$ Antagonist Bicuculline on Glucose Induced Insulin Secretion in vitro

The $GABA_A$ receptor antagonist, bicuculline (10^{-4}M) significantly inhibited ($p<0.001$) insulin secretion at all concentrations, 10^{-9} & 10^{-4}M in presence of 4mM glucose (Fig. - 45). In presence of 20mM glucose, Insulin secretion was significantly inhibited ($p<0.01$) at lower GABA concentration (10^{-9}M) and in presence of 10^{-4}M concentration of bicuculline. Secretion of insulin was unaltered in presence of 10^{-8}M GABA and was inhibited by 10^{-7}M to 10^{-4}M GABA concentrations ($p<0.01$ and $p<0.001$) at 10^{-4}M concentration of bicuculline (Fig. - 46).

Effect of Muscimol on Glucose Induced Insulin Secretion in vitro

There was a significant ($p<0.001$) increase in insulin secretion observed at 10^{-9}M muscimol when cells incubated with different concentrations of muscimol at 4mM Glucose. A significant decrease ($p<0.01$) in insulin secretion was observed at 10^{-8}M to 10^{-5}M muscimol concentrations. No change was observed at 10^{-4}M

muscimol (Fig. – 47). In the presence of 20mM glucose, a significant increase in insulin secretion was observed at 10^{-7}M and 10^{-4}M muscimol concentration but insulin secretion decreased at 10^{-8}M muscimol ($p<0.01$). No change in the secretion of insulin was observed at other concentrations of muscimol (Fig. - 48).

Effect of Baclofen on Glucose Induced Insulin Secretion in vitro

Baclofen (10^{-9}M, 10^{-7}M and 10^{-4}M) decreased insulin secretion ($P<0.01$) in presence of 4mM glucose. There was also a significant increase ($p<0.001$) in insulin secretion at 10^{-5}M baclofen. There was no change in the secretion of insulin observed at 10^{-9}M baclofen (Fig. - 49). Insulin secretion decreased significantly at baclofen concentrations from 10^{-8}M to 10^{-4}M ($p<0.01$; $p<0.001$) when cells were incubated in the presence of 20mM glucose (Fig. - 50).

24 hrs *in vitro* insulin synthesis

Effect of GABA on Glucose induced Insulin Secretion in 24 hrs Islet Cultures

Islets were incubated with 10^{-9} to 10^{-4} M concentrations of GABA and two different concentrations of glucose, 4mM and 20mM in 24 hrs *in vitro* cultures. GABA decreased insulin secretion significantly at all concentrations from 10^{-9} to 10^{-4} M ($p<0.01$ and $p<0.001$) in the presence of 4mM glucose (Fig. - 51). A similar and significant result decreasing the secretion of insulin from the incubated islets with different concentrations of GABA (10^{-9} to 10^{-4}M) was obtained in the presence of 20mM glucose (Fig.- 52).

Effect of GABA in The Presence of $GABA_A$ Antagonist Bicuculline on Glucose Induced Insulin Secretion In vitro

GABA at 10^{-9} & 10^{-4}M concentrations significantly ($p<0.001$) decreased glucose (4mM) induced insulin secretion in presence of 10^{-4}M bicuculline in the long

term incubation study (Fig. - 53). A significant decrease in insulin secretion was observed at all GABA concentrations ($p<0.001$) in the presence of 20mM glucose (Fig. - 54).

Effect of Muscimol on Glucose Induced Insulin Secretion in vitro

Muscimol (10^{-4}M) significantly increased glucose induced insulin secretion at 10^{-7} ($p<0.001$), 10^{-5} ($p<0.01$) and 10^{-4}M ($p<0.001$) concentrations in the presence of 4mM glucose (Fig. - 55). No significant change in insulin secretion was observed at other concentrations. Significant decrease in insulin secretion was observed at 10^{-9}, 10^{-8}, 10^{-6} ($p<0.001$) and 10^{-7}M ($p<0.01$) concentrations of muscimol in the presence of 20mM glucose. There was no significant change in insulin secretion observed at 10^{-5}M and 10^{-4}M baclofen concentrations (Fig. - 56).

Effect of Baclofen on Glucose Induced Insulin Secretion in vitro

Baclofen significantly decreased insulin secretion at 10^{-9} and 10^{-7}M($p<0.001$) when incubated with different concentrations (10^{-9} to 10^{-4}M) in presence of 4mM glucose. A significant increase in insulin secretion was observed at 10^{-5}M concentration of baclofen and there was no significant change with other baclofen concentrations (Fig. - 57). There was significant decrease ($p<0.01$ and $p<0.001$) in insulin secretion at all concentrations of baclofen ranging from 10^{-9} to 10^{-4}M when incubated in the presence of 20mM glucose (Fig. - 58).

IN VITRO DNA SYNTHESIS STUDIES IN PANCREATIC ISLETS

Effect of GABA 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 ($p<0.001$) in the islet DNA synthesis. GABA at 10^{-6}M concentration caused no

significant change in the DNA synthesis from basal level. Additional of 4mM glucose also could not stimulate a significant DNA synthesis, but at 20mM glucose concentration, significant [^{3}H]thymidine incorporation was observed. Addition of EGF in presence of 10^{-6}M GABA stimulated islet DNA synthesis ($p<0.01$). 10^{-6}M GABA in presence of 4mM glucose and 20mM glucose also significantly increased [^{3}H]thymidine incorporation in the cultured islets ($p<0.01$ and $p<0.001$) (Fig. 59).

Effect of Muscimol on Islet DNA Synthesis

Addition of muscimol (10^{-6}M) caused no significant change in the DNA synthesis when compared with control. Addition of 10^{-6}M muscimol along with EGF caused a significant increase ($p<0.001$) in DNA synthesis when compared with EGF alone group. The [^{3}H]thymidine incorporation in the cultured islets was increased significantly in the presence of both 4mM glucose and 20mM glucose in the medium along with EGF ($p<0.001$) (Fig. 60).

Effect of Baclofen on islet DNA Synthesis

Addition of baclofen (10^{-6}M) caused a significant increase ($p<0.001$) in the [^{3}H]thymidine incorporation in the cultured islets. The EGF mediated DNA synthesis in presence of 10^{-6}M baclofen also increased significantly ($p<0.001$). Glucose mediated [^{3}H]thymidine incorporation in the cultured islets in presence of 4mM and 20mM glucose concentrations in presence of EGF and 10^{-6}M baclofen also increased significantly ($p<0.001$) (Fig. 61).

Dose-dependent Response of Islet DNA Synthesis to Muscimol

Muscimol did not alter the DNA at its lower concentrations (10^{-8} and 10^{-7}) but inhibited significantly ($p<0.01$) the DNA synthesis of primary islet in culture at concentrations of 10^{-6} to 10^{-4} (Fig. 62).

Dose-dependent Response of EGF Induced islet DNA synthesis to Muscimol

Addition of muscimol at a concentration from 10^{-8}M to 10^{-4}M significantly decreased ($p<0.01$ & $p<0.001$) the EGF mediated DNA synthesis of cultured islets. Maximum DNA synthesis was observed at 10^{-8}M muscimol (Fig. 62).

Dose-dependent Response of islet DNA Synthesis to Baclofen

Baclofen simulated significantly the DNA synthesis of primary islets in at concentrations varying from 10^{-8}M to 10^{-4}M (($p<0.01$ & $p<0.05$) (Fig. 63).

Dose-dependent Response of EGF Induced islet DNA synthesis to Baclofen

Addition of baclofen 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^{-4}M baclofen (Fig. 63).

***Effect of Pertussis Toxin on Baclofen Mediated DNA synthesis*:**

Pertussis toxin significantly inhibited potentiation of EGF effect induced by baclofen at all concentrations (Fig. 64).

DISCUSSION

Functional pancreatic β-cell mass is dynamic and although fully differentiated, β-cells are capable of re-entering the cell cycle upon appropriate stimuli. Stimulating regeneration-competent cells *in situ* is clearly the most desirable way to restore damaged tissue. (Bouckenooghe *et al.*, 2005). A large number of growth factors and growth-stimulating peptides are expressed in or have stimulatory effect in the growing islets (Corbett *et al.*, 1997). The presence of GABA in the cells of the islets of Langerhans is well documented in various species, particularly rats, on the basis of immunohistochemical and biochemical data (Okada *et al.*, 1976; Vincent *et al.*, 1983; Sakaue *et al.*, 1987; Garry *et al.*, 1987, 1988; Gilon *et al.*, 1988; Sorenson *et al.*, 1991; Reetz *et al.*, 1991; Michalik & Erecinska, 1992; Michalik *et al.*, 1993)

DNA synthesis in pancreas after partial pancreatectomy

Partial pancreatectomy was used as a tool to provide the stimulus to regenerate the β-cells of the islets of Langerhans in the rats to study the regeneration. The study was performed by incorporating [3H]thymidine into the replicating DNA as a biochemical index to measure the DNA synthesis during the pancreatic regeneration. An increase in the DNA synthesis was observed after 12 hours after partial pancreatectomy. The rate of [^{3}H]thymidine incorporation was maximum when observed at 72 hours and declined after 7 days. The observation of the peak of the DNA synthesis at 72 hours is concordant with the previous reports (Pearson *et al.*, 1977; Brockenbrough *et al.*, 1988). Enhanced β-cell function and proliferation maintains the normoglycaemic level in rats during pancreatic regeneration (Leahy *et al.*, 1988).

There was an increase in the circulating insulin levels during the regeneration of pancreas. 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 enhanced the proliferation of the remnant pancreas and maintains endogenous insulin secretion for a long period, prolonging survival and promoting pancreatic regeneration (Ohashi, 1993).

There was no significant change in the body weight and blood glucose levels in the sham and experimental groups- P 72h and P 7d.

GABA content in brain regions during pancreatic regeneration

GABA is one of the most abundant neurotransmitters in the vertebrate central nervous system and is involved in neuroendocrine processes such as development, reproduction, feeding and stress (Martyniuk *et al.*, 2005). A decrease in GABA content was observed during active pancreatic proliferation in brain stem, cerebellum and hypothalamus. The decreased contents in the brain regions were reversed to basal level when pancreatic DNA synthesis declined to control level. The effect of regeneration in the peripheral tissues to the hypothalamic GABA content was already reported during the regeneration of liver (Biju *et al.*, 2001). This indicates the

decrease in brain GABA content is important in the DNA synthesis in pancreas. It may be a homeostatic feedback adjustment by the hypothalamus to trigger the sympathetic innervation and thereby DNA synthesis. The pancreas enhance the insulin secretion to compensate the insulin demand in the body during the loss of the cells. Brain GABAergic functional alterations are reported to regulate autonomic nerve function in rats (Martin *et al.*, 1998). GABA has been known to function as an autocrine/paracrine signal molecule in addition to its well-known inhibitory neurotransmitter function. Studies on the developing brain and on primary brain cell cultures showed that neuron formation was facilitated by GABA through $GABA_A$ ion channels during postmitotic differentiation, but not earlier during the phases of cell fate commitment (Jelitai *et al.*, 2004). These indicate that a decrease in the brain GABA content is important in the DNA synthesis in pancreas. Brain GABAergic changes are reported to regulate autonomic nerve functions in rats (Martin & Haywood, 1998). So the results show that a reduction in the GABA content in the brain regions may enhance DNA synthesis in pancreas by facilitating the sympathetic tone.

GABA content in pancreas during pancreatic regeneration

GABA is widely distributed in non-neural tissue including peripheral nervous and endocrine systems (Tillakaratne *et al.*, 1995). GABA was found by immunohistochemistry in glial cells in pancreatic ganglia (Nagamatsu *et al.*, 2001). GABA is stored in microvesicles in pancreatic islet cells (Chessler *et al.*, 2002). GABA released from β-cells functions as an autocrine inhibitor of insulin secretion in pancreatic islets and that the effect is principally due to direct suppression of exocytosis (Braun *et al.*, 2004). A decrease in GABA content was observed during active pancreatic proliferation in the pancreas of the experimental rats when compared with the sham. This effect was reversed to near normal level when the

DNA synthesis was completed during 7 days. Islets possess synaptic-like microvesicles, which have a secretory function and β-cell synaptic-like microvesicles are involved in the secretion of GABA (Thomas-Reetz *et al.*, 1993).

Brain $GABA_A$ and $GABA_B$ receptor alterations in the rats during pancreatic regeneration

Previous studies in the regeneration of liver have showed significant alterations in the $GABA_A$ receptor function in brain regions (Biju *et al.*, 2001_a; 2002_a). So we have studied the $GABA_A$ receptor alterations during the regeneration of pancreas of which the endocrine and exocrine secretions have a strong influence from the brain signals. Many gastrointestinal and pancreatic functions are under strong modulatory control by the brain via the vagus nerve (Berthoud *et al.*, 2001). Pancreatic polypeptide when microinjected into the dorsal vagal complex potentiates glucose-stimulated insulin secretion (Krowicki, 1996). Some of the neurons of dorsal motor nucleus of the vagus are presumed to play a role in the brain stem neural control of glycemic homeostasis (Adachi *et al.*, 1995). Targeted pharmacological lesion of the adrenergic innervation of dorsal motor nucleus of the vagus nerve causes hypersecretion by pancreatic β-cells, an effect, which requires an intact vagus nerve (Siaud *et al.*, 1990; 1995). Also, the hypothalamic neurons producing oxytocin that densely project to the dorsal vagal complex are proposed to involve in an inhibitory control of the vagal preganglionic neurons that innervate the pancreas (Siaud *et al.*, 1991). These all suggest the control of brain from hypothalamus and brain stem over pancreas by the vagal innervation. GABA and the hormonal functional studies will elucidate the functional integrity of their control on peripheral tissues including pancreas. A study in our lab in the regeneration of liver has already explained the importance of on the GABAergic receptor function and gene expression (Biju *et al.*, 2001_a; 2002_a).

In our present study we analysed the receptor binding parameters and expression of the GABA 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 Real Time-PCR technique using specific primers. $GABA_A$ receptor binding parameters were analysed using the receptor specific agonist [^{3}H]bicuculline (Kurioka *et al.*, 1981). $GABA_A$ receptor has two affinity sites and the double affinity status of the receptor was confirmed by displacement analysis using bicuculline.

It is well established that the autonomic fibres supplying the pancreas travel via the vagus and splanchnic nerves. These nerves are clearly related to the ventral hypothalamus. The ventro-medial hypothalamic nuclei are 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 $GABA_A$ receptor binding observed in the hypothalamus reduces the sympathetic nerve stimulation thus reducing the inhibitory effect of EPI on insulin secretion

Pancreatic β-cells express glutamate decarboxylase (GAD), which is responsible for the production and release of GABA. Increased cytoplasmic ATP levels can suppress GAD activity in β-cells, and hence GABA production and release, is compatible with previous findings on ATP suppression of brain GAD activity (Winnock *et al.*, 2002).

$GABA_A$ and $GABA_B$ receptor alterations in the brain stem

Brain stem region has direct connection with pancreas through the vagal innervation. Autonomic regulation of GABA is reported to mediate through $GABA_A$

and $GABA_B$ receptors (Sved, 1990; Coldman, 1998). We studied the brain stem GABAergic receptor subtypes functional regulation to elucidate its role during pancraetic regeneration. Previous reports have referred [^{3}H]GABA as a high affinity $GABA_A$ receptor agonist (Paulose & Dakshinamurti, 1984). The decreased B_{max} in partial pancreatectomised rats denotes the decreased receptor density. The displacement analysis of the [^{3}H]GABA against GABA indicates a shift in affinity towards the low-affinity. This suggests an altered receptor function during the pancreatic regeneration.

Bicuculline has a higher affinity for rapidly dissociating low-affinity $GABA_A$ sites. So we have used [^{3}H]bicuculline to study the status of $GABA_A$ low-affinity receptors. The decreased B_{max} of [^{3}H]bicuculline binding indicates a reduction in receptor density. The high-affinity sites of this receptor shifted to low-affinity in P 72 hrs rats denotes a decreased functioning of the receptor. Since GABA has a sympatho-inhibitory effect, these changes may be responsible for the increased sympathetic activity observed.

[^{3}H]baclofen were used to study the $GABA_B$ receptor. In P 72 hrs rats the receptor number of $GABA_B$ receptor increased. $GABA_B$ binding shift towards high-affinity in P 72 hrs treated rats indicates increased functioning of this receptor. $GABA_B$ receptor activation in central nervous system is reported to stimulate the sympathetic nervous system (Nonogaki *et al.*, 1994, Takenaka *et al.*, 1996).

Brain $GABA_A$ and $GABA_B$ receptor systems differentially regulate the sympathetic neural activity (Takenaka *et al.*, 1995). It is clear from our results that the brain stem $GABA_A$ and $GABA_B$ receptors functions were in the opposite manner. $GABA_A$ receptor demonstrated to have an inhibitory effect on sympathetic stimulation while $GABA_B$ receptor activates sympathetic stimulation. The changes in brain stem GABAergic function favoured the pancreatic cell proliferation mediated through sympathetic regulation.

$GABA_A$ and $GABA_B$ receptor alterations in hypothalamus

Hypothalamus is the centre of autonomic nervous system reinforcement. Lateral lesions of hypothalamus caused an increase in DNA synthesis during liver regeneration. Sympathectomy and vagotomy blocked this effect (Kiba *et al.*, 1994, 1995). Hypothalamic $GABA_B$ergic innervation is reported have a stimulatory effect on sympathetic nervous system. The receptor number decreased in P 72 hrs rats. This indicates a decreased high-affinity $GABA_A$ receptor activity in P 72 hrs group. The affinity change was confirmed by displacement analysis with GABA against [^{3}H]GABA in P 72 hrs rats where we have found a shift in the high-affinity towards low-affinity.

The low-affinity $GABA_A$ receptor binding parameters as determined by [^{3}H]bicuculline against bicuculline indicate a decrease in number and affinity of the receptor in P 72 hrs rats. Displacement analysis showed a significant shift in the high-affinity site to low-affinity site in P 72 hrs group.

$GABA_B$ receptor density increased in the P 72 hrs group. The affinity change in P 72 hrs group was confirmed by displacement analysis where we have observed a shift in affinity towards high-affinity.

The results showed that hypothalamic high-affinity and low-affinity $GABA_A$ receptor activity decreased during pancreatic regeneration. It is already reported that intrahypothalamic administration of $GABA_A$ receptor antagonist bicuculline methoiodide decreased the sympathetic innervation and blood pressure in a dose dependent manner (Tellioglu *et al.*, 1996). So the decreased $GABA_A$ receptor activity may facilitate sympathetic innervation. $GABA_B$ receptor activity was increased in P 72 hrs rats. This kind of differential functioning of $GABA_A$ and $GABA_B$ receptor system and its importance in sympathetic innervation is already reported (Takenaka *et al*, 1995).

GABA$_A$ and GABA$_B$ receptor alterations in cerebellum

GABA has been considered as a post-synaptic inhibitory neurotransmitter in the central nervous system particularly in the cerebellum (DeFeudis, 1977). The high affinity GABA$_A$ receptor number decreased in P 72 hrs rats. Slowly dissociating low-affinity GABA$_A$ receptor also decreased in p 72 hrs rats. Displacement analysis of the receptor with [^{3}H]bicuculline against bicuculline showed a shift in the low-affinity to very low-affinity.

GABA$_B$ receptor affinity was increased in P 72 hrs rats. The displacement data are in accordance with the affinity change obtained from Scatchard plot.

Pancreatic GABA$_A$ and GABA$_B$ receptor alterations in the rats during pancreatic regeneration

Pancreatic β-cells rank among the few nonneuronal cell types that express GAD and contain its product, GABA (Okada *et al.*, 1976; Reetz *et al.*, 1991). Pancreatic β-cells release GABA in amounts that correspond to the cellular GABA production (Smismans *et al.*, 1997). Gastropancreatic neuroendocrine cells synthesize large amounts of GABA. Results of electrophysiliological studies proved the presence of GABA$_A$ receptor in the pancreas depolarizing response of GABA (Sha *et al.*, 2001). Studies showed that expression of GABA$_A$ receptors is abundant in gastropancreatic neuroendocrine cells (von Blankenfeld *et al.*, 1995).

Inhibitory effects of GABA in the endocrine pancreas are consistent with its well-known suppressive actions as a neurotransmitter in the nervous system (Kuffler & Edwards, 1958). Electrophysiological measurements in guinea pig islet cells have indicated the presence of a GABA-sensitive chloride channel (Rorsman *et al.*, 1989).

In electrophysiological studies GABA pressure microejection depolarized membrane potential. Electrically evoked fast excitatory postsynaptic potentials were

significantly inhibited after GABA application through $GABA_A$ receptors and suggest that endogenous GABA released from ganglionic glial cells acts on pancreatic ganglion neurons through $GABA_A$ receptors (Sha *et al.*, 2001).

GABAergic inhibition of insulin synthesis and secretion from pancreatic β-cells *in vitro*

A sophisticated interplay between glucose and a plethora of additional factors including other nutrients, neurotransmitters, islet generated factors and systemic growth factors regulate the signal- transduction in the pancreatic β-cell and thereby the insulin secretory process. 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 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). 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).

A reduction in cellular and medium GABA levels is more sensitive than insulin as a marker for the presence of dead β-cells in isolated preparations (Wang *et al.*, 2005). Upon glucose stimulation, GABA and insulin are released from β-cells (Anhert-Hilger *et al.*, 1996). GABA is reported to inhibit glucagon secretion from α-cells (Rorsman *et al.*, 1989) as well as to inhibit insulin secretion from β-cells (Gu *et*

al., 1993) via $GABA_A$ and $GABA_B$ receptor mediated mechanisms, respectively. Most immunocytochemical studies have shown that GABA is found selectively in β-cells (Vincent *et al.*, 1983; Garry *et al.*, 1986; Garry *et al.*, 1988; Reetz *et al.*, 1991). Earlier studies, using exogenous GABA, have reported a wide variety of effects on β-cells function, including a general inhibition of both phases of insulin secretion, a stimulation of insulin secretion, or no effect at all (Michalik *et al.*, 1992; Satin *et al.*, 1998).

Isolated pancreatic islets were incubated for one hour with different concentrations of GABA, bicuculline, muscimol and baclofen separately in presence of 4mM and 20mM glucose, which would represent normal and diabetic conditions respectively. GABA has inhibited the insulin secretion in a dose dependent manner in presence of both glucose concentrations. This effect was not antagonized in presence on bicuculline, the $GABA_A$ receptor antagonist. The GABA agonist muscimol also inhibited the insulin secretion except in the lowest muscimol concentration of 10^{-9}M in presence of 4mM glucose. In presence of 20mM glucose, at 10^{-7} and 10^{-4}M muscimol enhanced insulin secretion was found but other concentrations either inhibited or did not significantly change the secretion status. Baclofen at 10^{-5}M concentration enhanced insulin secretion, but inhibited at 10^{-9}, 10^{-7}M and 10^{-4} molar concentrations and remained unaffecting at 10^{-8}M and 10^{-6}M baclofen concentrations. In presence of 20mM glucose, insulin secretion was significantly inhibited at all concentrations except 10^{-9}M baclofen, which did not change the insulin levels *in vitro*. Pancreas perfusion experiments suggest that GABA generated by GAD65 may function as a negative regulator of first-phase insulin secretion in response to glucose by affecting a step proximal to or at the K_{ATP}^+ channel (Yuguang *et al.*, 2000).

During the 24 hour secretion, results obtained were much similar to that obtained in the 1 hour insulin secretion study. GABA significantly inhibited the

insulin secretion at normal and hyper glycaemic conditions. Addition of bicuculline would not alter this secretion status. Muscimol gave an enhanced secretion of insulin in presence of 4mM glucose at 10^{-7}, 10^{-5} and 10^{-4}M muscimol concentrations, but remained unvoiced at other concentrations. But, in presence of 20mM glucose muscimol significantly inhibited the insulin secretion. PK 11195 [1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline-carboxami de], a potent and selective ligand for peripheral benzodiazepine binding sites, was shown to inhibit insulin release from rat pancreatic islets (Pujalte *et al.*, 2000). Baclofen also showed an inhibitory effect on insulin secretion in most of the concentrations in normal as well as hyperglycaemic conditions *in vitro*.

GABA released from β-cells is reported as an autocrine inhibitor of insulin secretion in pancreatic islets acting through $GABA_B$ receptors and this effect is presumed to be due to direct suppression of exocytosis (Braun *et al.*, 2004). L-glutamine is metabolized preferentially to GABA and L-aspartate, which accumulate in islets, thus preventing its complete oxidation in the Krebs cycle, which accounts for its failure to stimulate insulin secretion (Fernandez-Pascual *et al.*, 2004). When analyzing in terms of GABA release in presence of high glucose, a recent investigation showed inhibition (40%) of GABA release from reaggregated rat β-cells after a 2 hrs culture period in high when compared with low glucose conditions. A threefold increase in insulin secretion was observed in parallel (Winnock *et al.*, 2001). In agreement with this finding, a second study demonstrated a twofold increase in GABA release from rat islets after 30 min when islets were transferred from high to low glucose conditions (Hayashi *et al.*, 2003). A concurrent decrease in insulin secretion can be confirmed from these studies. When compared with the earlier studies GABA can be said to certainly inhibit insulin secretion through its both receptors $GABA_A$ and $GABA_B$.

Effect of GABA, muscimol and baclofen on pancreatic DNA synthesis

Many different types of glutamate and GABA receptor subunits show differential expression that some have prominent expression in the embryonic and/or postnatal brain, whereas others are mainly present in the adult brain (Lujan *et al.*, 2005). We have studied the effect of GABA, the $GABA_A$ receptor agonist muscimol and $GABA_B$ receptor agonist baclofen on pancreatic cell proliferation in *in vitro* cultures. Addition of EGF caused a marked increase in DNA synthesis from basal level. EGF is a known mitogen 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). The 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. The addition of GABA (1ng) did not elicit any significant change in pancreatic DNA synthesis. There was significant increase in DNA synthesis when islets were incubated with 20mM glucose, without EGF but no change was reported in presence of 4mM glucose, which is normal physiological level. The addition of EGF to the cultures containing 10^{-6}M GABA in presence of both the glucose concentrations obtained marked increase in DNA synthesis.

Muscimol is the specific agonist of the $GABA_A$ receptor. We used muscimol to study the $GABA_A$ receptor mediated effect on DNA synthesis of islets kept in primary culture. The addition of 10^{-6}M muscimol also did not elicit any marked difference in DNA replication in cultures. But the DNA synthesis was enhanced in the presence of EGF and with the addition of 4mM and 20mM glucose along with EGF. Also, it was found that there was a dose dependent inhibition of DNA

synthesis in the presence of different concentrations of muscimol when incubated with and without EGF. $GABA_A$ receptors are peripheral benzodiazepine receptor ligands, which are reported to inhibit the proliferation of various tumors. Peripheral benzodiazepine receptor ligands inhibited the proliferation of hepatocellular carcinoma cell lines by inducing apoptosis and cell cycle arrest (Sutter *et al.*, 2004). Peripheral benzodiazepine receptor is involved in numerous biological functions, including steroid biosynthesis, mitochondrial oxidative phosphorylation and cell proliferation. Recent studies support the idea that the subcellular localisation of peripheral benzodiazepine receptor defines its function and this receptor could be a possible target for new strategies against cancer (Corsi *et al.*, 2005). Apoptosis of GABAergic interneurons was demonstrated in the molecular layer and white matter of the cerebellar cortex during the first two weeks of development (Yamanaka *et al.*, 2004). Exposure of cultured Schwann cells to (-)-baclofen inhibits their proliferation and reduces the synthesis of specific myelin proteins providing evidence for a physiological role of $GABA_B$ receptors in the glial cells of the peripheral nervous system (Magnaghi *et al.*, 2004). Brown rice extracts with enhanced levels of GABA have an inhibitory action on leukemia cell proliferation and have a stimulatory action on the cancer cell apoptosis (Oh & Oh., 2004). Receptor functional alterations studied during hepatic proliferation in hepatocyte cultures showed that $GABA_A$ receptor agonist, muscimol, dose dependently inhibited epidermal growth factor induced DNA synthesis and enhanced the transforming growth factor β_1 mediated DNA synthesis suppression in primary hepatocyte cultures suggesting $GABA_A$ receptor acts as an inhibitory signal for hepatic cell proliferation (Biju *et al.*, 2001_b).

Baclofen is the specific agonist of $GABA_B$ receptor and we have studied the effect of baclofen in primary pancreatic cultures to learn about the $GABA_B$ receptor mediated changes during pancreatic DNA synthesis in detail. Baclofen has obtained a ranked increase in the DNA replication in primary pancreatic cultures with and

without EGF. The result was similar and significant when cells were incubated in the presence of 4mM and 20mM glucose also. The dose-dependent increase in $GABA_B$ receptor mediated EGF mitogenicity was abolished by G_i protein inhibitor pertsussis toxin. $GABA_B$ receptor is coupled to G_i protein. The stimulation of these G_i protein coupled receptors induces cell proliferation in various tissues (Biesen *et al*, 1996). Several lines of evidence suggest that activation of receptors that couple to heterotrimeric G-proteins is important in regulating proliferation in regenerating liver cells following a partial hepatectomy. The expression of the stimulating and inhibitory α subunits of G proteins that couple various receptors to their effector targets like adenylyl cyclase is differentially regulated during the early pre-replicative period in the liver (Mahler & Wilce, 1988). ERK activation via endogenous IGF-I receptor and G_i -coupled LPA receptor is sensitive to pertussis toxin treatment suggesting a cross talk between leading mitogenic effect as reported in rat fibroblasts. Such a cross talk between $GABA_B$ receptor α-subunit and for EGF receptor may be responsible for the triggering of pancreatic DNA synthesis. Neurotransmitter receptors like α_1 adrenergic and S2 class serotonin receptors act as co-mitogenic signals in EGF mediated DNA synthesis in hepatocyte cultures by the same mechanism (Sudha & Paulose, 1997, Michalopoulos *et al.*, 1997). A similar result was obtained during the primary culture of pancreatic islets, pertussis toxin inhibited potentiation of EGF effect induced by 8-OH DPAT, where an inhibition of ERK2 activation by the $5\text{-}HT_{1A}$ receptor-selective agonist is suggested (Mohanan, 2005_b). Studies on functional alterations of receptors during hepatic proliferation in hepatocyte cultures showed that $GABA_B$ receptor enhancement induce hepatic neoplasia. Also, baclofen is seen to act as a potent co-mitogen, triggering DNA synthesis in primary cultures of rat hepatocytes, mediated through the Gi protein coupled $GABA_B$ receptors (Biju *et al.*, 2002_b).

Our studies have revealed the significance of $GABA_A$ and $GABA_B$ receptors functional regulation during pancreatic rgeneration and insulin secretion in rats. This will have immense clinical significance in the management of diabetes.

SUMMARY

1. Pancreatic regeneration after partial pancreatectomy was used as a model system to study pancreatic β-cell proliferation in rats.
2. Primary cultures of pancreatic islets were used as the *in vitro* system to study pancreatic islet cell proliferation and insulin secretion.
3. $[^3H]$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. GABA content was analysed using displacement method. It decreased in the brain regions during active islet cell proliferation.
5. GABA receptor functional status was analysed by Scatchard and displacement analysis using $[^3H]$ ligands. Receptor kinetic parameters data were confirmed by studying the mRNA status of the corresponding receptor using Real Time- PCR. $GABA_A$ receptors were down regulated and $GABA_B$ receptors were up regulated in brain regions during active islet cell proliferation.
6. Pancreatic islet GABA content was decreased in 72 hrs pancreatectomised rats. Pancreatic islet $GABA_A$ receptor down regulation was observed during islet DNA synthesis. $GABA_B$ receptor up regulation was found during pancreatic regeneration.
7. *In vitro* insulin secretion study during 1 hour showed that GABA has inhibited the insulin secretion in a dose dependent manner in normal and hyperglycaemic conditions. Bicuculline did not antagonize this effect. $GABA_A$ agonist, muscimol inhibited glucose stimulated insulin secretion from pancreatic islets except in the lowest concentration of 10^{-9}M in presence of 4mM glucose and at 10^{-7} and 10^{-4}M muscimol in presence of 20mM glucose. $GABA_B$ agonist. Baclofen also inhibited glucose induced insulin secretion. Baclofen enhanced

glucose induced insulin secretion at the concentrations of 10^{-5}M at 4mM glucose and at 10^{-9}M in presence of 20mM glucose.

8. During 24 hours *in vitro* insulin secretion study it was observed that all concentrations of GABA has inhibited glucose stimulated insulin secretion from pancreatic islets. Muscimol, the $GABA_A$ agonist, inhibited the insulin secretion but, gave an enhanced secretion of insulin in presence of 4mM glucose at 10^{-7}, 10^{-5} and 10^{-4}M muscimol at 4mM glucose concentration. But in presence of 20mM glucose, muscimol significantly inhibited the insulin secretion. $GABA_B$ agonist, baclofen also inhibited glucose induced insulin secretion in presence of both 4mM and 20mM glucose.
9. *In vitro* DNA synthesis studies showed that activation of $GABA_A$ receptor by adding muscimol, a specific agonist, inhibited islet DNA synthesis. Also, the addition of baclofen, a specific -agonist of $GABA_B$ receptor resulted in the stimulation of DNA synthesis.

Thus, the regulation of $GABA_A$ and $GABA_B$ receptors in the brain and pancreatic islets plays an important role in insulin secretion and islet cell proliferation during pancreatic regeneration.

CONCLUSION

Our results demonstrate the functional alterations of the $GABA_A$ and $GABA_B$ receptors and the gene expression during the regeneration of pancreas following partial pancreatectomy. The role of these receptors in insulin secretion and pancreatic DNA synthesis using the specific agonists and antagonists also are studied *in vitro*. The alterations of $GABA_A$ and $GABA_B$ receptor function and gene expression in the brain stem, crebellum and hypothalamus play an important role in the sympathetic regulation of insulin secretion during pancreatic regeneration. Previous studies have given much information linking functional interaction between GABA and the peripheral nervous system. The involvement of specific receptor subtypes functional regulation during pancreatic regeneration has not given emphasis and research in this area seems to be scarce. We have observed a decreased GABA content, down regulation of $GABA_A$ receptors and an up regulation of $GABA_B$ receptors in the cerebral cortex, brain stem and hypothalamus. Real Time-PCR analysis confirmed the receptor data in the brain regions. These alterations in the $GABA_A$ and $GABA_B$ receptors of the brain are suggested to govern the regenerative response and growth regulation of the pancreas through sympathetic innervation. In addition, receptor binding studies and Real Time-PCR analysis revealed that during pancreatic regeneration $GABA_A$ receptors were down regulated and $GABA_B$ receptors were up regulated in pancreatic islets. This suggests an inhibitory role for $GABA_A$ receptors in islet cell proliferation i.e., the down regulation of this receptor facilitates proliferation. Insulin secretion study during 1 hour showed GABA has inhibited the insulin secretion in a dose dependent manner in normal and hyperglycaemic conditions. Bicuculline did not antagonize this effect. $GABA_A$ agonist, muscimol inhibited glucose stimulated insulin secretion from pancreatic islets except in the lowest concentration of 10^{-9}M in presence of 4mM glucose.

Muscimol enhanced insulin secretion at 10^{-7} and 10^{-4}M muscimol in presence of 20mM glucose- 4mM glucose represents normal and 20mM represent hyperglycaemic conditions. $GABA_B$ agonist, baclofen also inhibited glucose induced insulin secretion and enhanced at the concentration of 10^{-5}M at 4mM glucose and at 10^{-9}M baclofen in presence of 20mM glucose. This shows a differential control of the $GABA_A$ and $GABA_B$ receptors over insulin release from the pancreatic islets. During 24 hours *in vitro* insulin secretion study it showed that low concentration of GABA has inhibited glucose stimulated insulin secretion from pancreatic islets. Muscimol, the $GABA_A$ agonist, inhibited the insulin secretion but, gave an enhanced secretion of insulin in presence of 4mM glucose at 10^{-7}, 10^{-5} and 10^{-4}M muscimol. But in presence of 20mM glucose muscimol significantly inhibited the insulin secretion. $GABA_B$ agonist, baclofen also inhibited glucose induced insulin secretion in presence of both 4mM and 20mM glucose. This shows the inhibitory role of GABA and its specific receptor subtypes over insulin synthesis from pancreatic β-islets. *In vitro* DNA synthesis studies showed that activation of $GABA_A$ receptor by adding muscimol, a specific agonist, inhibited islet DNA synthesis. Also, the addition of baclofen, a specific agonist of $GABA_B$ receptor resulted in the stimulation of DNA synthesis.

Thus, we conclude that brain and pancreatic $GABA_A$ and $GABA_B$ receptor gene expression differentially regulates pancreatic insulin secretion and islet cell proliferation during pancreatic regeneration. This will have immense clinical significance in therapeutic applications in the management of Diabetes mellitus.

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LIST OF PUBLICATIONS

Papers published

Mohanan V, **Kaimal S.B** & Paulose C.S. (2005) Decreased 5-HT1A receptor gene expression and 5-HT1A receptor protein in the cerebral cortex and brain stem during pancreatic regeneration in rats. Neurochem Res. **30(1)**:25-32

Papers presented in scientific meetings

1. **Balarama Kaimal S**, Philip Augustine & C. S. Paulose "decreased $GABA_A$ receptor activity in the brain stem and cerebellum during pancreatic regeneration in rats and decreased insulin secretion from rat pancreatic islets *in vitro*". International Conference on Biotechnology and Neuroscience, December 29-31, 2004, Cochin
2. **Balarama Kaimal S** & C. S. Paulose, Decreased GABA receptor activity in the brain stem and cerebellum during pancreatic regeneration in rats (poster). International Symposium, National Brain Research Centre, New Delhi (December, 2003)
3. Reas Khan S, **S. Balarama Kaimal** & C.S.Paulose Decreased glutamate dehydrogenase activity in the liver of rats in alcoholism International Medical Science Academy - Annual Conference (IMSACON) Kochi (Sept. 2003).
4. **Balarama Kaimal S**, Pyroja S & C.S Paulose. "Decreased glutamate dehydrogenase activity during hepatic and pancreatic regeneration in the liver of rats". National Symposium on Medical, Plant And Industrial Biotechnology Dec. 1-2, Cochin University Of Science And Technology, Cochin (2000)
5. **Balarama Kaimal S** "Biofertilizers", 9th Swadeshi Science Congress, 7-9 November, 1999, Fatima College, Kollam.

Table – 1

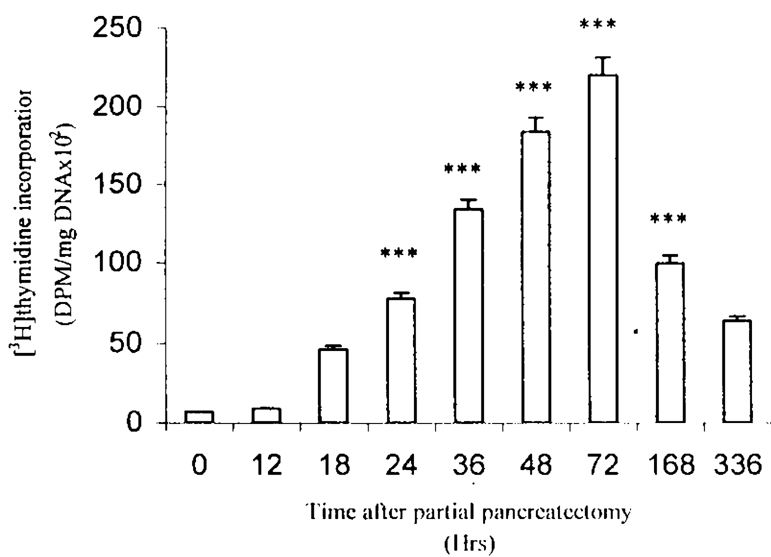
Body weight and blood glucose level of sham and experimental rats

Animal	Body weight (g)	Glucose level (mg/dL)
Sham	89 ± 2	99.6 ± 6.6
P 72hrs	84 ± 3	85.3 ± 6.1
P 7 days	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
***p<0.001 when compared with sham

Table- 2

GABA content in the brain regions and pancreas of the sham and experimental rats during partial pancreatectomy

(μmoles/g wt. of the tissue)

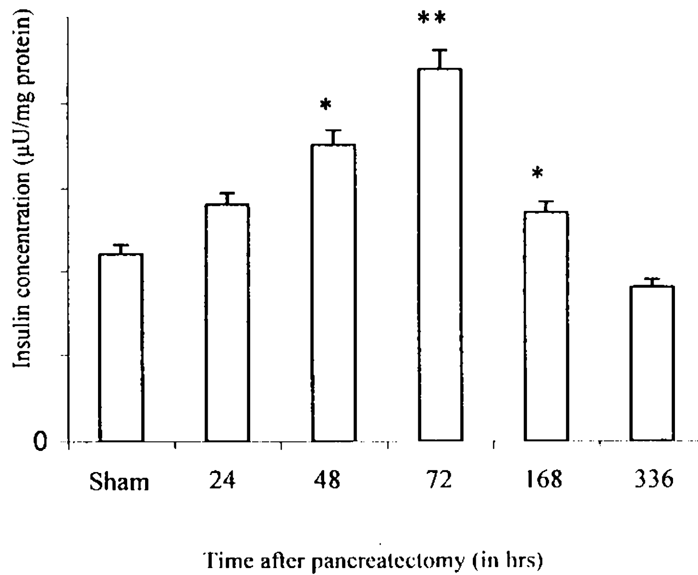
Regions	Sham	P 72 h	P 7D
Hypothalamus	4.56 ± 0.06	$2.99 \pm 0.13^{***}$	$3.38 \pm 0.10^{***\dagger}$
Brain stem	2.45 ± 0.12	$0.84 \pm 0.04^{***}$	$1.69 \pm 0.01^{***}$
Cerebellum	4.06 ± 0.10	$2.36 \pm 0.12^{***}$	$3.12 \pm 0.67^{***\dagger\dagger}$
Pancreas	$1.93 \pm .01$	$0.83 \pm 0.03^{***}$	$1.55 \pm 0.09^{***\dagger\dagger}$

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

$^{\dagger}p<0.05$ when compared with P 72h
$^{\dagger\dagger}p<0.01$ when compared with P 72h
*** $p<0.001$ when compared with control

Figure - 2

Circulating insulin levels of the sham and pancreatectomised rats



Values are mean ± SEM of 4-6 separate experiments
*$p<0.05$ when compared with sham
**$p<0.01$ when compared with sham

Figure- 3

Scatchard analysis of GABA receptor using [^{3}H]GABA against GABA in the brain stem of rats

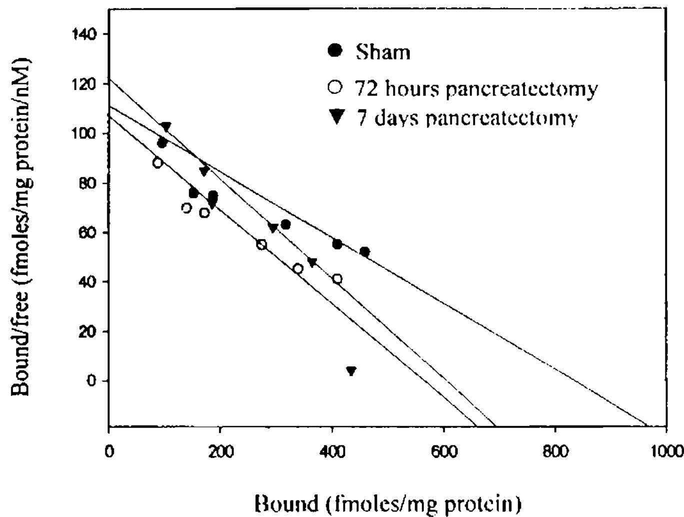


Table- 3

[^{3}H]GABA binding parameters in the brain stem of rats

Experimental group	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	983 ± 14.53	8.93 ± 0.72
72 hrs pancreatectomy	640 ± 15.26***	5.13 ± 0.46**
7 days pancreatectomy	717 ± 10.14***†	6.07 ± 0.32†

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

† $p<0.05$ when compared with 72 hours after pancreatectomy, ** $p<0.01$ when compared with sham, *** $p<0.001$ when compared with sham

Table- 4

Binding parameters of [^{3}H]GABA against GABA 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	-8.66	-9.77	1.4×10^{-10}	3.5×10^{-5}	-0.41
72 hrs pancreatectomy	Two-site	-4.69	-4.36	2.1×10^{-10}	2.2×10^{-5}	-0.21
7 days pancreatectomy	Two-site	-9.59	-4.56	2.1×10^{-10}	2.3×10^{-5}	-0.20

alues 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.

Fig. 4

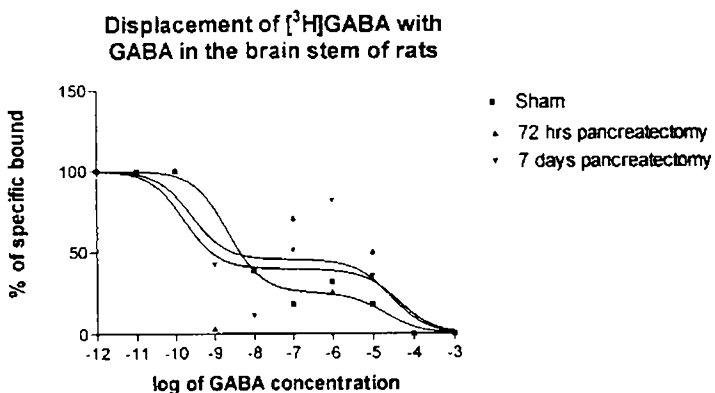


Figure- 5

Scatchard analysis of $GABA_A$ receptor using [^{3}H]bicuculline against bicuculline in the brain stem of rats

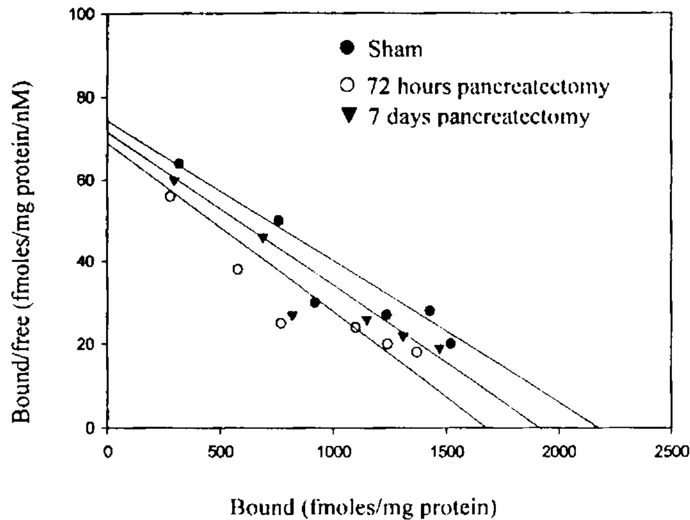


Table- 5

[^{3}H]bicuculline binding parameters in the brain stem of rats

Experimental group	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	2180 ± 11.55	29.30 ± 0.92
72 hrs pancrealectomy	1690 ± 20.28***	24.53 ± 0.80**
7 days pancreatectomy	1900 ± 14.53***†††	26.67 ± 0.43†

Values are mean ± S.E.M. of 4-6 separate experiments
† $p<0.05$ when compared with sham
** $p<0.01$ when compared with sham, *** $p<0.001$ when compared with sham
††† $p<0.001$ when compared with 72 hours after pancreatectomy

Table- 6

Binding parameters of [^{3}H]bicuculline against bicuculline 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	-8.68	-4.72	1.54×10^{-9}	1.38×10^{-5}	-0.40
72 hrs pancreatectomy	Two-site	-9.90	-4.55	9.28×10^{-11}	2.04×10^{-5}	-0.20
7 days pancreatectomy	Two-site	-9.59	-4.56	1.89×10^{-10}	2.02×10^{-5}	-0.20

lues are mean of 4-6 separate experiments

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

Fig. 6

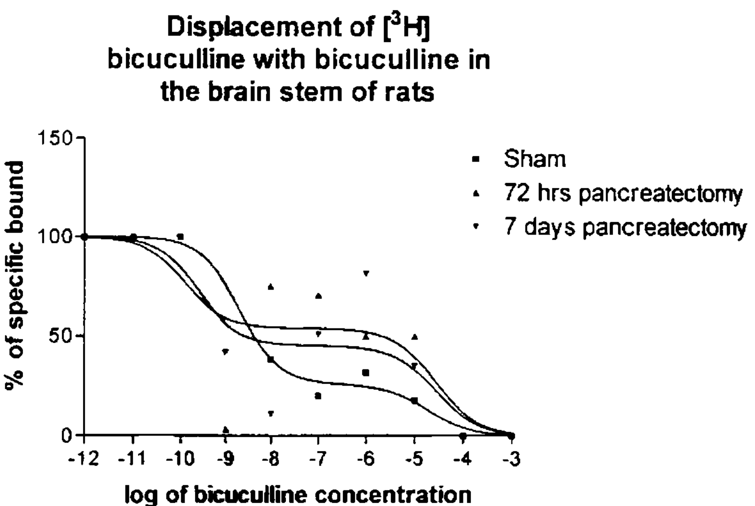


Figure 7

Real Time PCR amplification of the α_2 sub unit of $GABA_A$ receptor mRNA from the brain stem of experimental rats

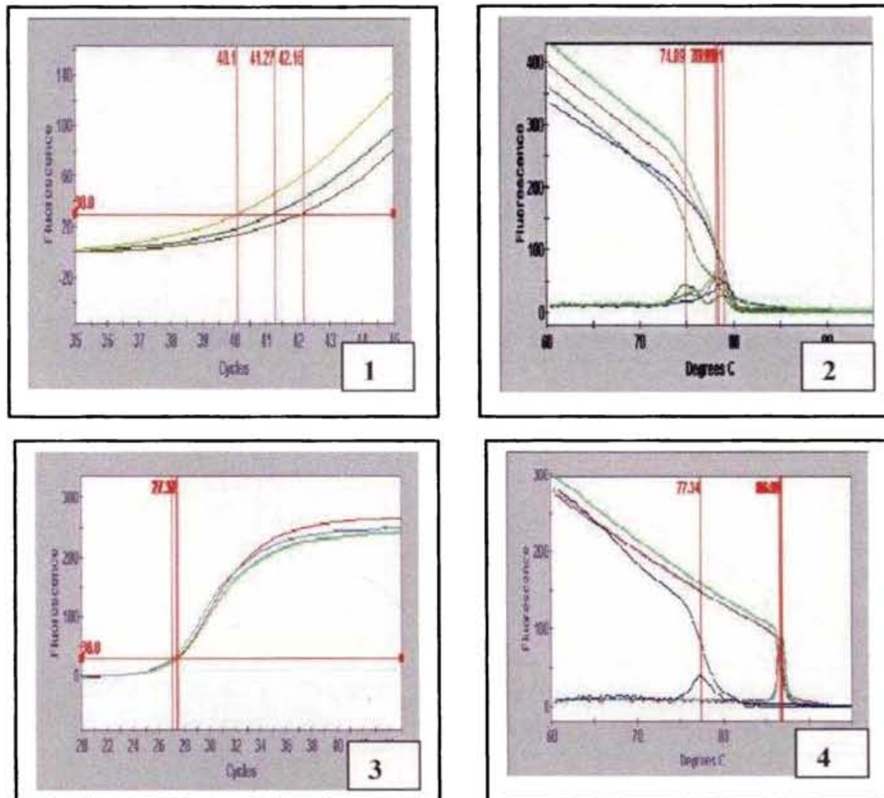


Table 7

No.	Experimental group	Ct Value
1	Sham	40.10
2	72 hrs pancreatectomy	42.16
3	7 days pancreatectomy	41.27

1.Graph representing the crossing threshold (Ct) of sample, 2. Melt curve of the sample of the amplicon obtained after the reaction, 3. Graph representing the crossing threshold of the house keeping gene (β-actin), 4. Melt curve of the house keeping gene obtained after the reaction.

Ct value represents the cycle number at which the fluorescence crosses the set threshold.

Figure 8

Real Time PCR amplification of the β_2 sub unit of $GABA_A$ receptor mRNA from the brain stem of experimental rats

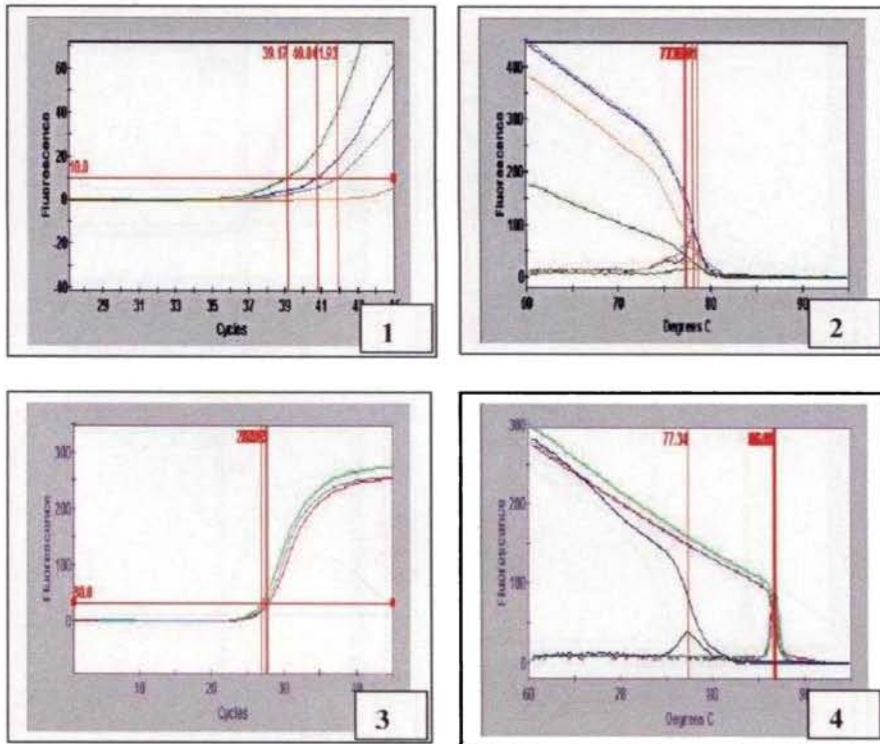


Table 8

No.	Experimental group	Ct Value
1	Sham	39.17
2	72 hrs pancreatectomy	41.93
3	7 days pancreatectomy	40.81

1.Graph representing the crossing threshold (Ct) of sample, 2. Melt curve of the sample of the amplicon obtained after the reaction, 3. Graph representing the crossing threshold of the house keeping gene (β-actin), 4. Melt curve of the house keeping gene obtained after the reaction.

Ct value represents the cycle number at which the fluorescence crosses the set threshold.

Figure 9

Real Time PCR amplification of the γ_1 sub unit of $GABA_A$ receptor mRNA from the brain stem of experimental rats

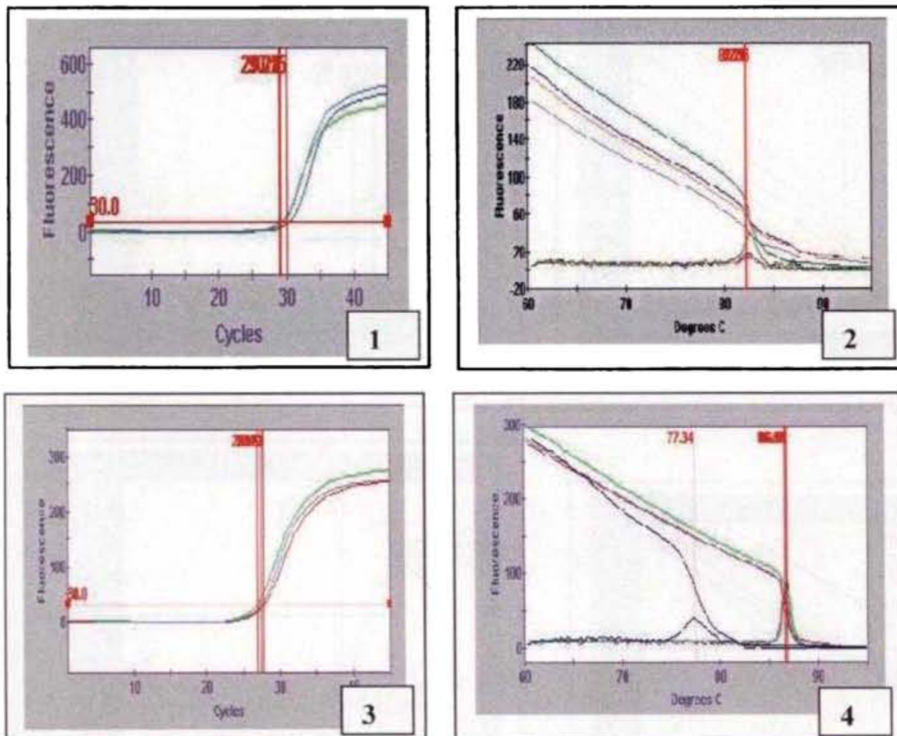


Table 9

No.	Experimental group	CT Value
1	Sham	29.07
2	72 hrs pancreatectomy	29.24
3	7 days pancreatectomy	30.66

1.Graph representing the crossing threshold (Ct) of sample, 2. Melt curve of the sample of the amplicon obtained after the reaction, 3. Graph representing the crossing threshold of the house keeping gene (β-actin), 4. Melt curve of the house keeping gene obtained after the reaction.

Ct value represents the cycle number at which the fluorescence crosses the set threshold.

Figure 10

Real Time PCR amplification of the γ_2 sub unit of $GABA_A$ receptor mRNA from the brain stem of experimental rats

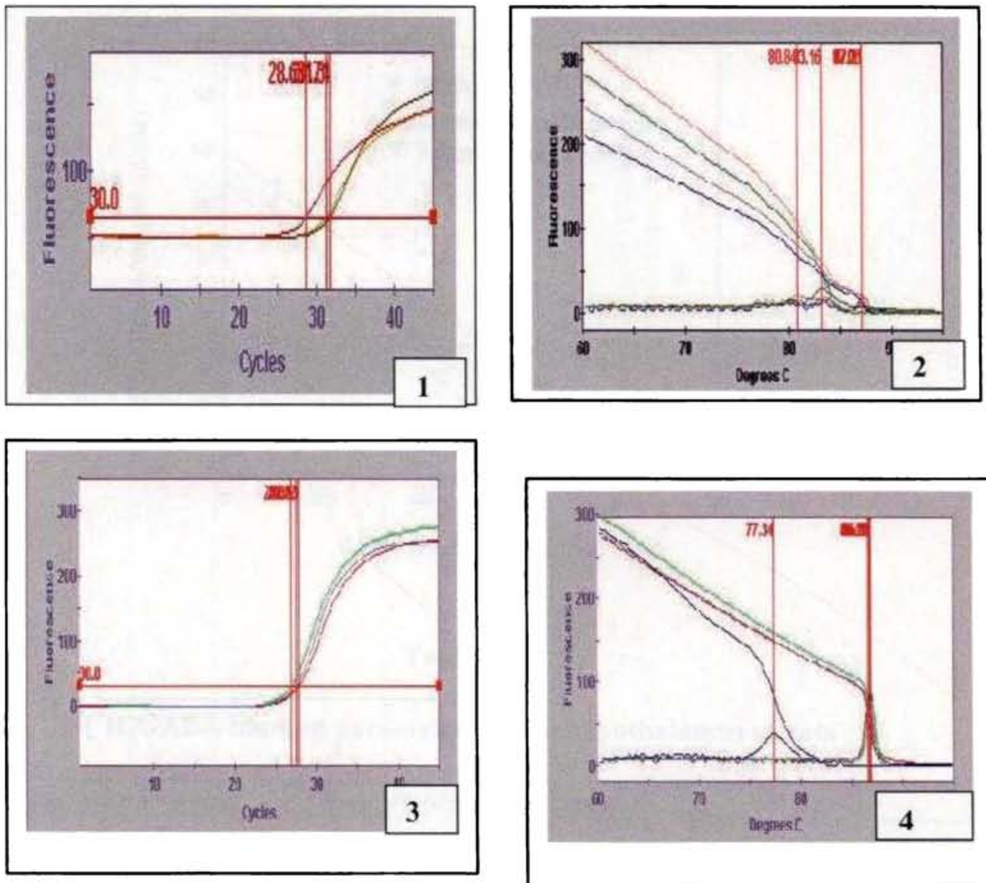


Table 10

No.	Experimental group	CT Value
1	Sham	31.30
2	72 hrs pancreatectomy	31.74
3	7 days pancreatectomy	28.86

1.Graph representing the crossing threshold (Ct) of sample, 2. Melt curve of the sample of the amplicon obtained after the reaction, 3. Graph representing the crossing threshold of the house keeping gene (â-actin), 4. Melt curve of the house keeping gene obtained after the reaction.

Ct value represents the cycle number at which the fluorescence crosses the set threshold.

Figure- 11

Scatchard analysis of GABA receptor using [^{3}H]GABA against GABA in the hypothalamus of rats

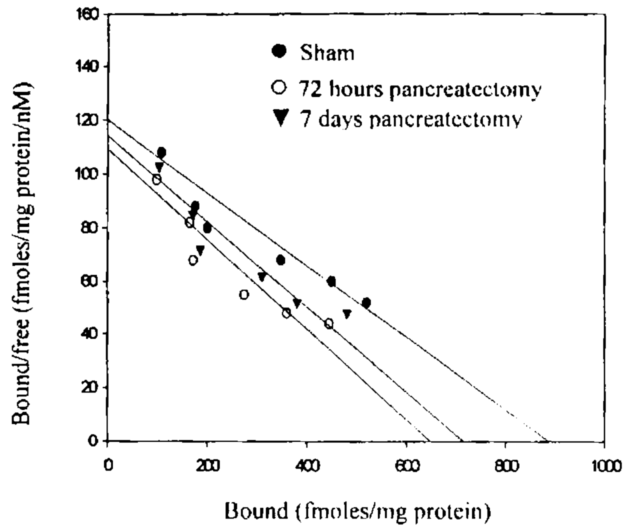


Table-11

[^{3}H]GABA binding parameters in the hypothalamus of rats

Experimental group	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	893 ± 10.14	7.70 ± 0.15
72 hrs pancreatectomy	653 ± 19.22***	5.67 ± 0.19*
7 days pancreatectomy	706 ± 11.67***†	6.50 ± 0.22*†

Values are mean ± S.E.M. of 4-6 separate experiments
†p<0.05 when compared with 72 hours after pancreatectomy, * p<0.05 when compared with sham, *** p<0.001 when compared with sham.

Table- 12

Binding parameters of $[^3H]$GABA against GABA 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	-8.34	-4.71	4.55×10^{-9}	1.93×10^{-5}	-0.40
72 hrs pancreatectomy	Two-site	-9.29	-5.18	5.09×10^{-10}	6.54×10^{-6}	-0.30
7 days pancreatectomy	Two-site	-9.44	-5.13	4.90×10^{-10}	6.29×10^{-6}	-0.29

ues are mean of 4-6 separate experiments

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

Fig. 12

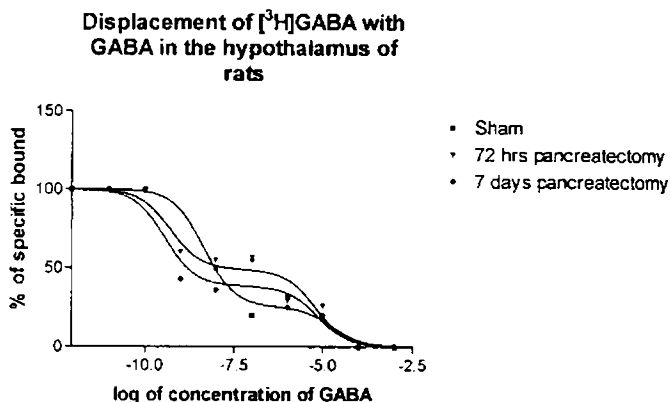


Figure- 13

Scatchard analysis of $GABA_A$ receptor using $[^3H]$bicuculline against bicuculline in the hypothalamus of rats

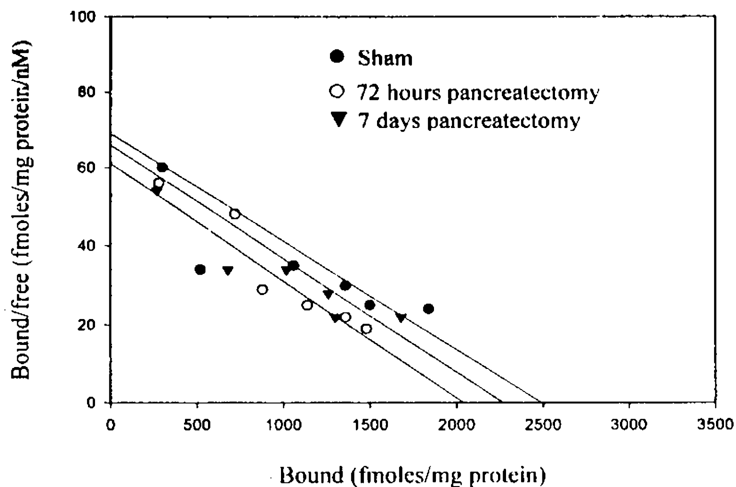


Table- 13

$[^3H]$bicuculline binding parameters in the hypothalamus of rats

Experimental group	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	2470 ± 24.03	35.26 ± 1.86
72 hrs pancreatectomy	2080 ± 32.83***	31.08 ± 2.53**
7 days pancreatectomy	2240 ± 18.55***††	36.06 ± 2.49††

Values are mean ± S.E.M. of 4-6 separate experiments
** $p<0.01$ when compared with sham, ††$p<0.01$ when compared with 72 hours after pancreatectomy, *** $p<0.001$ when compared with sham

Table- 14

Binding parameters of [^{3}H]bicuculline against bicuculline 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
;ham	Two-site	-9.25	-5.48	4.63×10^{-10}	$2.7 6 \times 10^{-6}$	-0.43
'2 hrs pancreatectomy	Two-site	-9.79	-5.00	1.34×10^{-10}	8.17×10^{-6}	-0.24
' days pancreatectomy	Two-site	-9.50	-4.59	$2.6 2 \times 10^{-10}$	2.12×10^{-5}	-0.24

ıes are mean of 4-6 separate experiments

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

Fig. 14

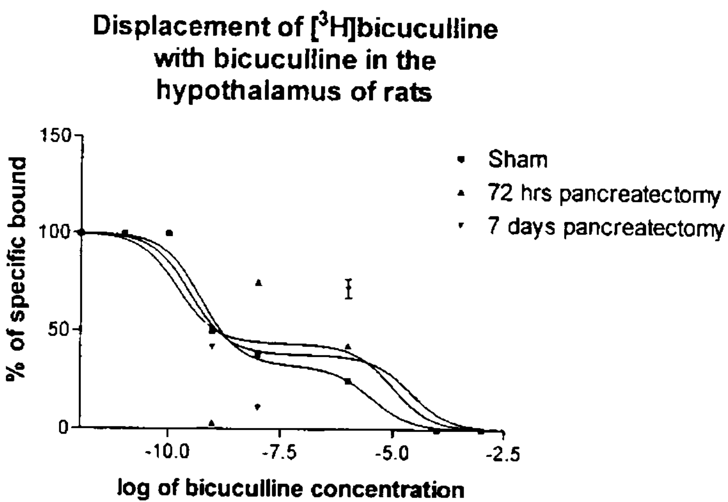


Figure 15

Real Time PCR amplification of the α_2 sub unit of $GABA_A$ receptor mRNA from the hypothalamus of experimental rats

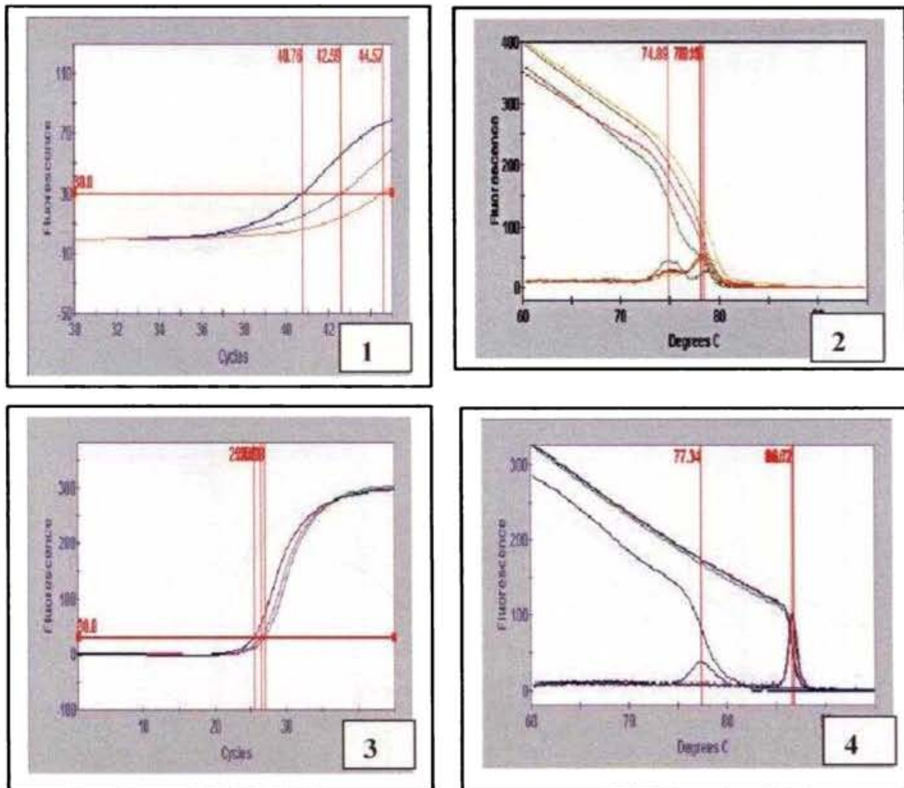


Table 15

No.	Experimental group	Ct Value
1	Sham	40.76
2	72 hrs pancreatectomy	44.57
3	7 days pancreatectomy	42.59

1.Graph representing the crossing threshold (Ct) of sample, 2. Melt curve of the sample of the amplicon obtained after the reaction, 3. Graph representing the crossing threshold of the house keeping gene (β-actin), 4. Melt curve of the house keeping gene obtained after the reaction.

Ct value represents the cycle number at which the fluorescence crosses the set threshold.

Figure 16

Real Time PCR amplification of the β_2 sub unit of $GABA_A$ receptor mRNA from the hypothalamus of experimental rats

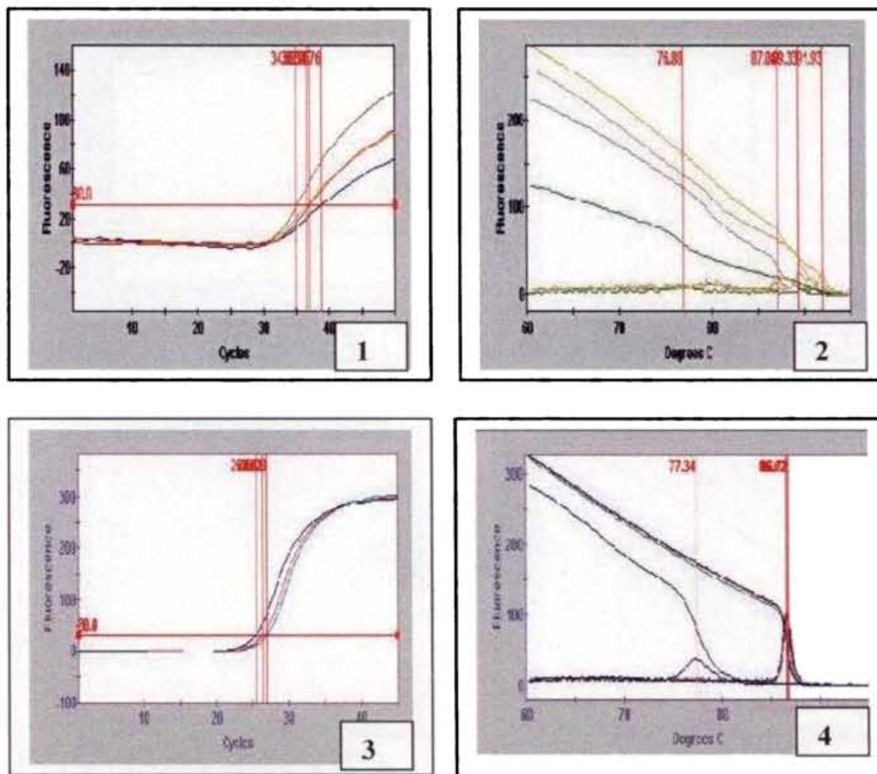


Table 16

No.	Experimental group	CT Value
1	Sham	34.36
2	72 hrs pancreatectomy	37.38
3	7 days pancreatectomy	37.21

1.Graph representing the crossing threshold (Ct) of sample, 2. Melt curve of the sample of the amplicon obtained after the reaction, 3. Graph representing the crossing threshold of the house keeping gene (β-actin), 4. Melt curve of the house keeping gene obtained after the reaction.

Ct value represents the cycle number at which the fluorescence crosses the set threshold.

Figure 17

Real Time PCR amplification of the γ_1 sub unit of $GABA_A$ receptor mRNA from the hypothalamus of experimental rats

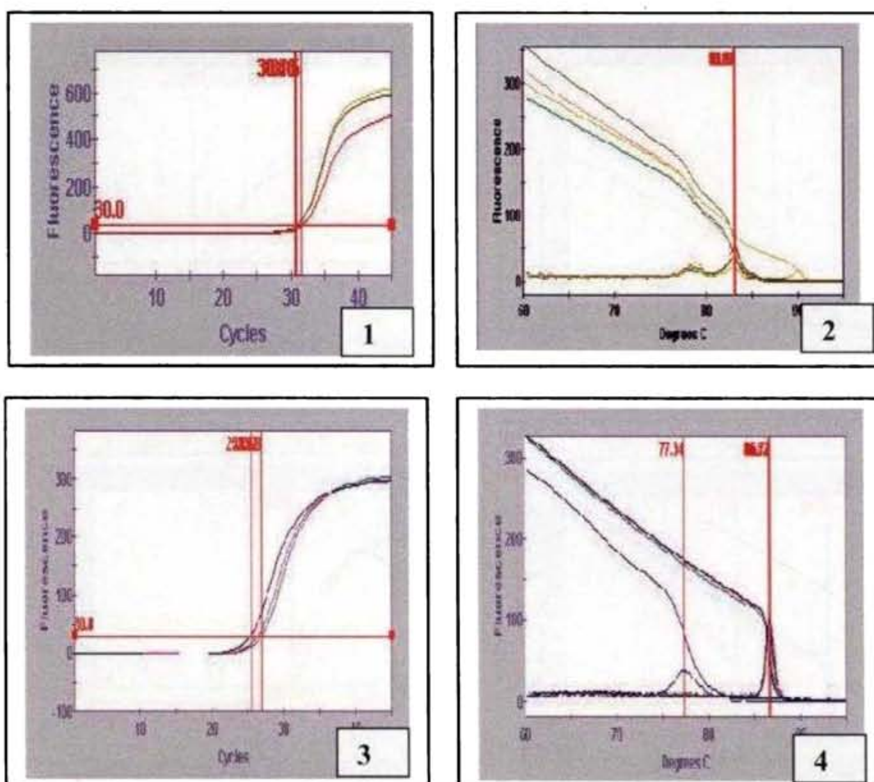


Table 17

No.	Experimental group	CT Value
1	Sham	30.26
2	72 hrs pancreatectomy	30.61
3	7 days pancreatectomy	31.35

1.Graph representing the crossing threshold (Ct) of sample, 2. Melt curve of the sample of the amplicon obtained after the reaction, 3. Graph representing the crossing threshold of the house keeping gene (â-actin), 4. Melt curve of the house keeping gene obtained after the reaction.

Ct value represents the cycle number at which the fluorescence crosses the set threshold.

Figure 18

Real Time PCR amplification of the γ_2 sub unit of $GABA_A$ receptor mRNA from the hypothalamus of experimental rats

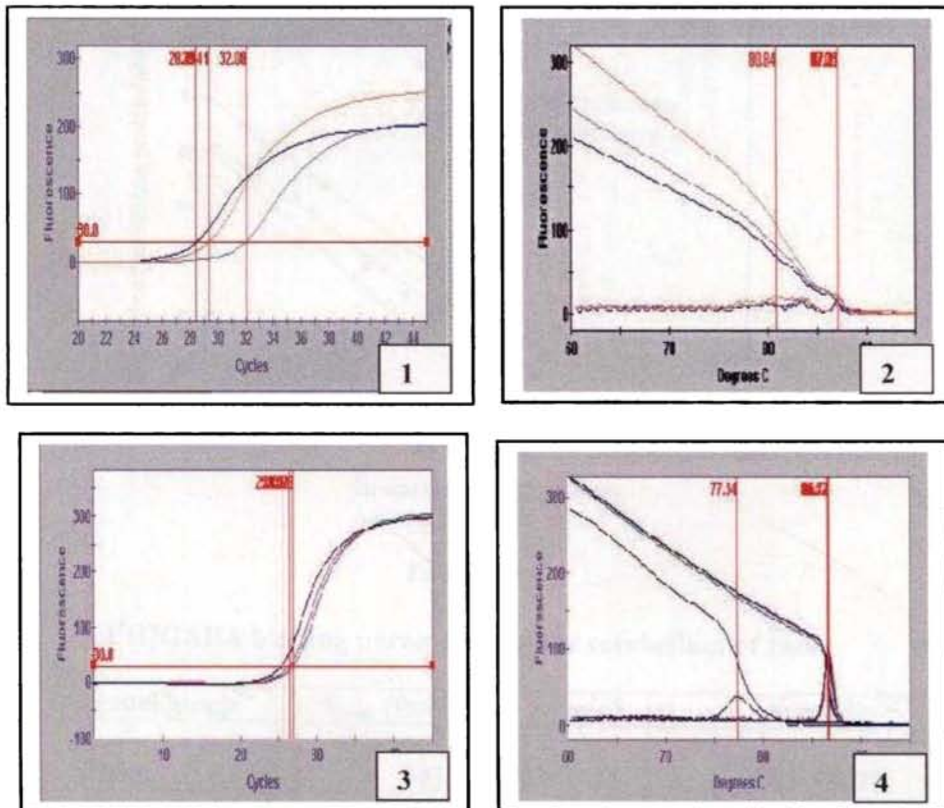


Table 18

No.	Experimental group	Ct Value
1	Sham	28.28
2	72 hrs pancreatectomy	32.08
3	7 days pancreatectomy	29.41

1.Graph representing the crossing threshold (Ct) of sample, 2. Melt curve of the sample of the amplicon obtained after the reaction, 3. Graph representing the crossing threshold of the house keeping gene (β-actin), 4. Melt curve of the house keeping gene obtained after the reaction.

Ct value represents the cycle number at which the fluorescence crosses the set threshold.

Figure- 19

Scatchard analysis of GABA receptor using [^{3}H]GABA against GABA in the cerebellum of rats

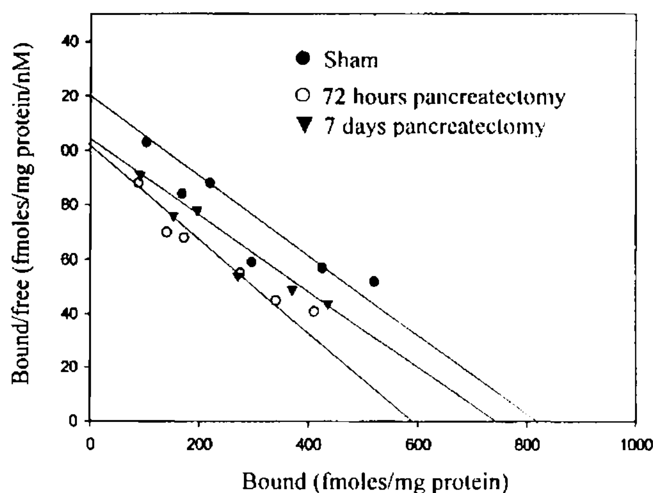


Table- 19

[^{3}H]GABA binding parameters in the cerebellum of rats

Experimental group	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	803 ± 11.87	8.93 ± 0.72
72 hrs pancreatectomy	596 ± 10.68***	5.13 ± 0.46*
7 days pancreatectomy	720 ± 9.24**††	6.07 ± 0.32†

Values are mean ± S.E.M. of 4-6 separate experiments
*$p<0.05$ when compared with Sham, † $p<0.05$ when compared with 72 hours after pancreatectomy, **$p<0.001$ when compared with Sham, †† $p<0.05$ when compared with 72 hours after pancreatectomy, ** $p<0.001$ when compared with Sham.

Table- 20

Binding parameters of [^{3}H]GABA against GABA in the cerebellum of experimental rats

xperimental Group	Best-fit model	log (EC_{50})-1	log (EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
ham	Two-site	-9.0	-4.79	2.81×10^{-10}	5.37×10^{-6}	-0.37
2 hrs pancreatectomy	Two-site	-9.31	-4.54	1.6×10^{-10}	9.5×10^{-5}	-0.26
days pancreatectomy	Two-site	-9.53	-4.59	9.7×10^{-11}	8.53×10^{-6}	-0.22

ıes are mean of 4-6 separate experiments

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

Fig. 20

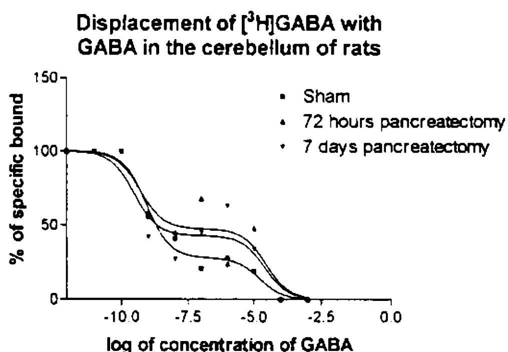


Figure- 21

Scatchard analysis of $GABA_A$ receptor using [^{3}H]bicuculline against bicuculline in the cerebellum of rats

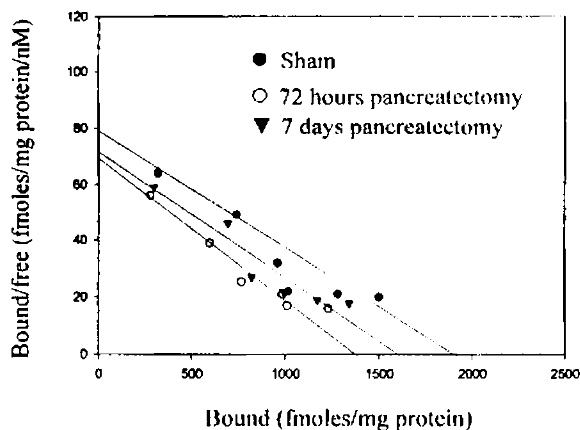


Table- 21

[^{3}H]bicuculline binding parameters in the cerebellum of rats

Experimental group	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	1896 ± 20.27	24.27 ± 0.89
72 hrs pancreatectomy	1416 ± 17.64***	21.13 ± 0.27*
7 days pancreatectomy	1610 ± 20.82***†††	22.56 ± 0.63

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

* $p < 0.01$ when compared with sham

*** $p < 0.001$ when compared with sham

††† $p < 0.01$ when compared with 72 hours after pancreatectomy

Table- 22

Binding parameters of [^{3}H]bicuculline against bicuculline in the cerebellum 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	-8.83	-4.56	1.47×10^{-8}	2.73×10^{-8}	-0.46
72 hrs pancreatectomy	Two-site	-9.85	-4.49	1.40×10^{-10}	3.22×10^{-5}	-0.20
7 days pancreatectomy	Two-site	-9.55	-4.59	2.80×10^{-10}	2.56×10^{-5}	-0.21

lues are mean of 4-6 separate experiments

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

Fig. 22

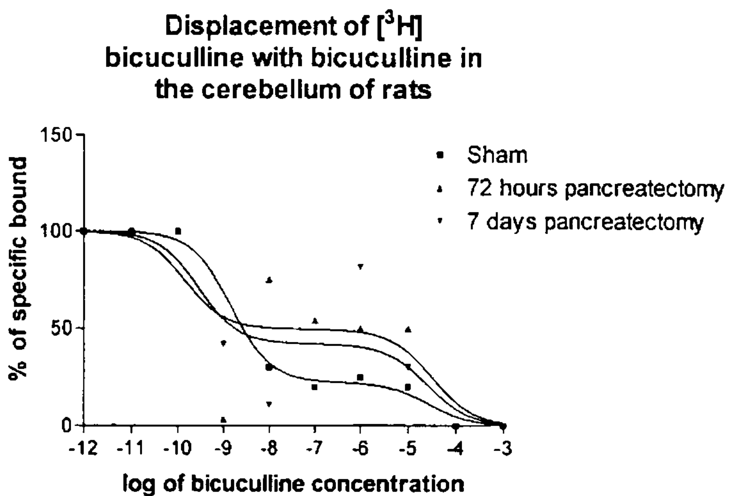


Figure 23

Real Time PCR amplification of the α_2 sub unit of $GABA_A$ receptor mRNA from the cerebellum of experimental rats

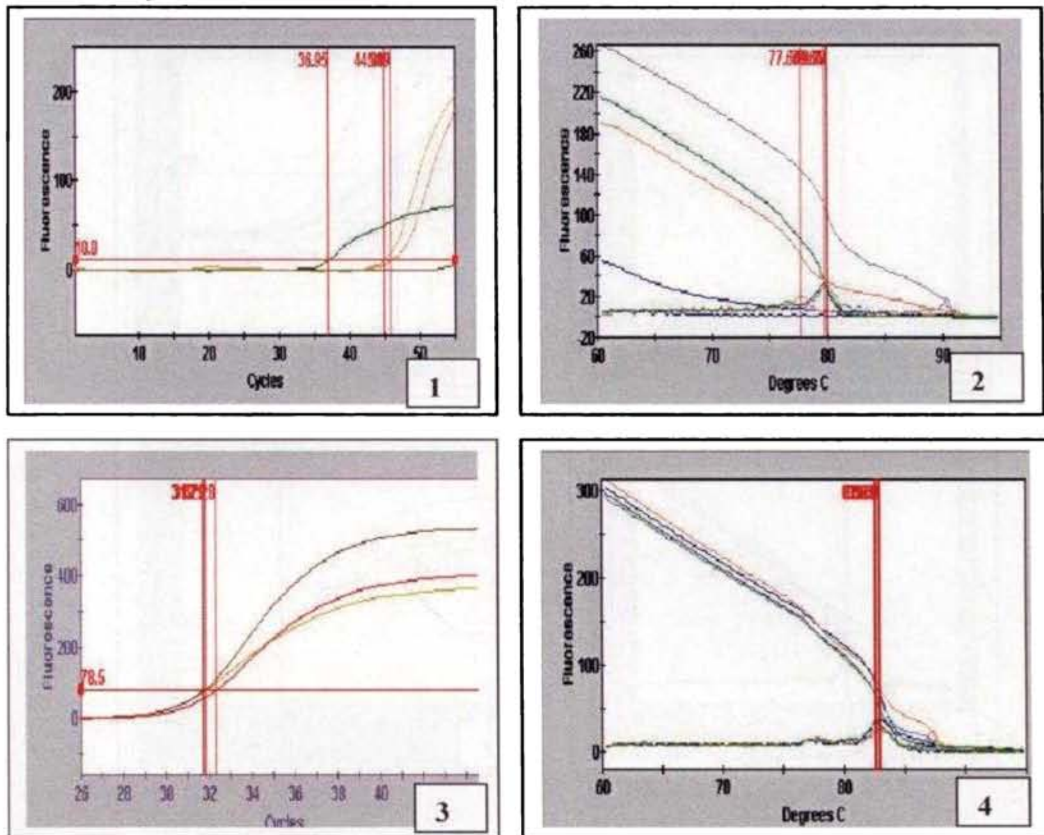


Table 23

No.	Experimental group	Ct Value
1	Sham	36.95
2	72 hrs pancreatectomy	44.58
3	7 days pancreatectomy	44.79

1.Graph representing the crossing threshold (Ct) of sample, 2. Melt curve of the sample of the amplicon obtained after the reaction, 3. Graph representing the crossing threshold of the house keeping gene (β-actin), 4. Melt curve of the house keeping gene obtained after the reaction.

Ct value represents the cycle number at which the fluorescence crosses the set

Figure 24

Real Time PCR amplification of the β_2 sub unit of $GABA_A$ receptor mRNA from the cerebellum of experimental rats

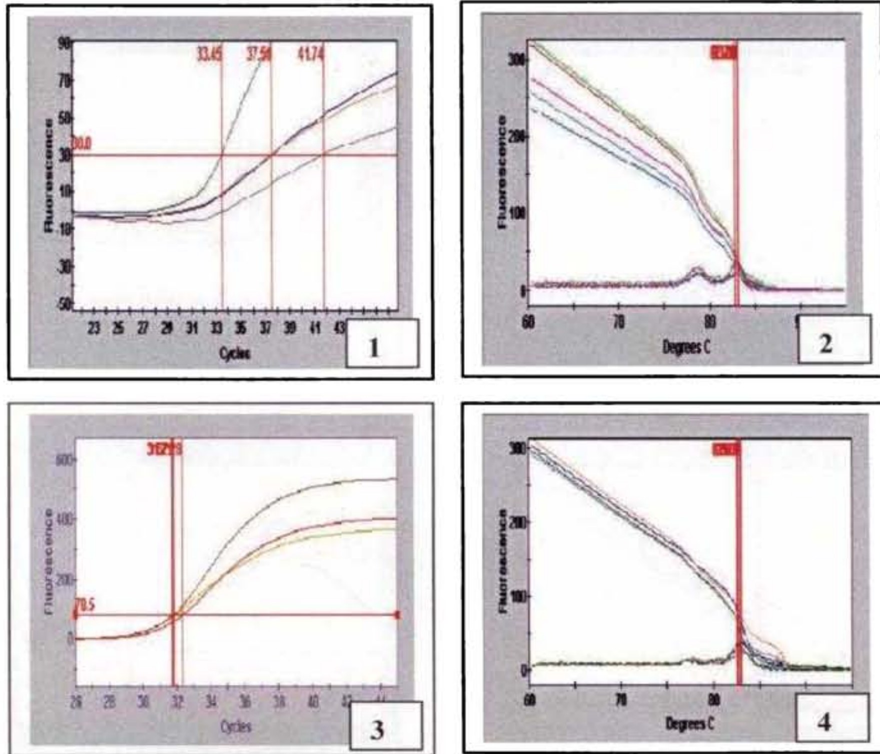


Table 24

No.	Experimental group	Ct Value
1	Sham	33.45
2	72 hrs pancreatectomy	41.74
3	7 days pancreatectomy	37.56

1.Graph representing the crossing threshold (Ct) of sample, 2. Melt curve of the sample of the amplicon obtained after the reaction, 3. Graph representing the crossing threshold of the house keeping gene (β-actin), 4. Melt curve of the house keeping gene obtained after the reaction.

Ct value represents the cycle number at which the fluorescence crosses the set threshold.

Figure 25

Real Time PCR amplification of the γ_1 sub unit of $GABA_A$ receptor mRNA from the cerebellum of experimental rats

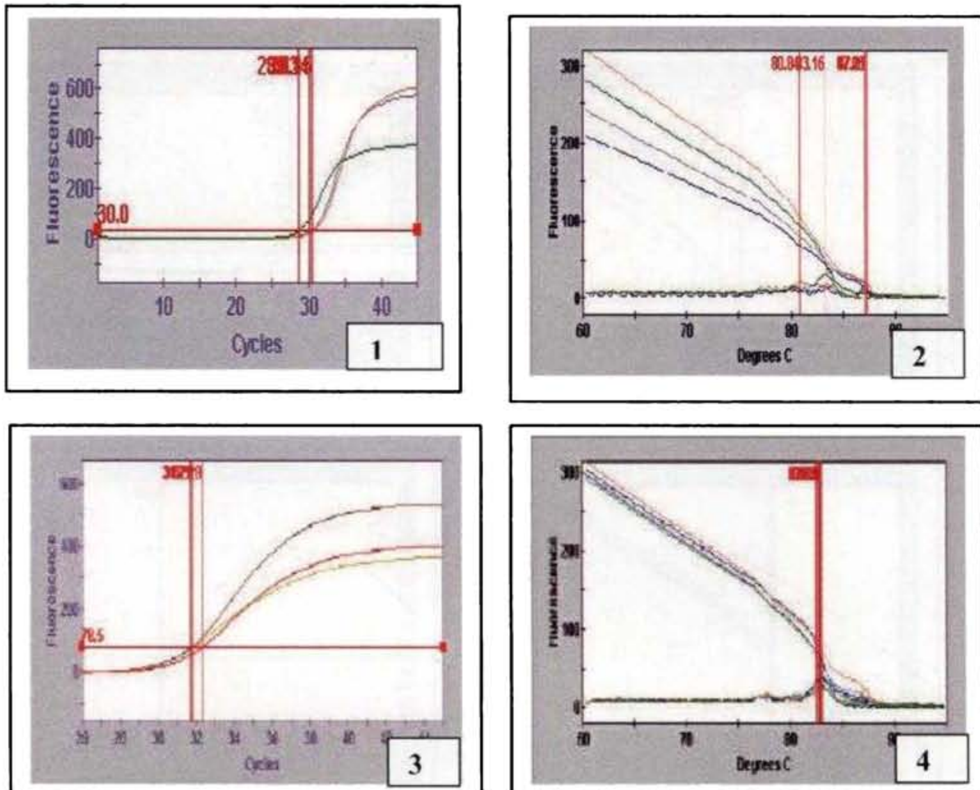


Table 25

No.	Experimental group	CT Value
1	Sham	28.53
2	72 hrs pancreatectomy	30.76
3	7 days pancreatectomy	30.66

1.Graph representing the crossing threshold (Ct) of sample, 2. Melt curve of the sample of the amplicon obtained after the reaction, 3. Graph representing the crossing threshold of the house keeping gene (â-actin), 4. Melt curve of the house keeping gene obtained after the reaction.

Ct value represents the cycle number at which the fluorescence crosses the set threshold.

Figure 26

Real Time PCR amplification of the γ_2 sub unit of $GABA_A$ receptor mRNA from the cerebellum of experimental rats

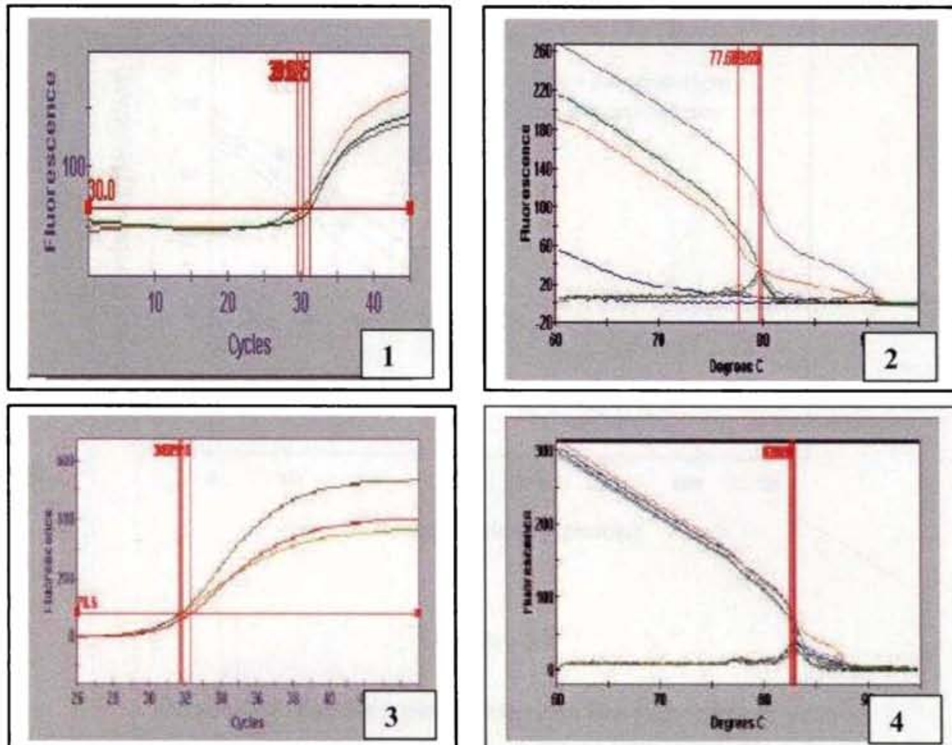


Table 26

No.	Experimental group	Ct Value
1	Sham	29.60
2	72 hrs pancreatectomy	30.18
3	7 days pancreatectomy	31.25

1.Graph representing the crossing threshold (Ct) of sample, 2. Melt curve of the sample of the amplicon obtained after the reaction, 3. Graph representing the crossing threshold of the house keeping gene (â-actin), 4. Melt curve of the house keeping gene obtained after the reaction.

Ct value represents the cycle number at which the fluorescence crosses the set threshold.

Figure- 27

Scatchard analysis of GABA receptor using [^{3}H]GABA against GABA in the pancreas of rats

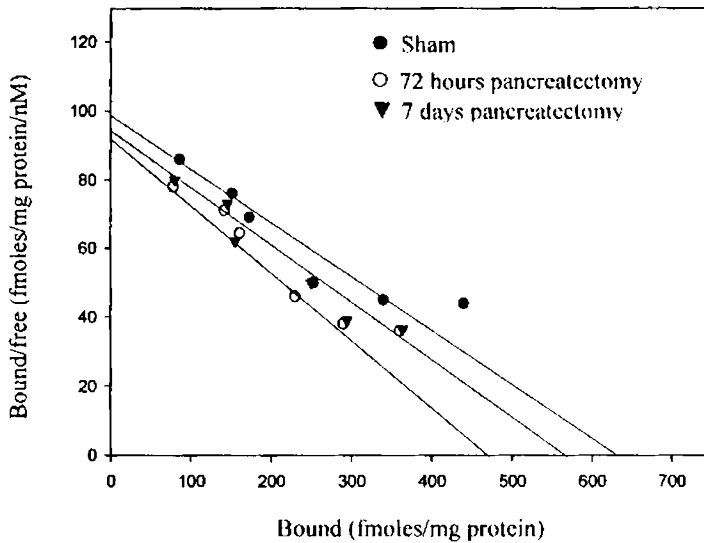


Table- 27

[^{3}H]GABA binding parameters in the pancreas of rats

Experimental group	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	629 ± 10.93	5.40 ± 0.36
72 hrs pancreatectomy	469 ± 6.64***	4.25 ± 0.15*
7 days pancreatectomy	562 ± 11.14**†††	4.60 ± 0.16

Values are mean ± S.E.M. of 4-6 separate experiments
p*<0.05 when compared with sham, **p<0.01 when compared with sham
*** p<0.01 when compared with sham, †††p<0.001 when compared with 72 hours pancreatectomy

Table 28

Binding parameters of [^{3}H]GABA against GABA in the pancreas 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	-9.17	-6.00	6.67×10^{-10}	9.80×10^{-7}	-0.48
72 hrs pancreatectomy	Two-site	-9.86	-5.63	1.35×10^{-10}	2.29×10^{-6}	-0.24
7 days pancreatectomy	Two-site	-9.41	-4.69	3.80×10^{-10}	2.04×10^{-5}	-0.28

alues 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.

Fig. 28

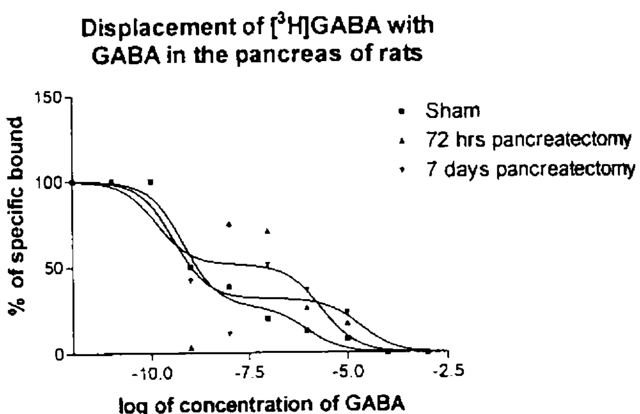


Figure- 29

Scatchard analysis of $GABA_A$ receptor using [^{3}H]bicuculline against bicuculline in the pancreas of rats

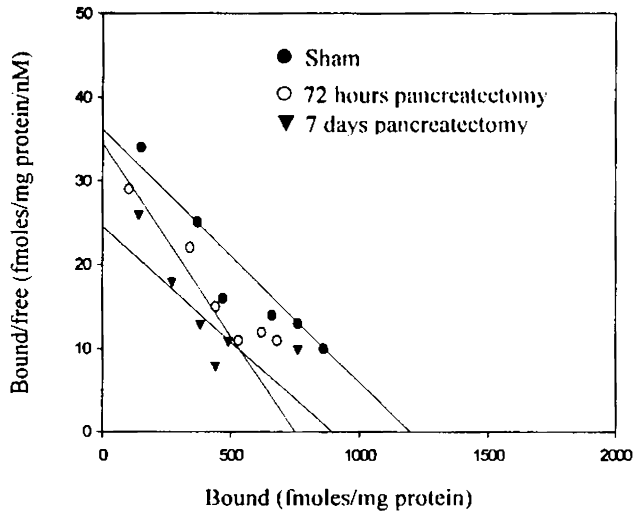


Table- 29

[^{3}H]bicuculline binding parameters in the pancreas of rats

Experimental group	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	1190 ± 12.02	31.33 ± 1.29
72 hrs pancreatectomy	790 ± 18.56***	23.34 ± 1.48***
7 days pancreatectomy	930 ± 17.64***††	35.55 ± 1.27††

Values are mean ± S.E.M. of 4-6 separate experiments
*** $p<0.001$ when compared with sham
††$p<0.01$ when compared with 72 hours after pancreatectomy

Table- 30

Binding parameters of [^{3}H]bicuculline against bicuculline in the pancreas 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	-8.97	-6.13	8.86×10^{-10}	6.09×10^{-7}	-0.41
72 hrs pancreatectomy	Two-site	-9.74	-5.56	1.48×10^{-10}	2.29×10^{-6}	-0.28
7 days pancreatectomy	Two-site	-8.87	-4.92	1.11×10^{-9}	9.94×10^{-6}	-0.38

lues are mean of 4-6 separate experiments

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

Fig. 30

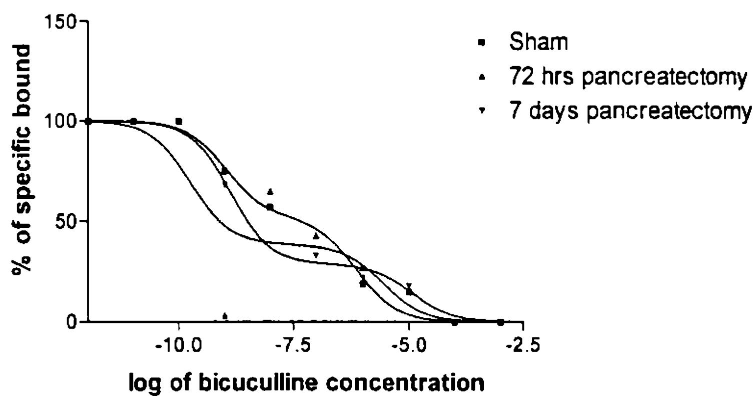


Figure 31

Real Time PCR amplification of the α_2 sub unit of $GABA_A$ receptor mRNA from the pancreas of experimental rats

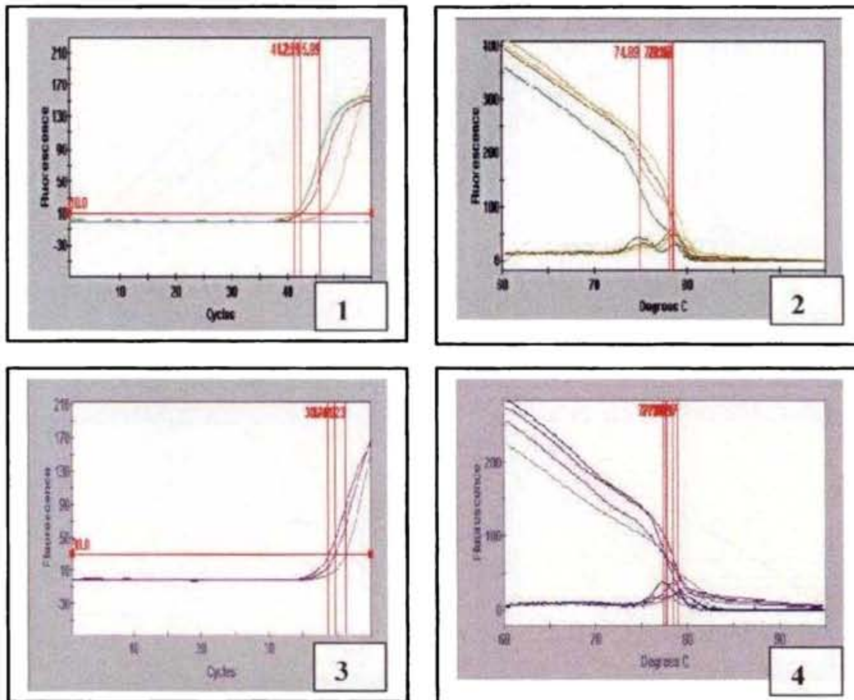


Table 31

No.	Experimental group	Ct Value
1	Sham	41.26
2	72 hrs pancreatectomy	45.89
3	7 days pancreatectomy	42.73

1.Graph representing the crossing threshold (Ct) of sample, 2. Melt curve of the sample of the amplicon obtained after the reaction, 3. Graph representing the crossing threshold of the house keeping gene (β-actin), 4. Melt curve of the house keeping gene obtained after the reaction.

Ct value represents the cycle number at which the fluorescence crosses the set threshold.

Figure 32

Real Time PCR amplification of the β_2 sub unit of $GABA_A$ receptor mRNA from the pancreas of experimental rats

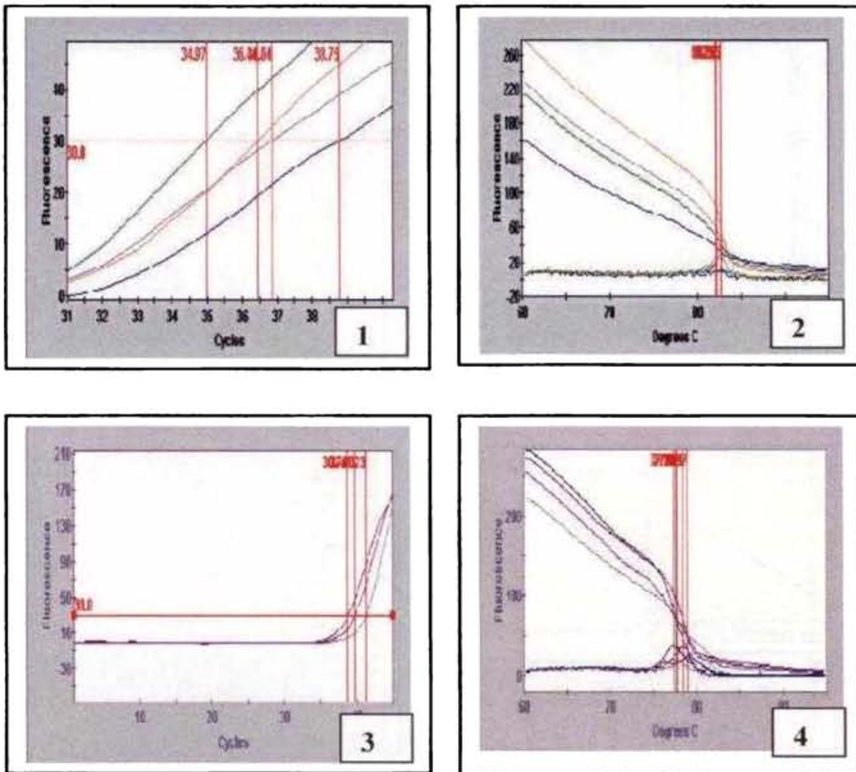


Table 32

No.	Experimental group	Ct Value
1	Sham	34.97
2	72 hrs pancreatectomy	37.84
3	7 days pancreatectomy	36.92

1.Graph representing the crossing threshold (Ct) of sample, 2. Melt curve of the sample of the amplicon obtained after the reaction, 3. Graph representing the crossing threshold of the house keeping gene (β-actin), 4. Melt curve of the house keeping gene obtained after the reaction.

Ct value represents the cycle number at which the fluorescence crosses the set threshold.

Figure 33

Real Time PCR amplification of the γ_1 sub unit of $GABA_A$ receptor mRNA from the pancreas of experimental rats

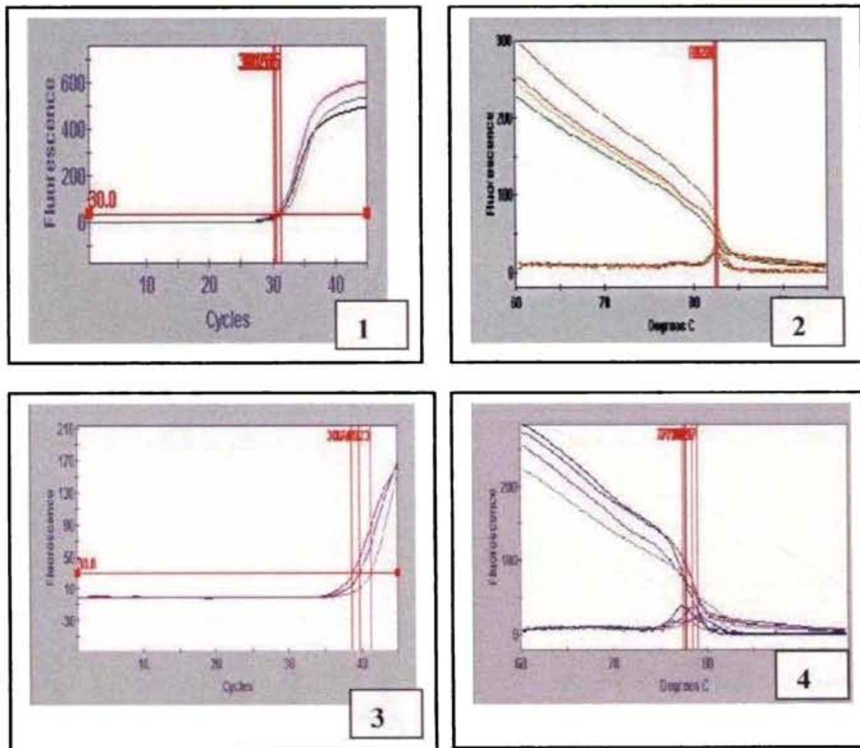


Table 33

No.	Experimental group	Ct Value
1	Sham	30.56
2	72 hrs pancreatectomy	31.61
3	7 days pancreatectomy	31.75

1.Graph representing the crossing threshold (Ct) of sample, 2. Melt curve of the sample of the amplicon obtained after the reaction, 3. Graph representing the crossing threshold of the house keeping gene (β-actin), 4. Melt curve of the house keeping gene obtained after the reaction.

Ct value represents the cycle number at which the fluorescence crosses the set threshold.

Figure 34

Real Time PCR amplification of the γ_2 sub unit of $GABA_A$ receptor mRNA from the pancreas of experimental rats

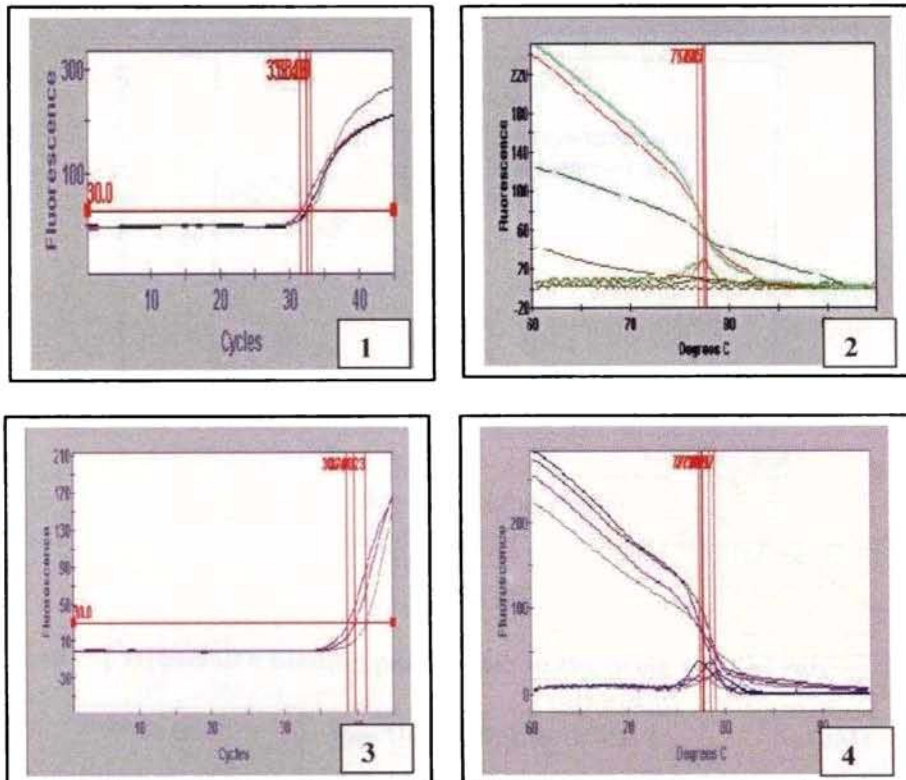


Table 34

No.	Experimental group	Ct Value
1	Sham	31.61
2	72 hrs pancreatectomy	32.48
3	7 days pancreatectomy	33.09

1.Graph representing the crossing threshold (Ct) of sample, 2. Melt curve of the sample of the amplicon obtained after the reaction, 3. Graph representing the crossing threshold of the house keeping gene (β-actin), 4. Melt curve of the house keeping gene obtained after the reaction.

Ct value represents the cycle number at which the fluorescence crosses the set threshold.

Figure- 35

Scatchard analysis of $GABA_B$ receptor using [^{3}H]baclofen against baclofen in the brain stem of rats

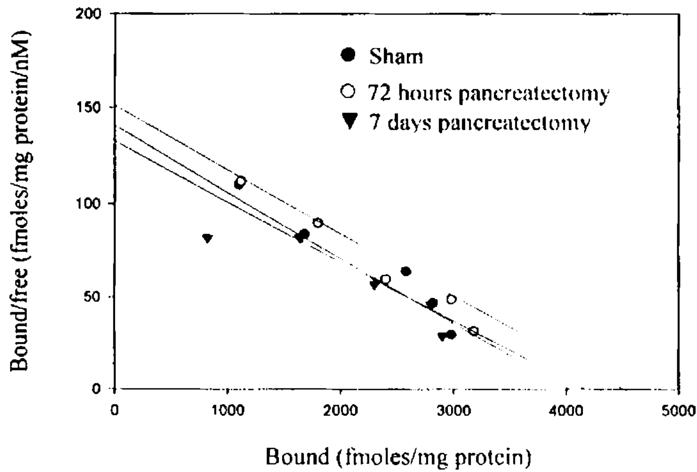


Table- 35

[^{3}H]baclofen binding parameters in the brain stem of rats

Experimental group	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	4080 ± 17.02	24.56 ± 0.47
72 hrs pancreatectomy	4490 ± 23.62***	29.35 ± 0.69**
7 days pancreatectomy	4180 ± 18.55***†††	26.73 ± 0.36*†

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

$^*p<0.05$ when compared with sham, $^\dagger p<0.05$ when compared with 72 hours after pancreatectomy, ** $p<0.01$ when compared with sham, *** $p<0.001$ when compared with sham, ††† $p<0.001$ when compared with 72 hours after pancreatectomy

Table- 36

Binding parameters of [^{3}H]baclofen against baclofen in the brain stem of experimental rats

Experimental Group	Best-fit model	Log (EC_{50})	Ki	Hill slope
Sham	One-site	-7.69	1.88×10^{-8}	-1.09
72 hrs pancreatectomy	One-site	-8.92	1.09×10^{-9}	-0.96
7 days pancreatectomy	One-site	-9.05	8.85×10^{-10}	-1.01

'alues are mean of 4-6 separate experiments

›ata were fitted with an iterative nonlinear regression oftware (Prism, GraphPad, San Diego, CA). Ki – The ffinity of the receptor for the competing drug. EC_{50} is the oncentration of the competitor that competes for half the pecific binding.

Fig. 36

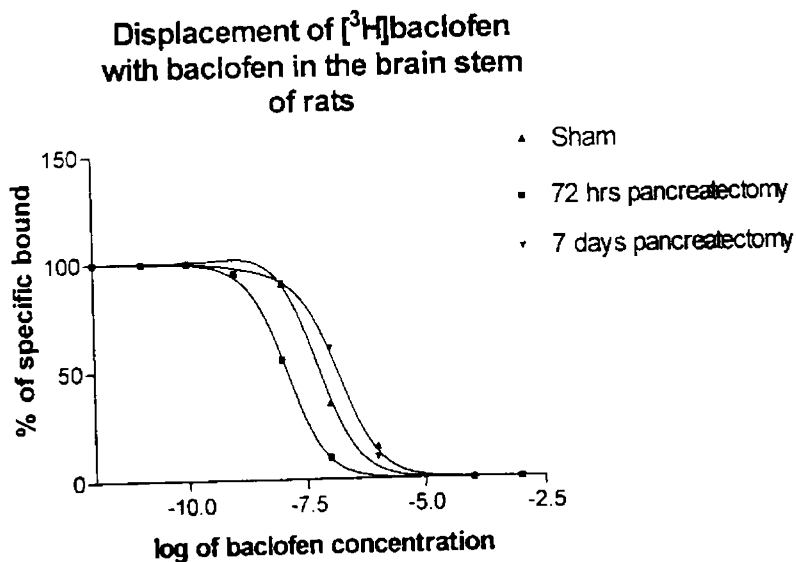


Figure- 37

Scatchard analysis of $GABA_B$ receptor using [^{3}H]baclofen against baclofen in the hypothalamus of rats

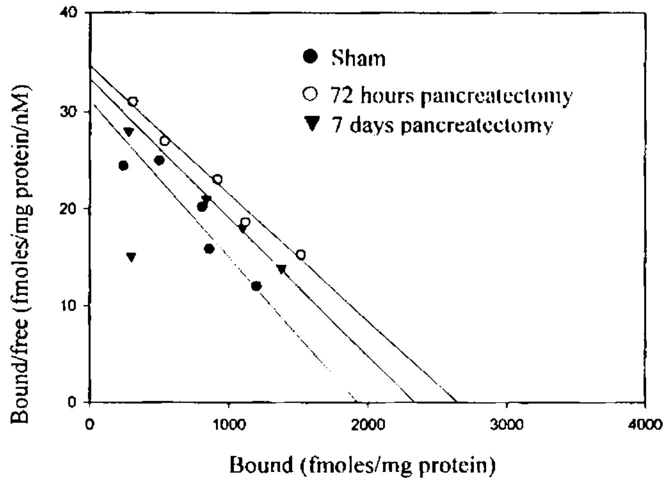


Table- 37

[^{3}H]baclofen binding parameters in the hypothalamus of rats

Experimental group	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	1970 ± 17.64	61.67 ± 2.60
72 hrs pancreatectomy	2630 ± 47.26***	76.00 ± 3.61**
7 days pancreatectomy	2190 ± 8.82**†††	65.33 ± 1.76†

Values are mean ± S.E.M. of 4-6 separate experiments
†$p<0.01$ when compared with 72 hours after pancreatectomy, **$p<0.01$ when compared with Sham, *** $p<0.01$ when compared with Sham, †††$p<0.001$ when compared with 72 hours after pancreatectomy.

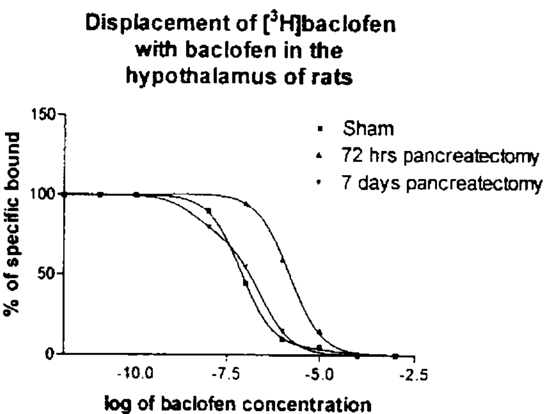
Table- 38

Binding parameters of $[^3H]$baclofen against baclofen in the hypothalamus of experimental rats

Experimental Group	Best-fit model	Log (EC_{50})	Ki	Hill slope
Sham	One-site	-7.12	7.57×10^{-8}	-1.04
72 hrs pancreatectomy	One-site	-8.83	1.45×10^{-9}	-1.12
7 days pancreatectomy	One-site	-8.37	4.25×10^{-9}	-0.68

Fig. 38

lues are mean of 4-6 separate experiments



ta were fitted with an iterative nonlinear regression tware (Prism. GraphPad. San Diego. CA). Ki – The inity of the receptor for the competing drug. EC_{50} is the icentration of the competitor that competes for half the cific binding.

Figure- 39

Scatchard analysis of $GABA_B$ receptor using [^3H]baclofen against baclofen in the cerebellum of rats

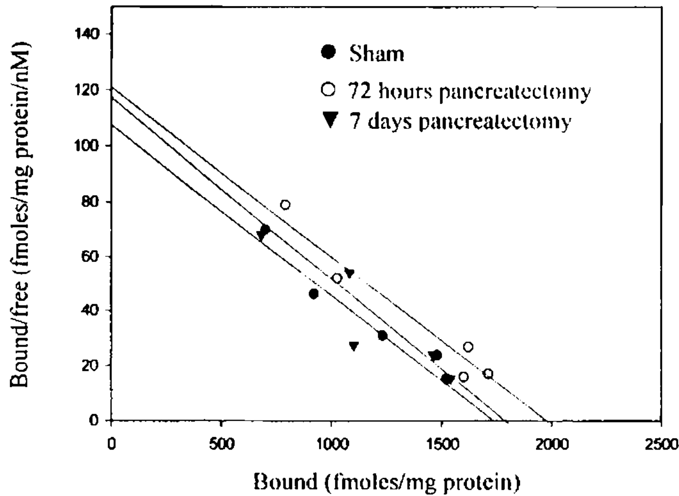


Table- 39

[^3H]baclofen binding parameters in the cerebellum of rats

Experimental group	B_{max} (nmoles/mg protein)	K_d (nM)
Sham	1610 ± 0.13	24.12 ± 3.35
72 hrs pancreatectomy	1940 ± 0.70***	29.93 ± 2.27*
7 days pancreatectomy	1780 ± 0.12***†††	28.97 ± 2.15*†

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

*p<0.05 when compared with sham, † p<0.05 when compared with 72 hours after pancreatectomy, *** p<0.01 when compared with Sham, †††p<0.001 when compared with 72 hours after pancreatectomy

Table- 40

Binding parameters of [^{3}H]baclofen against baclofen in the cerebellum of experimental rats

Experimental Group	Best-fit model	Log (EC_{50})	Ki	Hill slope
Sham	One-site	-7.32	4.78×10^{-8}	-0.85
72 hrs pancreatectomy	One-site	-6.14	7.12×10^{-7}	-1.13
7 days pancreatectomy	One-site	-9.17	7.12×10^{-10}	-0.86

alues are mean of 4-6 separate experiments

ata were fitted with an iterative nonlinear regression ftware (Prism, GraphPad, San Diego, CA). Ki – The finity of the receptor for the competing drug. EC_{50} is the ncentration of the competitor that competes for half the ecific binding.

Fig. 40

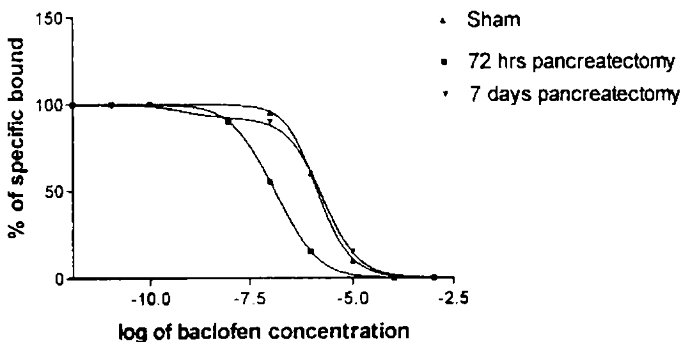


Figure- 41

Scatchard analysis of $GABA_B$ receptor using [^{3}H]baclofen against baclofen in the pancreas of rats

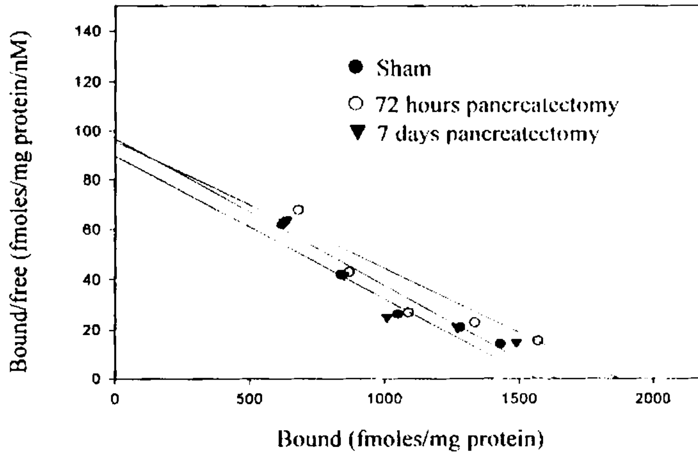


Table- 41

[^{3}H]GABA binding parameters in the pancreas of rats

Experimental group	B_{max} (fmoles/mg protein)	K_d (nM)
Sham	1570 ± 35.12	21.87 ± 2.98
72 hrs pancreatectomy	1930 ± 26.03***	23.92 ± 2.06*
7 days pancreatectomy	1730 ± 72.43***†††	17.63 ± 2.03*††

Values are mean ± S.E.M. of 4-6 separate experiments
*p<0.05 when compared with Sham, ††p<0.01 when compared with 72 hours after pancreatectomy, *** p<0.001 when compared with Sham
†††p<0.001 when compared with 72 hours after pancreatectomy

Table- 42

Binding parameters of [^{3}H]baclofen against baclofen in the pancreas of experimental rats

Experimental Group	Best-fit model	Log (EC_{50})	Ki	Hill slope
Sham	One-site	-7.85	1.39×10^{-8}	-1.57
72 hrs pancreatectomy	One-site	-7.33	4.62×10^{-8}	-0.79
7 days pancreatectomy	One-site	-8.01	9.56×10^{-9}	-0.89

lues are mean of 4-6 separate experiments

a were fitted with an iterative nonlinear regression ware (Prism, GraphPad. San Diego, CA). Ki – The nity of the receptor for the competing drug. EC_{50} is the centration of the competitor that competes for half the cific binding.

Fig. 42

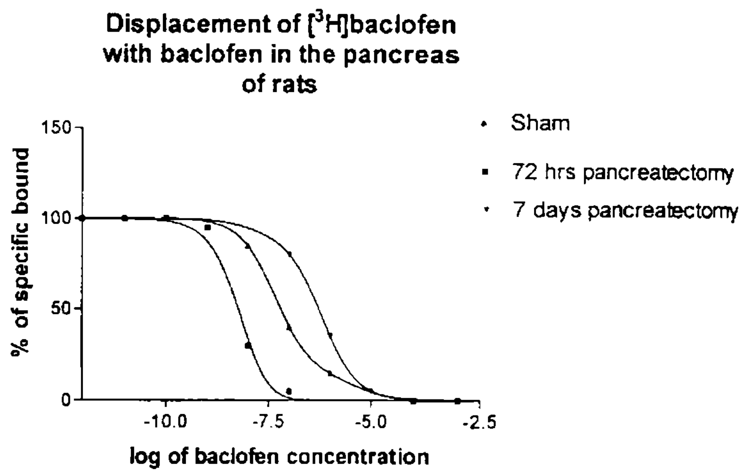
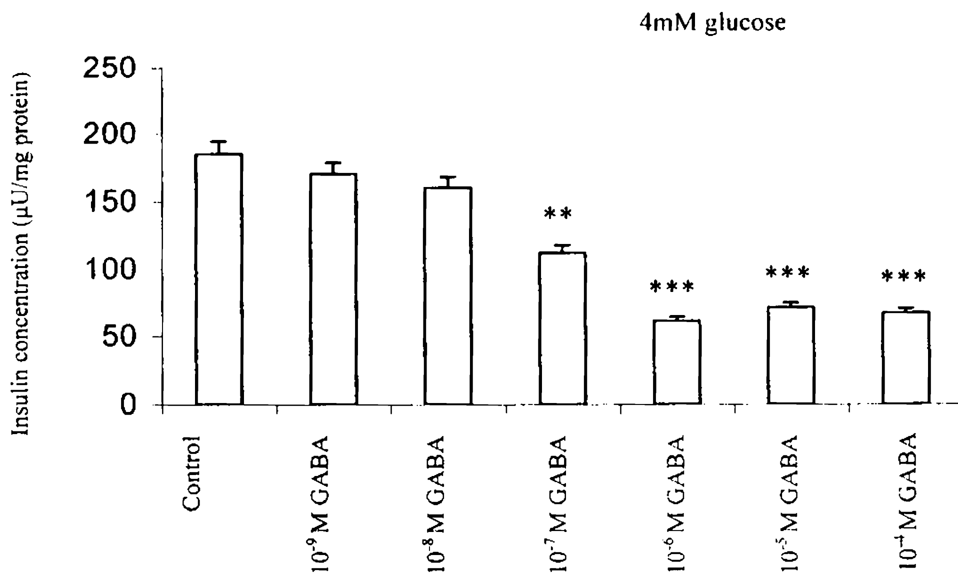


Figure- 43

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

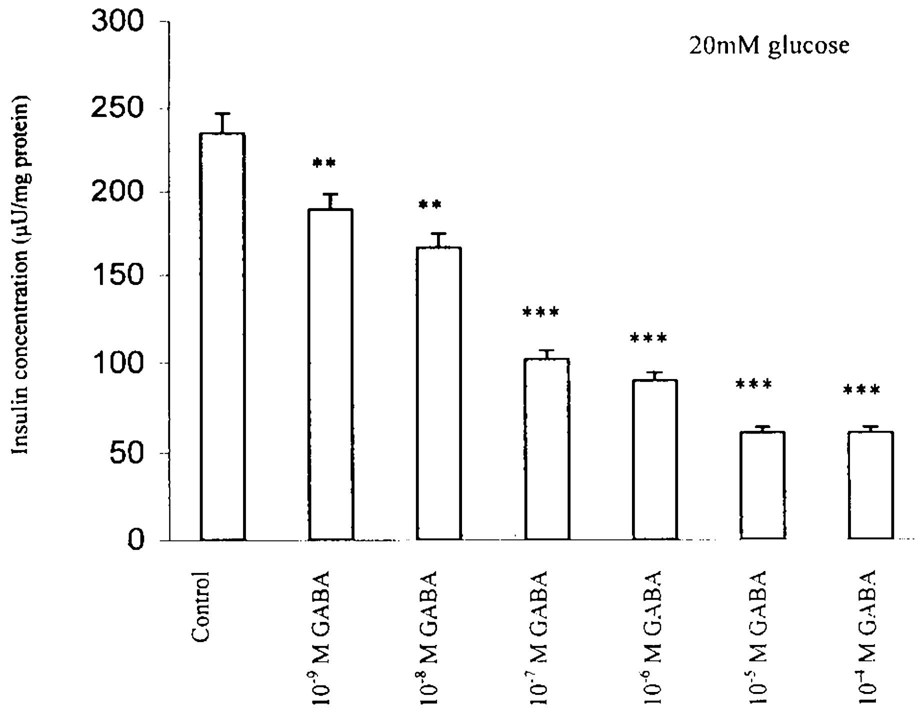


Values are mean ± S.E.M. of 4-6 separate experiments
** $p<0.01$ when compared with control
*** $p<0.001$ when compared with control

Islets were incubated in KRB buffer with different concentrations of GABA (10^{-9} to 10^{-4}M) and 4mM glucose for one hour.

Figure- 44

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

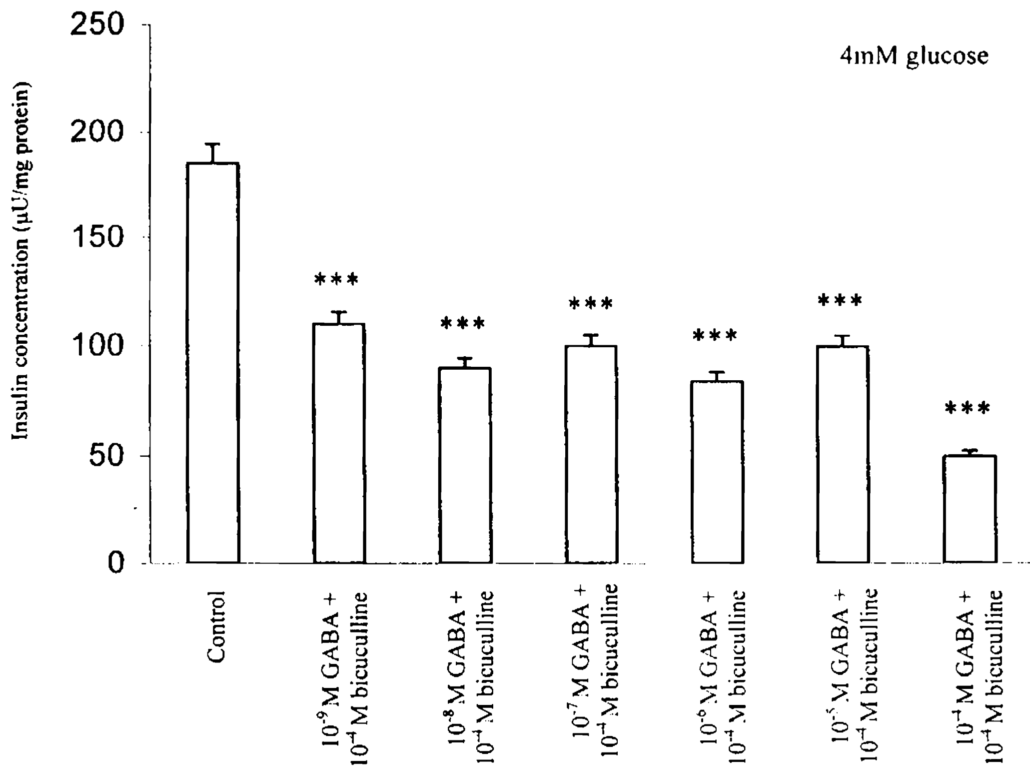


Values are mean ± S.E.M. of 4-6 separate experiments
** $p<0.01$ when compared with control
*** $p<0.001$ when compared with control

Islets were incubated in KRB buffer with different concentrations of GABA (10^{-9} to 10^{-4}M) (10^{-9} to 10^{-4}M) and 20mM glucose for one hour.

Figure- 45

Effect of GABA in presence of $GABA_A$ antagonist bicuculline on insulin secretion in pancreatic islets in one hour *in vitro* culture



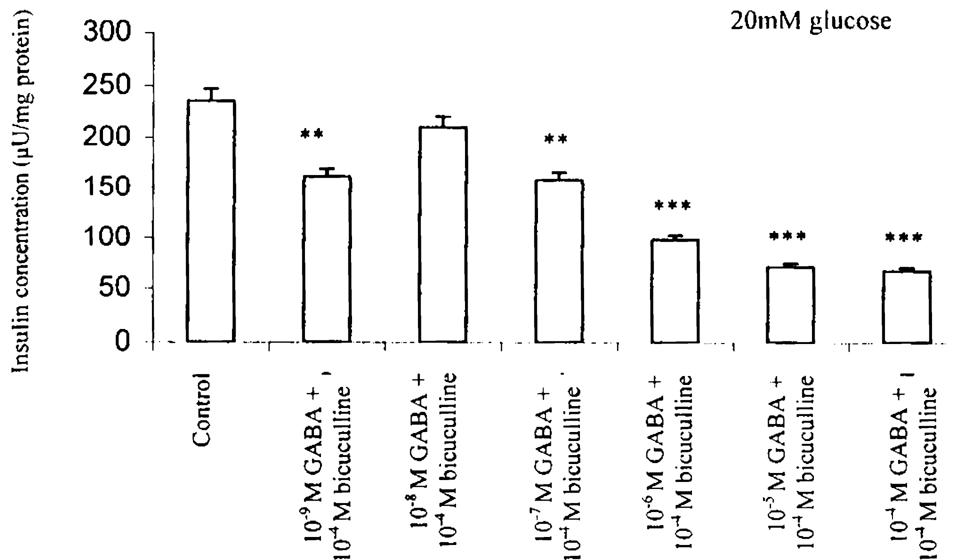
Values are mean ± S.E.M. of 4-6 separate experiments

*** $p<0.001$ when compared with control

Islets were incubated in KRB buffer with different concentrations of GABA (10^{-9} to 10^{-4}M), 10^{-4}M bicuculline and 4mM glucose for one hour.

Figure- 46

Effect of GABA in presence of $GABA_A$ antagonist bicuculline on insulin secretion in pancreatic islets in one hour *in vitro* culture

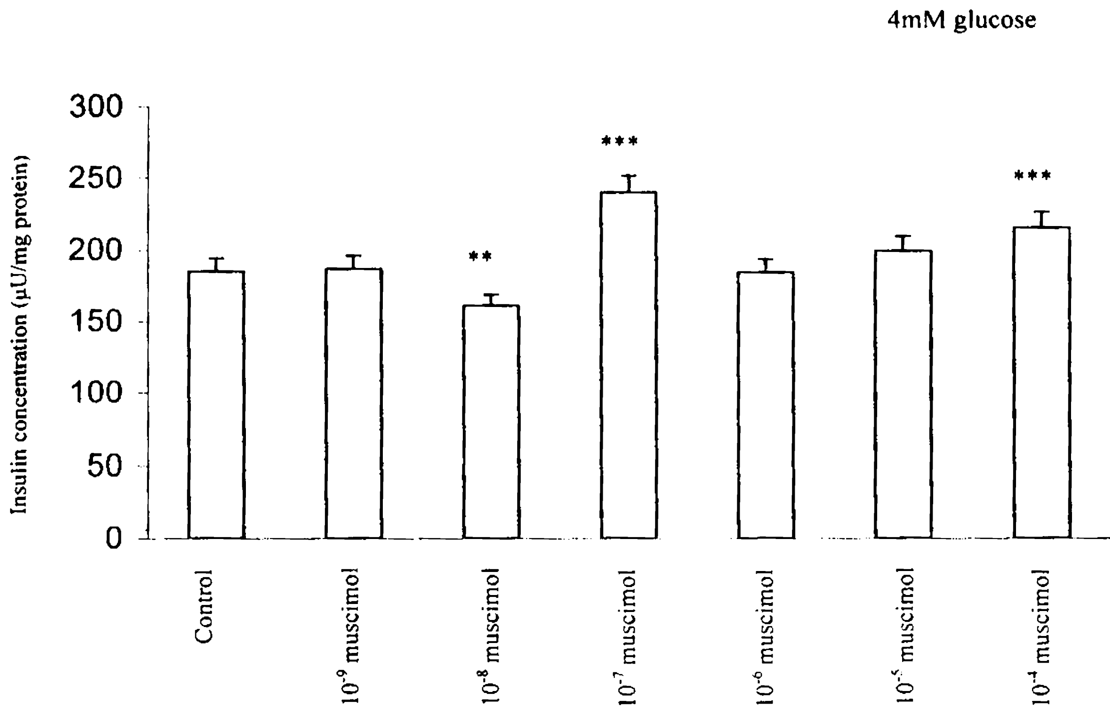


Values are mean ± S.E.M. of 4-6 separate experiments
** $p<0.01$ when compared with control
*** $p<0.001$ when compared with control

Islets were incubated in KRB buffer with different concentrations of GABA (10^{-9} to 10^{-4}M), 10^{-4}M bicuculline and 20mM glucose for one hour.

Figure- 47

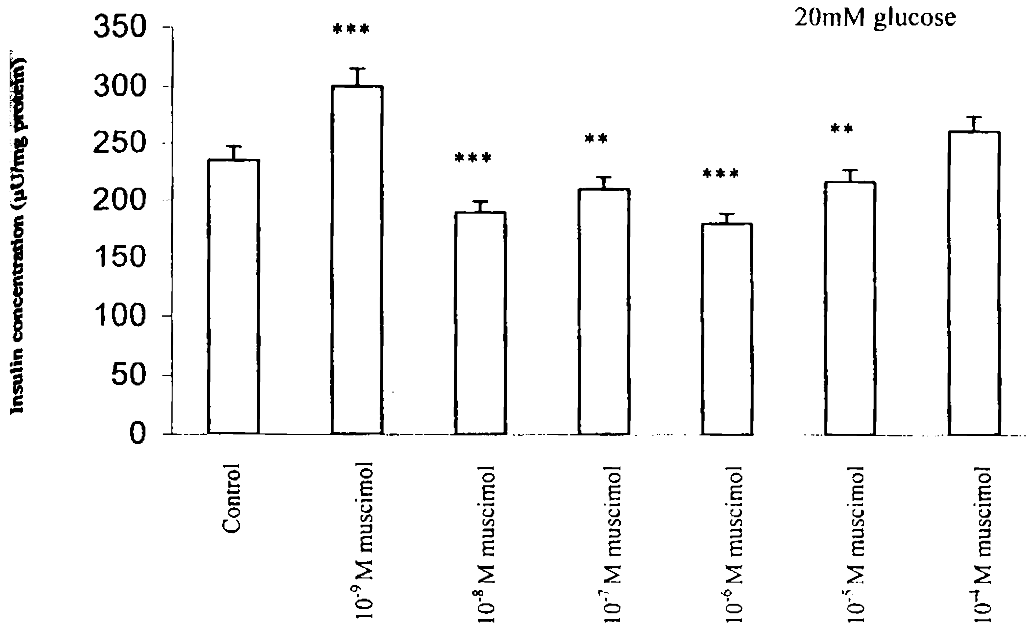
Effect of $GABA_A$ agonist muscimol on insulin secretion in pancreatic islets in one hour *in vitro* culture



Values are mean ± S.E.M. of 4-6 separate experiments
** $p<0.01$ when compared with control
*** $p<0.001$ when compared with control

Islets were incubated in KRB buffer with different concentrations of muscimol (10^{-9} to 10^{-4}M) and 4mM glucose for one hour.

Effect of $GABA_A$ agonist muscimol on insulin secretion in pancreatic islets in one hour *in vitro* culture

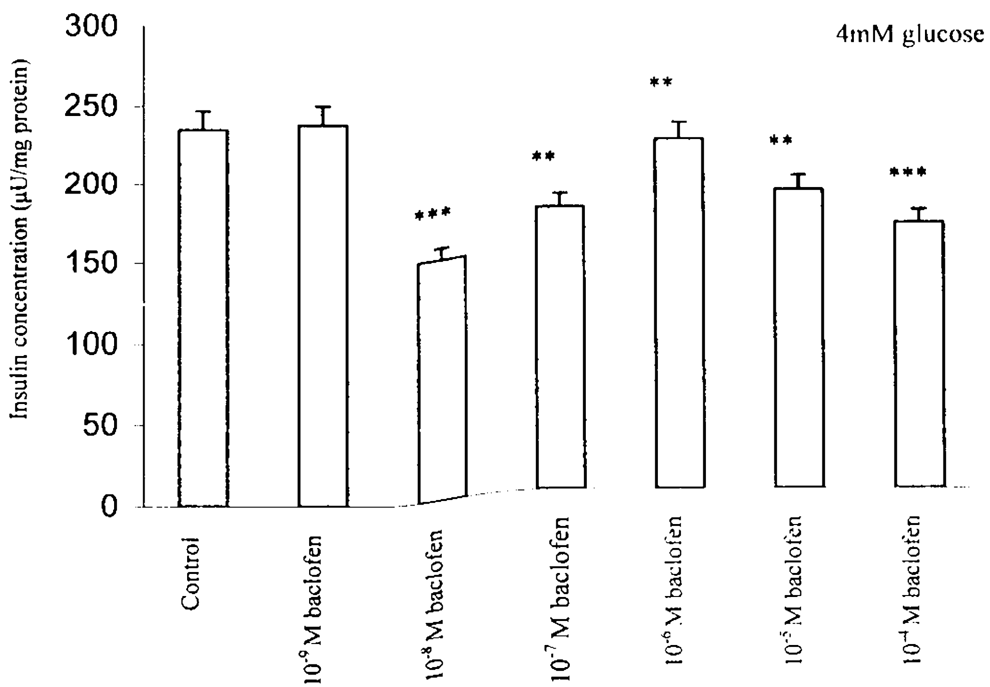


Values are mean ± S.E.M. of 4-6 separate experiments
** $p<0.01$ when compared with control
*** $p<0.001$ when compared with control

Islets were incubated in KRB buffer with different concentrations of muscimol (10^{-9} to 10^{-4}M) and 20mM glucose for one hour.

Figure- 49

Effect of GABA$_B$ agonist baclofen on insulin secretion in pancreatic islets in one hour *in vitro* culture

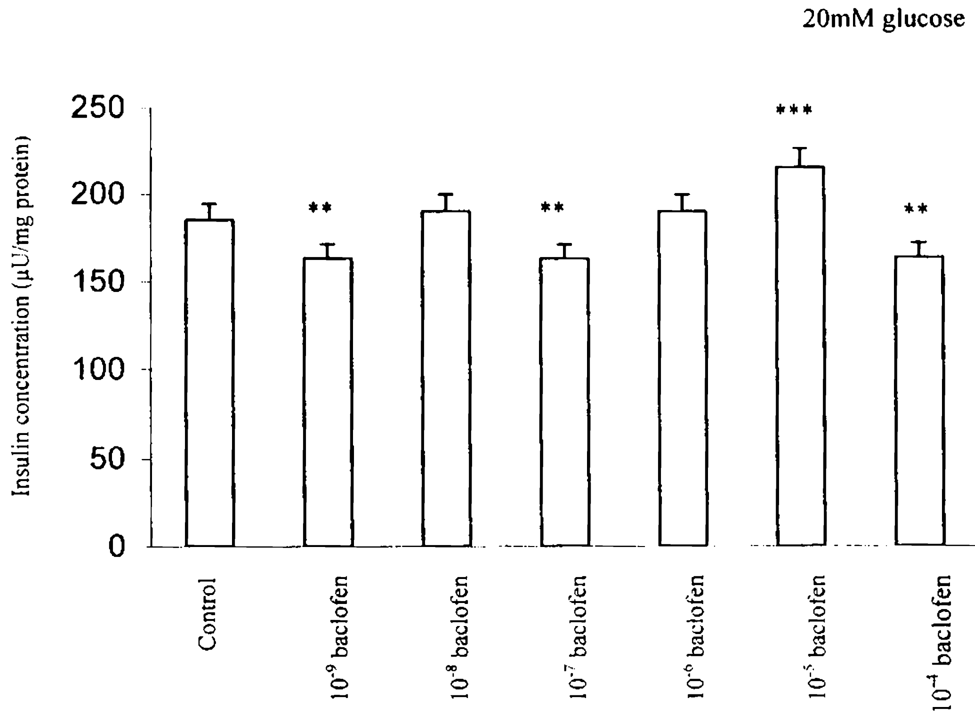


Values are mean ± S.E.M. of 4-6 separate experiments
** $p<0.01$ when compared with control
*** $p<0.001$ when compared with control

Islets were incubated in KRB buffer with different concentrations of baclofen (10^{-9} to 10^{-4}M) and 4mM glucose for one hour.

Figure- 50

Effect of $GABA_B$ agonist baclofen on insulin secretion in pancreatic islets in one hour *in vitro* culture

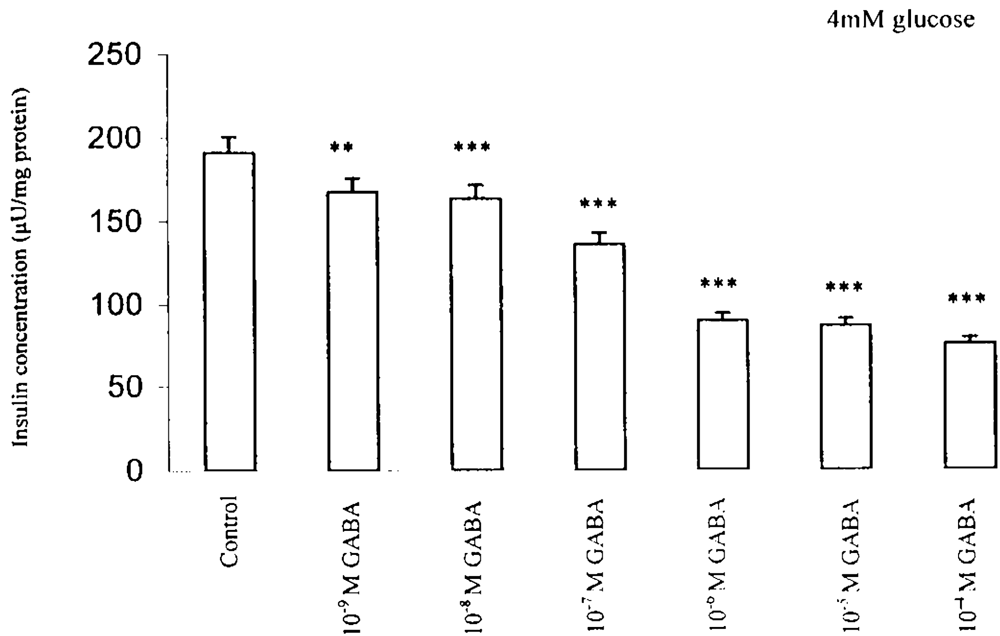


Values are mean ± S.E.M. of 4-6 separate experiments
** $p<0.01$ when compared with control
*** $p<0.001$ when compared with control

Islets were incubated in KRB buffer with different concentrations of baclofen (10^{-9} to 10^{-4}M) and 20mM glucose for one hour.

Figure- 51

Effect of GABA on insulin secretion in pancreatic islets in 24 hours *in vitro* culture

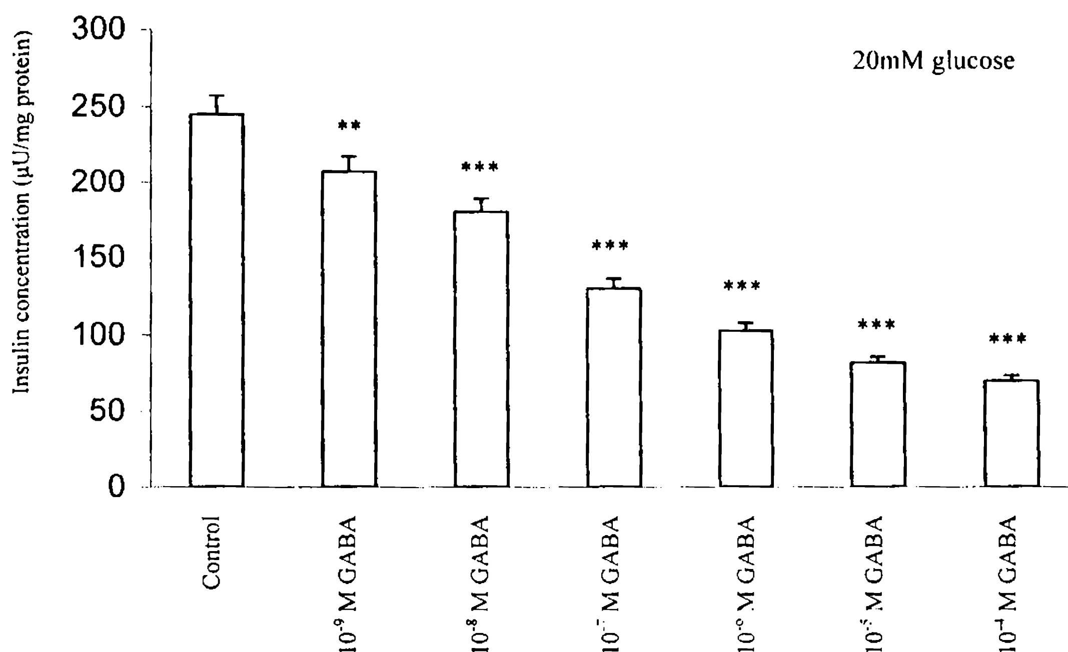


Values are mean ± S.E.M. of 4-6 separate experiments
** $p<0.01$ when compared with control
*** $p<0.001$ when compared with control

Islets were incubated in RPMI-1640 medium with different concentrations of GABA (10^{-9} to 10^{-4}M) and 4mM glucose for 24 hours.

Figure- 52

Effect of GABA on insulin secretion in pancreatic islets in 24 hours *in vitro* culture

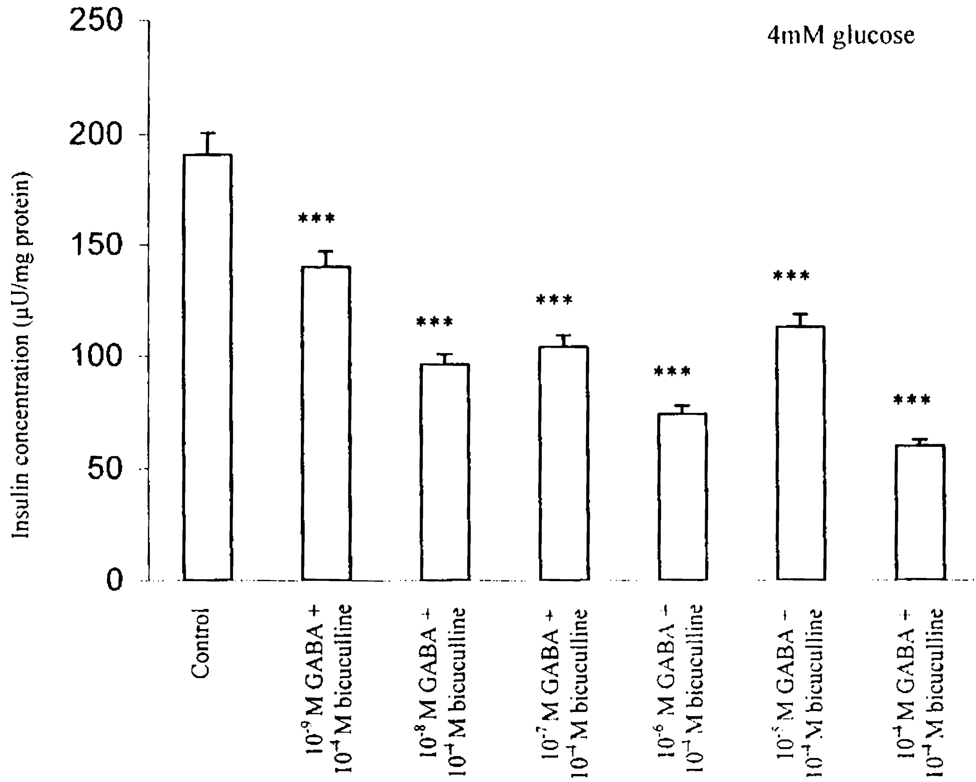


Values are mean ± S.E.M. of 4-6 separate experiments
** $p<0.01$ when compared with control
*** $p<0.001$ when compared with control

Islets were incubated in RPMI-1640 medium with different concentrations of GABA (10^{-9} to 10^{-4}M) and 20mM glucose for 24 hours.

Figure- 53

Effect of GABA in presence of $GABA_A$ antagonist bicuculline on insulin secretion in pancreatic islets in 24 hours *in vitro* culture

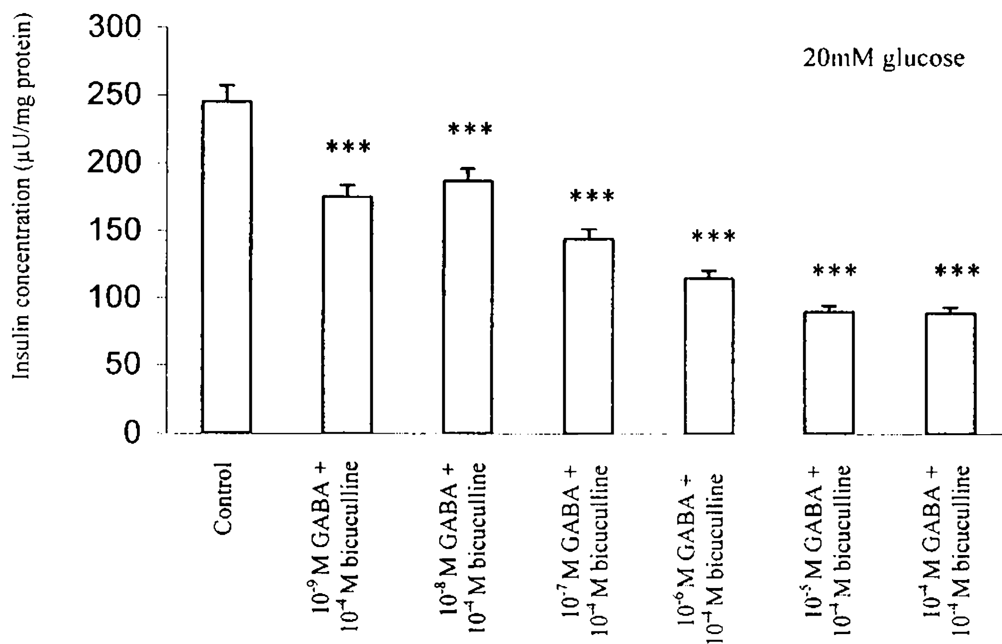


Values are mean ± S.E.M. of 4-6 separate experiments
*** $p<0.001$ when compared with control

Islets were incubated in RPMI-1640 medium with different concentrations of GABA, 10^{-4}M bicuculline (10^{-9} to 10^{-4}M) and 4mM glucose for 24 hours.

Figure- 54

Effect of GABA in presence of $GABA_A$ antagonist bicuculline on insulin secretion in pancreatic islets in 24 hours *in vitro* culture

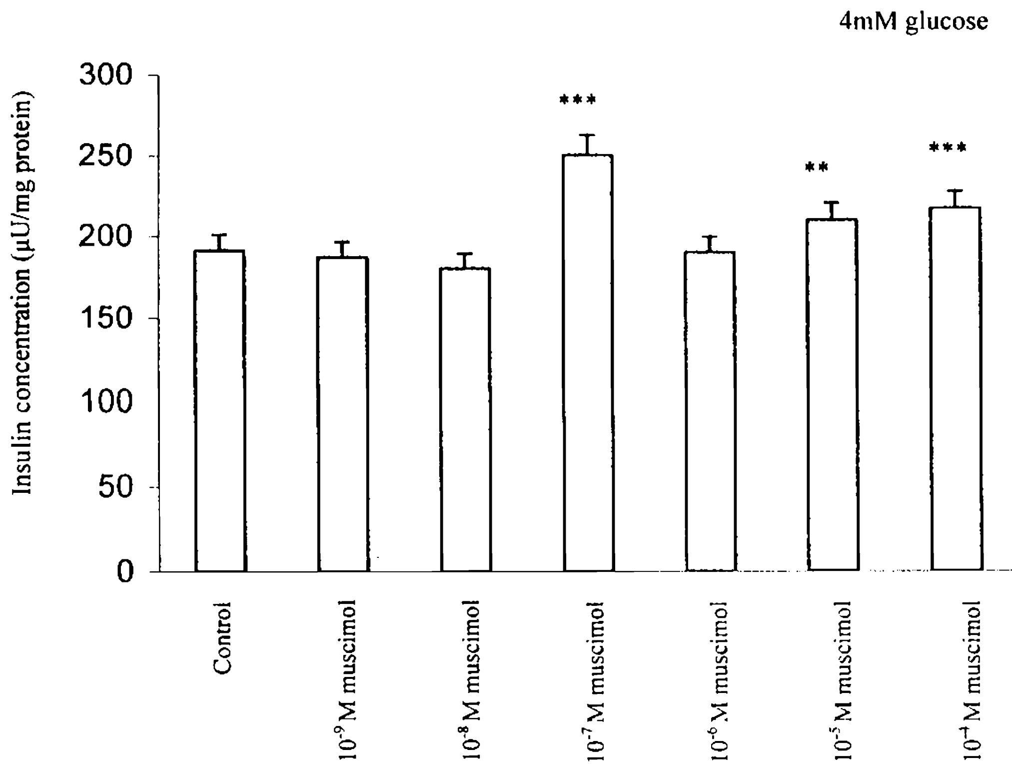


Values are mean ± S.E.M. of 4-6 separate experiments
*** $p<0.001$ when compared with control

Islets were incubated in RPMI-1640 medium with different concentrations of GABA, 10^{-4}M bicuculline (10^{-9} to 10^{-4}M) and 4mM glucose for 24 hours

Figure- 55

Effect of $GABA_A$ agonist muscimol on insulin secretion in pancreatic islets in 24 hours *in vitro* culture

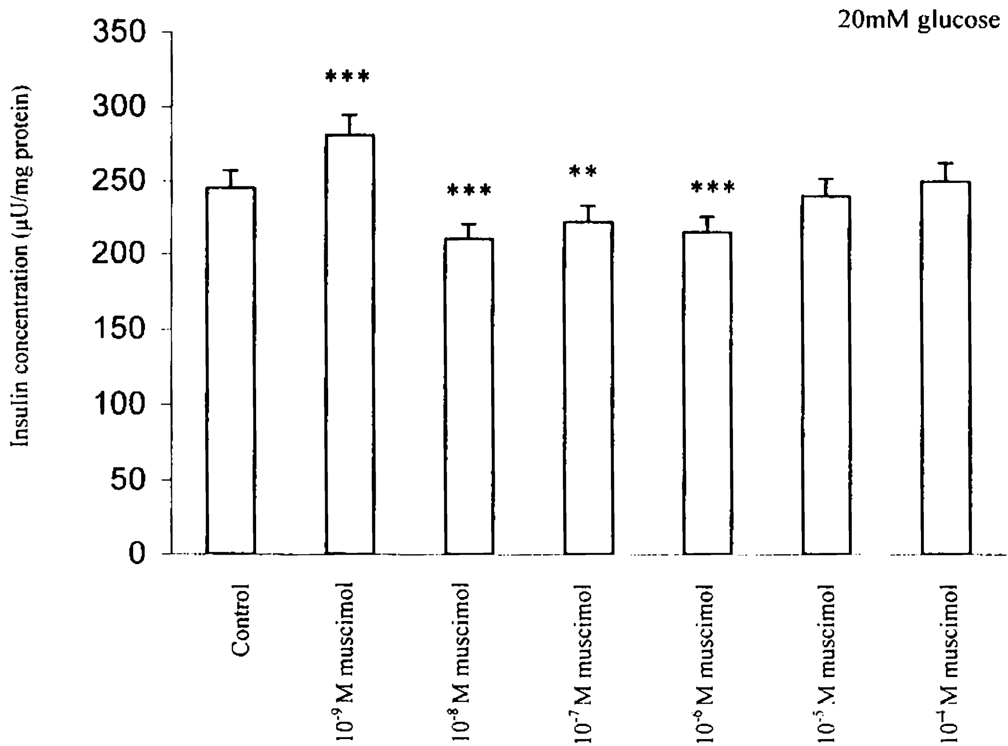


Values are mean ± S.E.M. of 4-6 separate experiments
** $p<0.01$ when compared with control
*** $p<0.001$ when compared with control

Islets were incubated in RPMI-1640 medium with different concentrations of muscimol (10^{-9} to 10^{-4}M) and 4mM glucose for 24 hours.

Figure- 56

Effect of $GABA_A$ agonist muscimol on insulin secretion in pancreatic islets in 24 hours *in vitro* culture

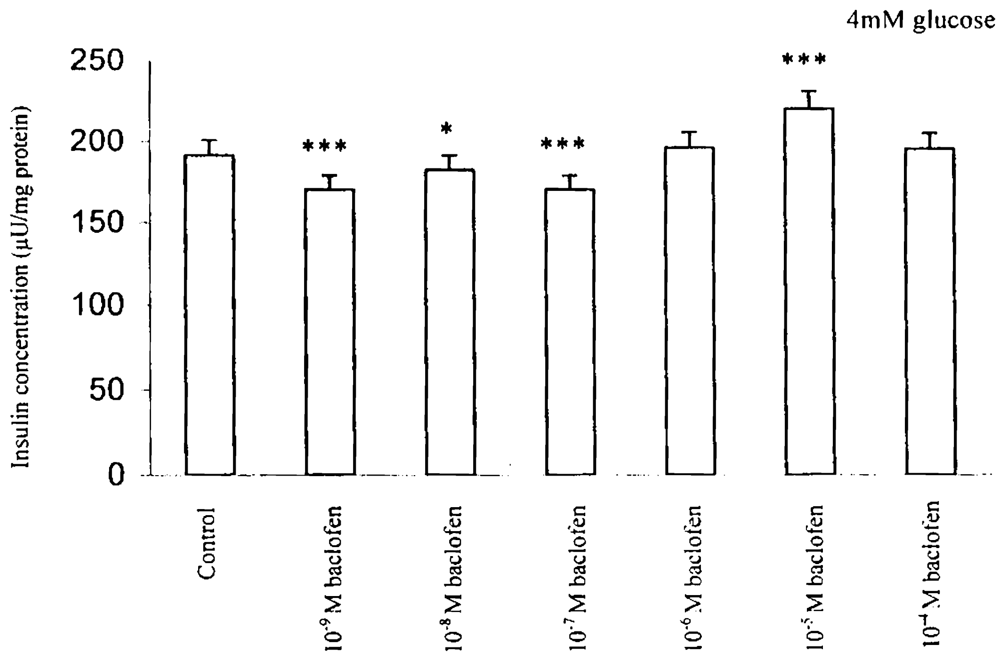


Values are mean ± S.E.M. of 4-6 separate experiments
** $p<0.01$ when compared with control
*** $p<0.001$ when compared with control

Islets were incubated in RPMI-1640 medium with different concentrations of muscimol (10^{-9} to 10^{-4}M) and 4mM glucose for 24 hours.

Figure- 57

Effect of $GABA_B$ agonist baclofen on insulin secretion in pancreatic islets in 24 hours *in vitro* culture

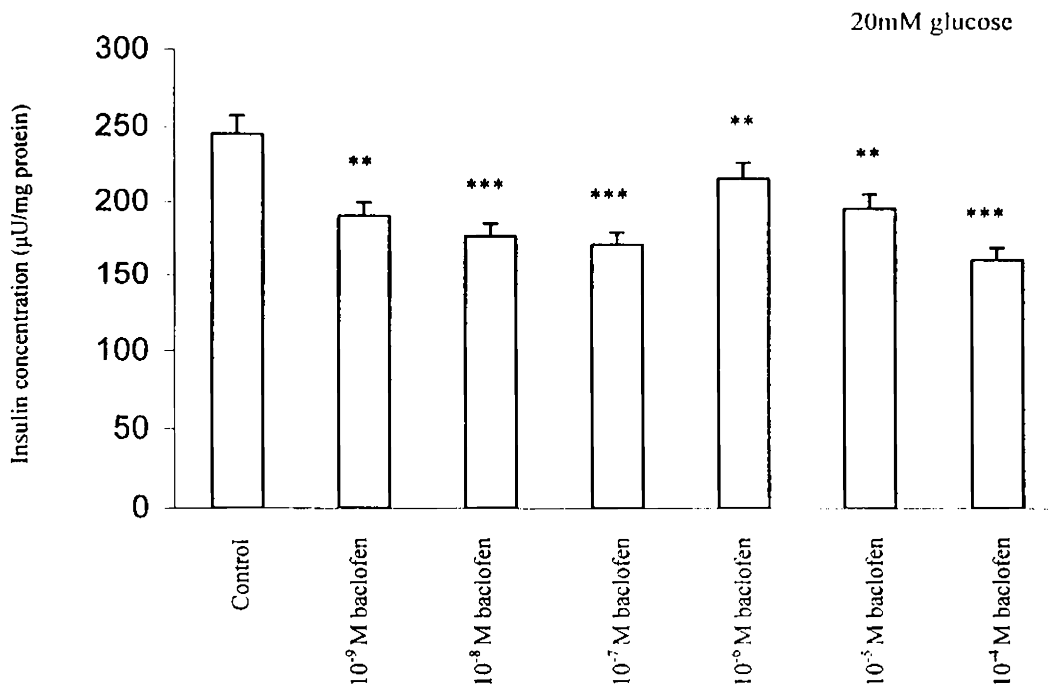


Values are mean ± S.E.M. of 4-6 separate experiments
* $p<0.05$ when compared with control
*** $p<0.001$ when compared with control

Islets were incubated in RPMI-1640 medium with different concentrations of baclofen (10^{-9} to 10^{-4}M) and 4mM glucose for 24 hours.

Figure- 58

Effect of $GABA_B$ agonist baclofen on insulin secretion in pancreatic islets in 24 hours *in vitro* culture

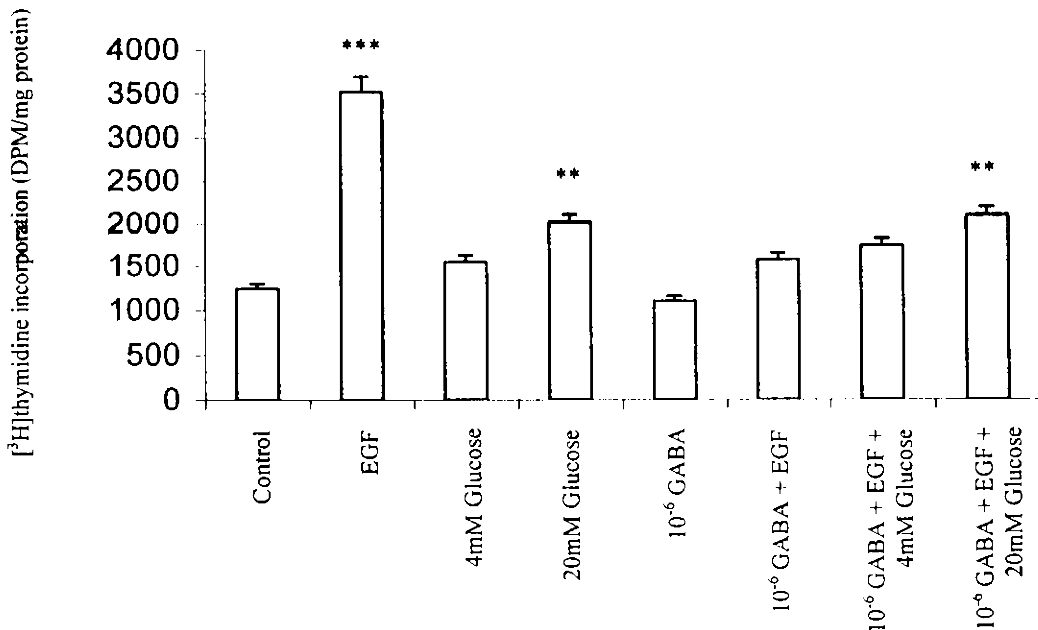


Values are mean ± S.E.M. of 4-6 separate experiments
** $p<0.01$ when compared with control
*** $p<0.001$ when compared with control

Islets were incubated in RPMI-1640 medium with different concentrations of baclofen (10^{-9} to 10^{-4}M) and 20mM glucose for 24 hours.

Figure - 59

Effect of GABA on glucose induced DNA synthesis in the pancreatic islets of rats

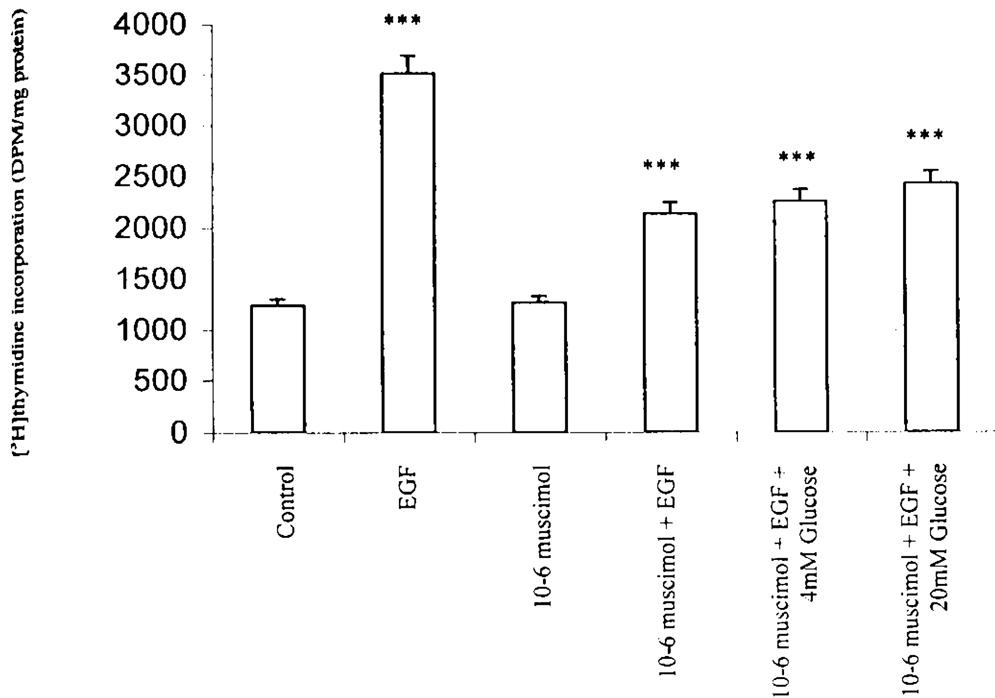


** $p<0.01$ when compared with control
*** $p<0.001$ when compared with control

EGF- epidermal growth factor
Islets were incubated in RPMI-1640 medium with EGF, GABA, 4mM glucose, 20mM glucose and 2.5 μCi of [^{3}H]thymidine.

Figure - 60

Effect of muscimol on glucose induced DNA synthesis in the pancreatic islets of rats



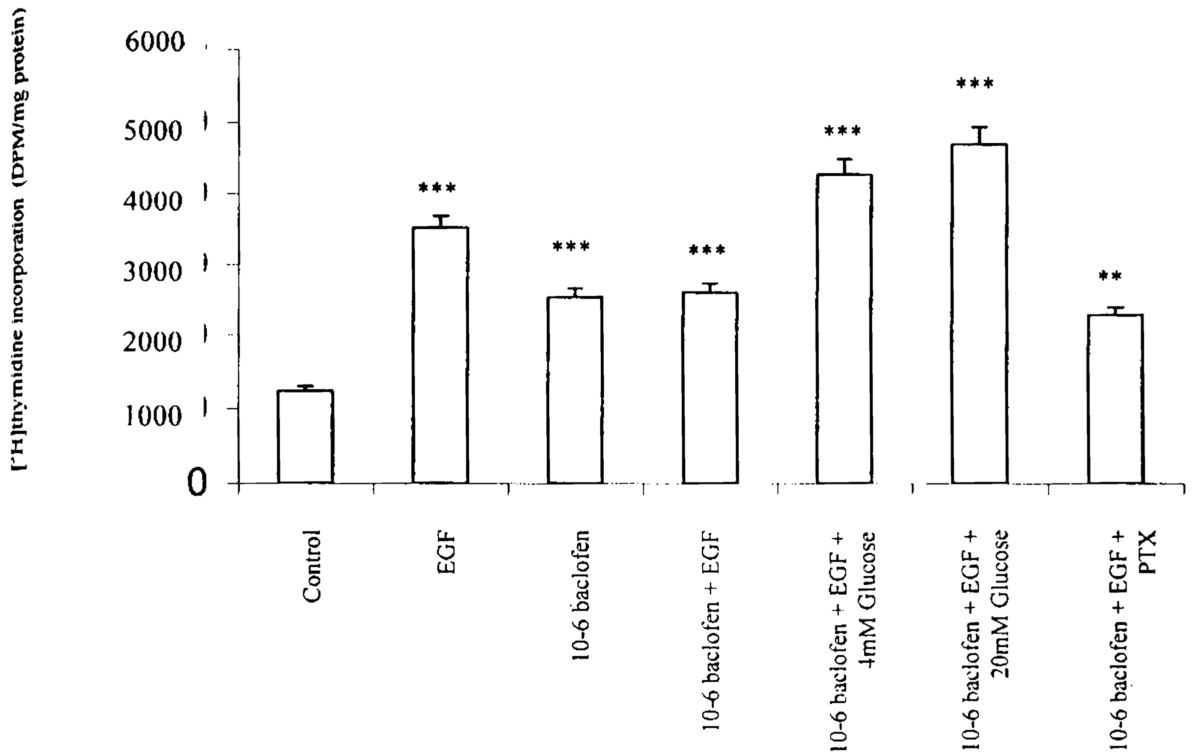
** $p<0.01$ when compared with control
*** $p<0.001$ when compared with control

EGF- epidermal growth factor

Islets were incubated in RPMI-1640 medium with EGF, 10^{-6}muscimol, 4mM glucose, 20mM glucose and 2.5 μCi of [^{3}H]thymidine.

Figure - 61

Effect of baclofen on glucose induced DNA synthesis in the pancreatic islets of rats



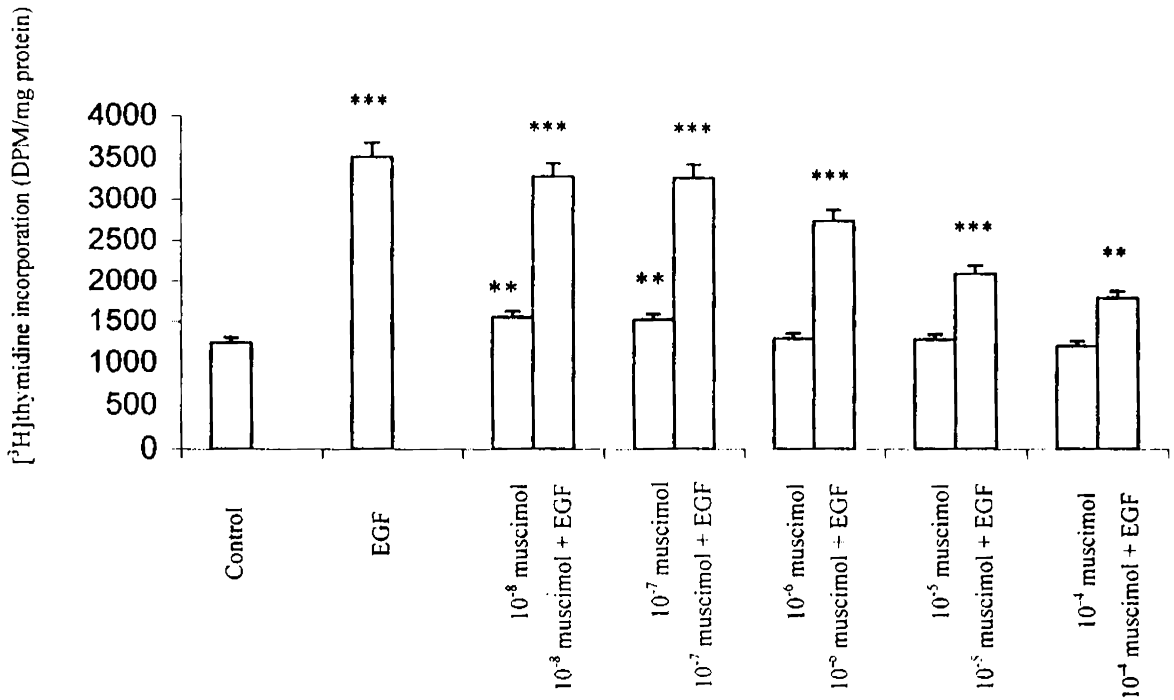
** $p<0.01$ when compared with control, *** $p<0.001$ when compared with control

EGF- epidermal growth factor, PTX- pertussis toxin

Islets were incubated in RPMI-1640 medium with EGF, 10-6M baclofen, 4mM glucose, 20mM glucose, pertussis toxin and 2.5 µCi of [^{3}H]thymidine.

Figure - 62

Dose dependent effect of pancreatic islet DNA synthesis to muscimol



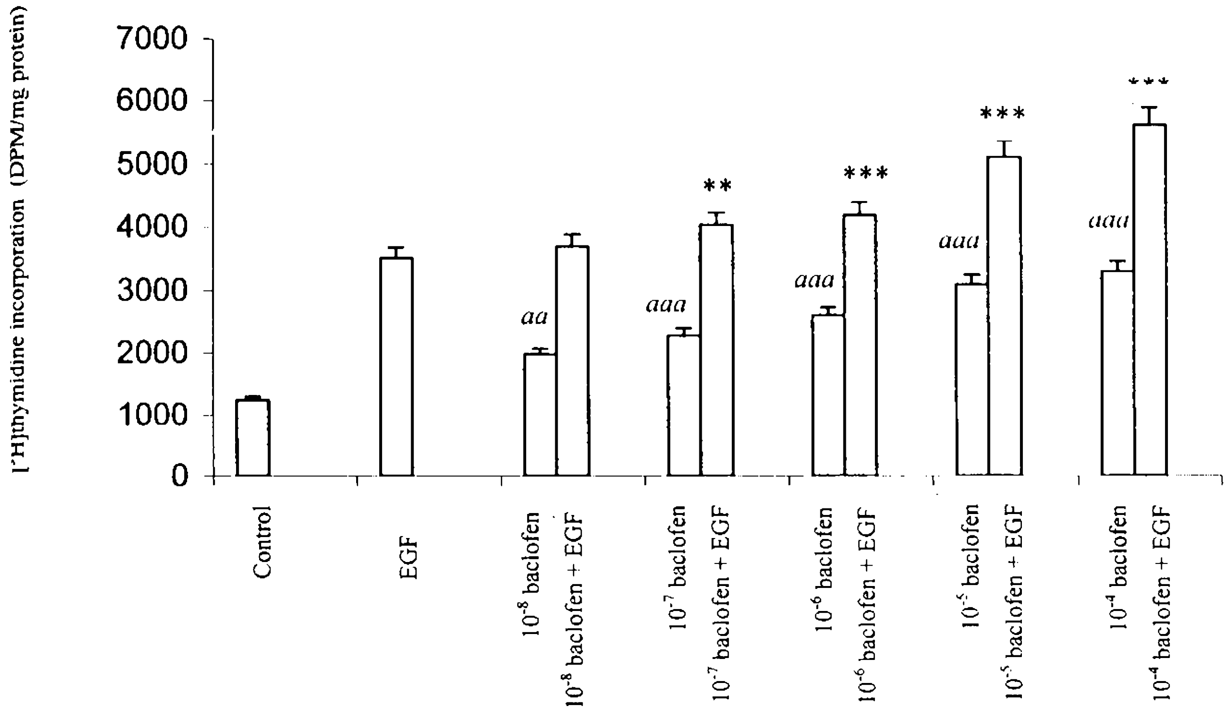
** $p<0.01$ when compared with control
*** $p<0.001$ when compared with control

EGF- epidermal growth factor

Islets were incubated in RPMI-1640 medium with EGF, different concentrations of muscimol and 2.5 μCi of [^{3}H]thymidine.

Figure - 63

Dose dependent effect of pancreatic islet DNA synthesis to baclofen



** $p<0.01$ when compared with control, *aa* $p<0.01$ when compared with control, *** $p<0.001$ when compared with control, *aaa* $p<0.001$ when compared with control

EGF- epidermal growth factor

Islets were incubated in RPMI-1640 medium with EGF, different concentrations of baclofen and 2.5 μCi of [^{3}H]thymidine.

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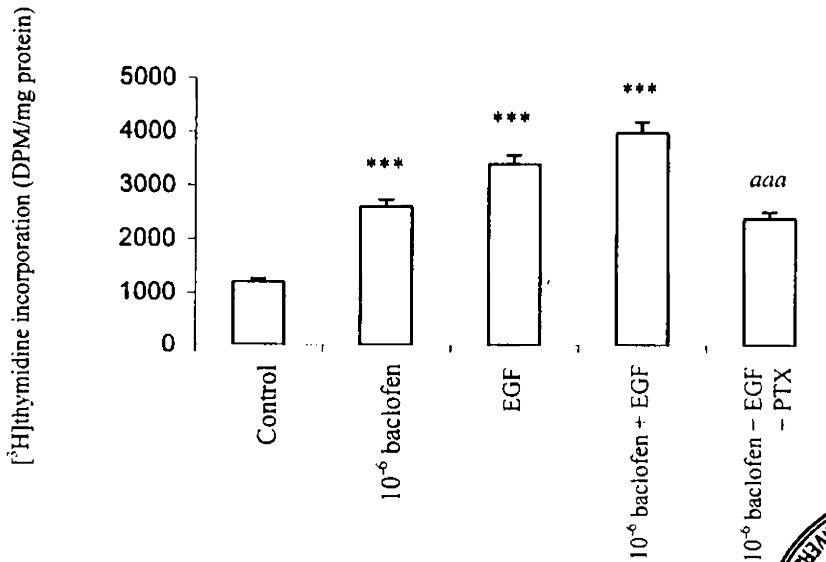
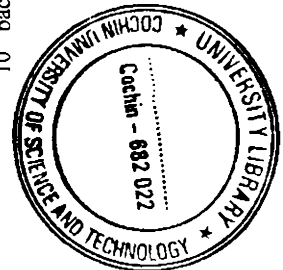
Figure - 64

Effect of pertussis toxin on DNA synthesis in the pancreatic islets of rats





*** $p<0.001$ when compared with control
aaa $p<0.001$ when compared with EGF

EGF- epidermal growth factor
PTX- pertussis toxin
Islets were incubated in RPMI-1640 medium with EGF, different concentrations of baclofen, pertussis toxin and 2.5 μCi of [^{3}H]thymidine.