SEROTONIN RECEPTOR GENE EXPRESSION AND INSULIN FUNCTION IN STREPTOZOTOCIN INDUCED DIABETIC RATS

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By

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CERTIFICATE

This is to certify that the thesis entitled **"SEROTONIN RECEPTOR GENE EXPRESSION AND INSULIN FUNCTION IN STREPTOZOTOCIN INDUCED DIABETIC RATS"** is a bonafide record of the research work carried out by **Mr JACKSON JAMES** under my guidance and supervision in the Department of Biotechnology, Cochin University of Science and Technology and that no part thereof has been presented for the award of any other degree.

Cochin - 682 022
Date 16 September, 1999

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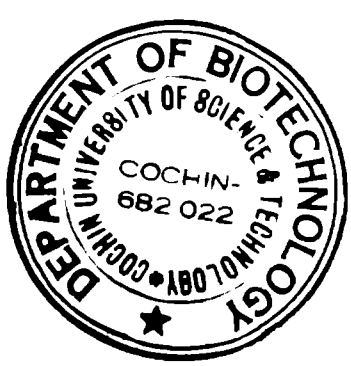
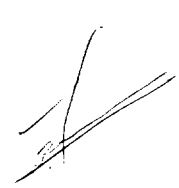


DECLARATION

I hereby declare that this thesis entitled **"SEROTONIN RECEPTOR GENE EXPRESSION AND INSULIN FUNCTION IN STREPTOZOTOCIN INDUCED DIABETIC RATS"** is based on the orginal research carried out by me at the Department of Biotechnology, Cochin University of Science and Technology, under the guidance of Dr C.S. Paulose, Reader, Department of Biotechnology, and no part thereof has been presented for the award of any other degree, diploma, associateship or other similar titles or recognition.



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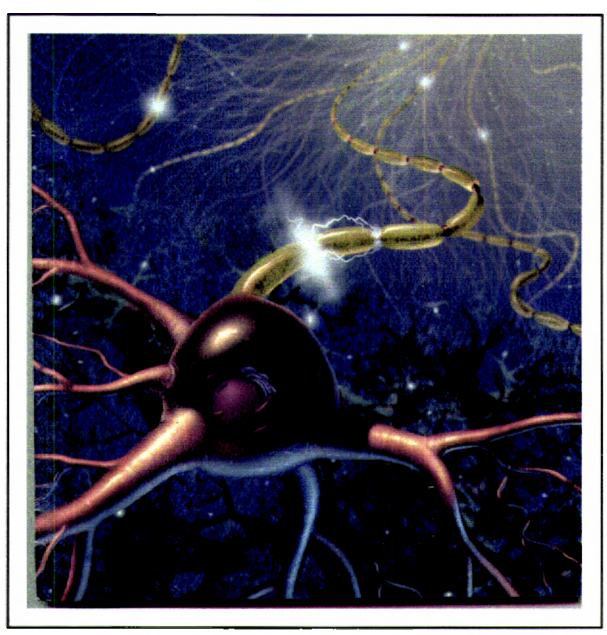


Dedicated to these wonderful living cells whose computing abilities and potentials are unparalleled by the most modern supercomputers. This is a modest approach in understanding the abilities of these cells and its constituents in the regulation of insulin secretion.

ABBREVIATIONS USED IN THE TEXT

AD2	Acivation Domain 2
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BBB	Blood-brain-barrier
bHLH	basic Helix-loop-helix protein
B_{max}	Maximal binding
BS	Brain stem
C/EBP β	CCAAT /Enhancer binding protein β
cAMP	cyclic Adenosine mono phosphate
CC	Cerebral cortex
CNS	Central nervous system
CSF	Cerbro spinal fluid
DA	Dopamine
DEPC	Di ethyl pyro carbonate
DOI	1-(2,5-di-methoxy-4-iodophenyl)-2-aminopropane
DTT	Dithiothreitol
ECD	Electro chemical detector
EPI	Epinephrine
GABA	Gamma aminobutyric acid
GOD	Glucose oxidase
Gpp[NH]p	5'-Guanylyl-imidodiphosphate
GTP	Guanosine triphosphate
5-HIAA	5-Hydroxyindole acetic acid
HPLC	High performance liquid chromatography
5-HT	5-Hydroxy tryptamine
5-HTP	5-hydroxy tryptophan
HVA	Homovanillic acid
HYPO	Hypothalamus
i.p	Intraperitoneally
IAPP	Islet amyloid polypeptide

K_d	Dissociation constant
MIF	Macrophage migration inhibiting factor
mRNA	messenger Ribonucleic acid
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NE	Norepinephrine
NMN	Normetanephrine
8-OH-DPAT	8-Hydroxy-2(di-n-propylamino)-tetralin
p	Level of significance
p-CPA	para-Chlorophenylalanine
PEG	Polyethylene glycol
Pi	Inorganic phosphate
POD	Peroxidase
RIA	Radioimmuno assay
RT-PCR	Reverse-transcription-polymerase chain reaction
S.E.M.	Standard error of mean
SDS-PAGE	Sodium dodecyl sulphate-poly acrylamide gel electrophoresis
STZ	Streptozotocin
T_3	Tri iodothyronine
T_4	Thyroxine
TRH	Thyrotropin releasing hormone
TSH	Thyroid stimulating hormone
VMH	Ventro medial hypothalamus

CONTENTS

INTRODUCTION

1. INTRODUCTION

Diabetes is a metabolic disorder associated with insulin deficiency, which not only affects the metabolism of carbohydrates but is also associated with central as well as peripheral neuropathy There are various factors, which regulate insulin secretion from the pancreatic β-cells, such as metabolic substrates, hormones and neurotransmitters. The central nervous system (CNS) neurotransmitters such as norepinephrine and serotonin play an important role in glucose homeostasis. These neurotransmitters mediate rapid intracellular communication within the nervous system by interacting with cell surface receptors. These receptors often trigger second messenger signalling pathways. Although neurotransmitter receptors by definition have been restricted to the nervous system, these receptors and second messenger systems have been observed in both neural and non-neural cells (Julius *et al.*, 1989).

Since the early seventies, the hypothesis for a control of circulating glucose and insulin levels by serotonin (5-HT) systems has been the matter of numerous works. There are reports on reductions in central nervous system (CNS) 5-hydroxytryptamine synthesis and turn over in chronically hyperglycaemic rats (Bellush & Reid, 1991, Trulson *et al.*, 1986). It has been well documented that long term hyperglycaemia in diabetic animals can lead to decreased functions of central 5-HT neurons leading to reduced brain tryptophan, 5-HT and 5-hydroxy indole acetic acid (5-HIAA). This decrease in brain 5-HT is due to the reduced uptake of tryptophan into the brain. One of the main determinants of brain tryptophan content is the circulating insulin level. An increase in the level of insulin can result in decreased plasma concentrations of large neutral amino acids, which compete with tryptophan for uptake into the brain. Streptozotocin (STZ) selectively destroys pancreatic β-cells and causes hypoinsulinemia leading to hyperglycaemia.

The effect of serotonin is mediated in different tissues by different subclasses of serotonin (5-HT) receptors, each of which are coded by a distinct gene and possess distinct pharmacological properties and physiological functions (Hoyer *et al.*, 1991). Moreover, serotonin receptor subtypes couple to different intracellular signalling systems. In neurons that express the 5-HT_{2A} receptors, receptor activation is likely to generate inositol polyphosphates that release intracellular calcium ions (Hoyer & Shoeffeter, 1988b). The

5-HT_{1A} receptors mediate inhibition of adenylate cyclase activity through G-protein coupling (Shenker *et al.*, 1987; Shenker *et al.*, 1985; Shenker *et al.*, 1983). Serotonin receptor subtypes, including 5-HT_{1A}, 1b, 1c, 5-HT_2 and 5-HT_3, have been defined on the basis of their pharmacological properties (Hoyer & Shoeffeter, 1988b). The existence of multiple receptor subtypes provides one mechanism by which a single neurotransmitter can elicit distinct cellular responses. The association of individual receptor subtypes with different G proteins and different signalling systems can achieve the variation in cellular response. Further flexibility is provided by the ability of distinct receptors for the same ligand to activate or inhibit the same second messenger system (Chung *et al.*, 1988).

Neurotransmitter changes in the hypothalamus lead to impairment of the hypothalamic-pituitary-end organ axis. The secretion of adrenocorticotropin, growth hormone, prolactin, thyroid stimulating hormone and the gonadotropins by the pituitary is governed by releasing factors from the hypothalamus. Regulation of the release factors from the hypothalamus involves complex neural circuit in which the serotonergic neurons represent one link in the control mechanism (Krulich, 1979). Hypothalamo-pituitary-thyroid-response, that is, the secretion of thyroid stimulating hormone (TSH) is directly controlled by two factors, a negative feed back signal indicating serum thyroid status and a stimulatory factor, thyrotropin releasing hormone (TRH) secreted by the hypothalamus (Chen & Ramirez, 1981). The postulate that 5-HT neurons stimulate TSH secretion in rats is supported by the observation that injection of 5-HT into the third ventricle caused rapid increase in serum TSH and the effect was completely reversed by pre-treatment of rats with cyproheptadine, a serotonin receptor antagonist. Dakshinamurti *et al.*, (1985) have reported that pyridoxine-deficiency can lead to hypothyroidism accompanied by decreased level of hypothalamic serotonin without any change in brain norepinephrine and dopamine. This decreased serotonin reduces the synthesis and release of TSH from the pituitary through TRH secretion (Dakshinamurti *et al.*, 1986). Smythe *et al.*, (1982) have also reported that TSH release is controlled by 5-HT neuronal activity

Recent studies have established a functional correlation of serotonergic and adrenergic function in the brain regions with insulin secretion in diabetic rats (Vahabzadeh *et al.*, 1995). Administration of 5-HT_{1A} agonist 8-OH-DPAT to conscious rats caused an

increase in blood glucose level. This increase in blood glucose is due to inhibition of insulin secretion by increased circulating EPI (Chaouloff *et al.*, 1990a; Chaouloff *et al.*, 1990d; Chaouloff & Jeanrenaud, 1987). The increase in EPI is brought about by increased sympathetic stimulation. This increase can lead to increased sympatho-medullary stimulation thereby inhibiting insulin release (Bauhelal & Mir, 1993, Bauhelal & Mir, 1990a; Chaouloff *et al.*, 1990d). Also, studies have shown that Gi protein in the liver has been decreased in diabetes which will increase gluconeogenesis and glycogenolysis thereby causing hyperglycaemia (Pennington, 1987). Serotonergic control is suggested to exert different effects on insulin secretion according to the activation of different receptor subclasses (Pontiroli *et al.*, 1975). In addition to this mechanism, the secretion of insulin is dependent on the turnover ratio of endogenous 5-hydroxy tryptophan (5-HTP) to 5-HT in the pancreatic islets (Jance *et al.*, 1980).

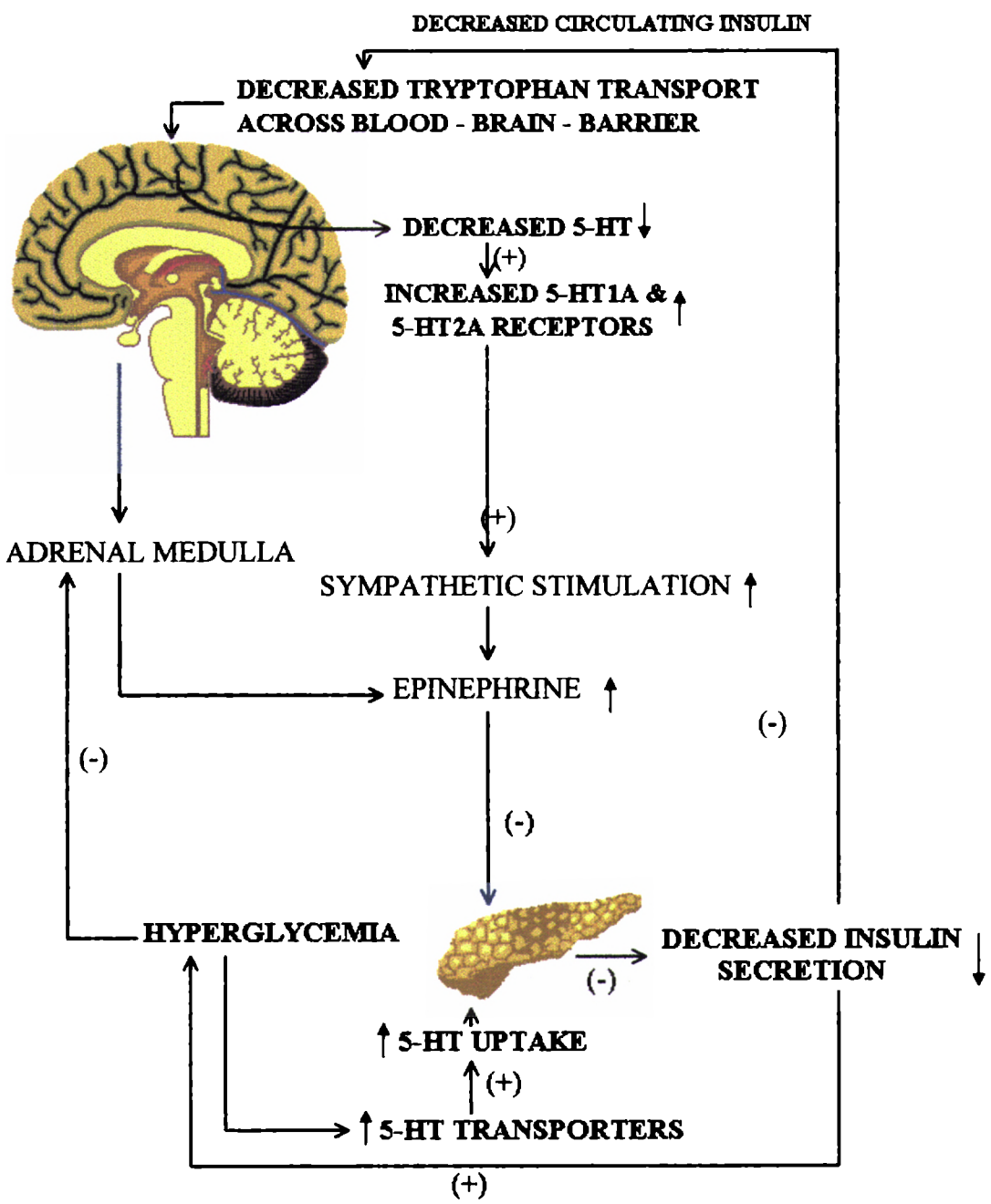
The reports so far stated does not explain the complete mechanism and the subclass of 5-HT receptors whose expression regulate insulin secretion in a diabetic state. Also, there is no report of a direct regulation of insulin secretion by 5-HT from the pancreatic islets even though there are reports stating that the pancreatic islets is a rich source of 5-HT (Bird *et al.*, 1980). Therefore, in the present study the mechanism by which 5-HT and its receptors regulate insulin secretion from pancreatic β-cells was investigated. Our results led to the following hypotheses by which 5-HT and its receptors regulate the insulin secretion.

- **Central nervous system control of insulin secretion:-** This pathway is triggered by a decrease in brain 5-HT content brought about by a decrease in transport of tryptophan across the blood-brain-barrier (BBB). This transport of tryptophan across the BBB depends on the circulating insulin and tryptophan levels. There are a number of reports which state that a tryptophan deficient diet can lead to decreased circulating tryptophan and decreased uptake of it into the brain leading to decreased brain 5-HT synthesis (Fernstrom & Fernstrom, 1995; Fernstrom, 1991, Fernstrom, 1979; Biggio *et al.*, 1974). This decreased brain 5-HT stimulates the over expression of 5-HT_{1A} and 5-HT_{2A} receptors in the brain region, which lead to sympathetic stimulation that inhibits insulin release. Once the circulating insulin content is reduced, it leads to an

increase in large neutral amino acids, which compete with tryptophan for uptake into brain.

- **Peripheral control of insulin secretion:-** This regulation occurs directly within the pancreatic β-cells. During diabetes the amount of 5-HT within the pancreatic islets increases. This excess 5-HT binds and down regulates the nuclear receptors and may directly alter the transcription of insulin gene from the β-cells.

SEROTONERGIC SYSTEM AND INSULIN REGULATION IN DIABETES MELLITUS



1.1 MAJOR OBJECTIVES

1. To study the changes in monoamines and their metabolites in cerebral cortex (CC), brain stem (BS), hypothalamus (Hypo) and pancreas by high performance liquid chromatography (HPLC) in control, diabetic and diabetic rats treated with insulin, tryptophan alone and in combination with insulin.

2. To study the role of brain serotonin and its receptors in insulin secretion from pancreatic β-cells.

3. To study the kinetic parameters of serotonin receptors with special emphasis on 5-HT_{1A} and 5-HT_{2A} receptors in brain regions and pancreatic islets of control, diabetic, diabetic + insulin, diabetic + tryptophan and diabetic + insulin + tryptophan treated rats.

4. To study alterations in G protein of 5-HT_{1A} receptors using Gpp[NH]p.

5. To establish a functional correlation of brain 5-HT_{1A} and 5-HT_{2A} receptors and insulin secretion during diabetes.

6. To study insulin secretion, *in vitro*, from isolated pancreatic β-cells in presence of 5-HT

7 To study the role of tryptophan on brain 5-HT_{1A} and 5-HT_{2A} receptors in diabetic state.

8. To establish the role and mechanism of peripheral 5-HT in regulating insulin release by its binding to nuclear proteins.

9. To study the expression of 5-HT_{2A} receptor during diabetes and in diabetic rats treated with insulin, tryptophan and insulin + tryptophan by RT-PCR technique.

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1. BRAIN NEUROTRANSMITTER CHANGES DURING DIABETES

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

In case of 5-HT there are contradicting reports which state that 5-HT content is increased in the brain regions and hypothalamic nuclei (Chen & Yang, 1991, Lackovic *et al.*, 1990; Bitar *et al.*, 1987), but majority of reports suggest a decrease in brain 5-HT content during diabetes (Jackson & Paulose, 1999; Sandrini *et al.*, 1997; Sumiyoshi *et al* 1997; Thorre *et al.*, 1997; Shimizu, 1991, Chu *et al.*, 1986; Kulikov *et al.*, 1986). 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 MHPG/NE and 5-HIAA/5-HT were increased. A similar observation was reported by

Ding *et al.*, (1992) with a decrease in 5-HT in entorhinal cortex (19%) and 5-HT turnover (5-HIAA/5-HT) that increased by 48%. Chu *et al.*, (1986) has reported lower 5-HT levels in both hypothalamus and brain stem but not in corpus striatum. Insulin treatment brought about an increase in the cerebral concentration of 5-HIAA and accelerated the cerebral 5-HT turnover (Juszkiewicz, 1985). The 5-HIAA concentration was reported to be approximately twice as high as the controls regardless of duration of treatment. Brain tryptophan, the precursor of 5-HT, was also reduced in brain regions during diabetes (Jamnicky *et al.*, 1991). Insulin treatment was reported to reverse this reduced tryptophan content to normal (Jamnicky *et al.*, 1993). It also produced a significant increase in 5-HIAA that was observed at 2, 3, 4 and 6 hours after insulin administration (Kwok & Juorio, 1987). There was no change in 5-HIAA content in the corpus striatum during diabetes (Chu *et al.*, 1986).

2.2. HYPERGLYCAEMIC EFFECT BY 5-HT RECEPTOR AGONISTS

The 5-HT_{1A} and 5-HT_{2A} receptor subclasses are the two main receptors that mediate a hyperglycaemic response when stimulated by their respective agonists.

2.2.1. Hyperglycaemia induced by 5-HT_{1A} receptor stimulation

8-OH-DPAT has been reported as a selective agonist for 5-HT_{1A} receptor It binds and activates the 5-HT_{1A} receptors and decreases plasma insulin and increases basal plasma glucose levels in several strains of rats and other species via a central mechanism of action (Laude *et al.*, 1990; Bauhelal *et al.*, 1990b; Chaouloff & Jeanrenard, 1988, Chaouloff & Jeanrenaud, 1987). The decrease in insulin is brought about by increased sympathetic activity that stimulates the adrenal gland to secrete EPI which in turn inhibits insulin secretion (Bauhelal & Mir, 1993, Bauhelal & Mir, 1990a). Both adrenal cortex and medulla can participate in glucose metabolism through their respective secretory products i.e., corticosteriods and catecholamines respectively The fact that 8-OH-DPAT increases plasma EPI and that selective α_2 and β_2 adrenoreceptor agonists suppress 8-OH-DPAT mediated hyperglycaemia supports the involvement of adrenal catecholamines in the metabolic effects of 8-OH-DPAT (Chaouloff *et al* 1990a; Bugdy *et al.*, 1989; Chaouloff & Jeanrenaud, 1987). The EPI releasing effect of 8-OH-DPAT are

blocked by both 5-HT_{1A} and β-adrenoreceptor antagonist (-)-pindolol. However, selective β_1 or β_2-adrenoreceptor antagonists do not block this effect (Chaouloff *et al.*, 1990a).

Plasma levels of corticosterone showed an increase with 8-OH-DPAT treatment in rats (Chaouloff *et al.*, 1990d; Aulakh *et al.*, 1988; Koenig *et al.*, 1987). An increase in corticosterone can be a potential contributing factor for 8-OH-DPAT mediated hyperglycaemia. Administration of 8-OH-DPAT also produced a sustained fall in blood pressure and heart rate that were preceded by transient (<5min) increase in blood pressure. All these symptoms were abolished in adrenalectomised rats (Bauhelal & Mir, 1993). At high concentrations, 8-OH-DPAT can bind to α_2-adrenoreceptors and inhibit insulin release via a mechanism similar to that of clonidine inhibition of insulin from pancreatic β-cells (Fozord *et al.*, 1987).

Corticotropin releasing factor (CRF) and ACTH release is stimulated by 5-HT and 8-OH-DPAT (Calogero *et al.*, 1989). CRF is reported to stimulate adrenal medulla via a central site of action manifested by increase in efferent adrenal nerve activity leading to increased plasma EPI concentration and blood pressure (Brown *et al.*, 1985). Also, intra-cerebroventricular injections of CRF have induced hyperglycaemia in rats and dogs (Brown *et al.*, 1982). EPI release and hyperglycaemia is induced by several other selective 5-HT_{1A} receptor agonists such as buspirone, ipsapirone and flesinoxan, thus implicating a key role for 5-HT_{1A} receptors in the regulation of glucose metabolism (Chaouloff *et al.*, 1990e).

2.2.2. Hyperglycaemia induced by 5-HT_{2A} receptor stimulation

Administration of a selective 5-HT_{2A} receptor agonist DOI produced a rapid increase in blood glucose level. Administration of DOI is also accompanied by an increase (1500%) in EPI concentration (Glennon, 1987). Pre-treatment of the animals with 5-HT_{2A} receptor antagonists i.e., ketanserin and LY53857, were able to reverse the increase in sympathetic nerve discharge produced by DOI (Chaouloff *et al.*, 1990b; Hoyer, 1988c; McCall & Hornis, 1988). These findings show that the central 5-HT_{2A} receptors stimulates sympathetic nerve discharge which in turn increases EPI release from adrenal medulla similar to the 5-HT_{1A} receptor activation.

Administration of the 5-HT_{2A} receptor agonist, α-methyl-5-HT, can also elicit hyperglycaemic effects which are blocked by 5-HT_{2A} antagonist ketanserin (Chaouloff *et al.*, 1990b). α-methyl-5-HT was able to significantly suppress food intake by food-deprived rats and also inhibited 2-deoxy-D-glucose induced hyperphagia in rats. α-methyl-5-HT induced hyperphagia was antagonised by ketanserin (Yamada *et al.*, 1997; Sugimoto *et al.*, 1996). Intraperitonial administration of 5-HT brought about hyperglycaemia mediated through a dose dependent increase in plasma EPI level. 5-HT induced hyperglycaemia was abolished by pre-treatment with ketanserin and also adrenodemedullation (Yamada *et al.*, 1995). This suggests that the hyperglycaemic effects of 5-HT are closely related to the decrease of EPI from the adrenal gland, mediated by $5HT_{2A}$ receptors. 5-HT_{2A} receptors may also be partly involved in the pharmacological effects of induction of hyperglycaemia induced by the 5-HT_4 receptor agonist, 5-methoxytryptamine (Yamada *et al.*, 1997).

2.3. FACTORS AFFECTING INSULIN REGULATION FROM PANCREATIC β-CELLS

2.3.1. Glucose

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

diabetes mellitus (Dukes *et al.*, 1997). Glucose induced insulin secretion is also partly dependent upon the activation of typical isoforms of protein kinase C (PKC) within the β-cell (Harris *et al.*, 1996). It is suggested that PKC 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).

2.3.2. Amino acids

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

2.3.3. Fatty acids

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).

2.3.4. Substrates derived from nutrients

This may involve indirect reflux stimulation triggered by food intake or local islet stimulation through the production of a metabolite common to several substrates like pyruvate (Lisa *et al* 1994), citrate, ATP (Tahani, 1979), NADH and NADPH (Iain *et al.*, 1994). Adenosine diphosphate acts as an intracellular regulator of insulin secretion. Mg-ADP is required for the stimulation of K^+-ATP channels in intact β-cells. Other intracellular factors such as arachidonate guanine nucleotides, small monomeric GTP-binding proteins such as rab 3A (Regazzi *et al.*, 1996) and the heterotrimeric GTP-binding protein $G_{\alpha i}$ are 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).

2.3.5. Glucagon

Glucagon is the hormone secreted by pancreatic α-cells. 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 (Kiefer, 1996). Intra-islet glucagon appears to be a paracrine regulator of cAMP *in vitro* (Schuit, 1996). Glucagon stimulates insulin release by elevating cAMP The 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).

2.3.6. 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).

2.3.7. Epinephrine and norepinephrine

These are secreted by the adrenal medulla. NE is a principal neurotransmitter of sympathetic nervous system. These hormones inhibit insulin secretion, both *in vivo* and *in vitro* (Renstrom *et al.* 1996; Porte, 1967). Epinephrine exerts opposite effects on peripheral glucose disposal and glucose stimulated insulin secretion (Avogaro *et al* 1996).

2.3.8. 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).

2.3.9. 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 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).

2.3.10. Adrenomedullin

Adrenomedullin is a novel hypotensive adrenal polypeptide isolated from a human pheochromocytoma 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).

2.3.11. 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 via activation of G-proteins by the mediation of activated galanin receptors (Renstrom *et al.*, 1996). However, galanin receptors are not as effective as α_2-adrenergic receptors in activating G-proteins.

2.3.12. Macrophage migration inhibitory factor (MIF)

MIF, originally identified as cytokines secreted by T lymphocytes 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 expresses MIF and its production is regulated

by glucose in a time and concentration dependent manner MIF and insulin co-localise 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).

2.3.13. Other agents

Coenzyme Q_{10} improved insulin release and it may also have a blood glucose lowering effect (Conget *et al.*, 1996). 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 GTP-ases 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).

2.4. ROLE OF NEUROTRANSMITTERS IN INSULIN REGULATION

2.4.1. Epinephrine and Norepinephrine

Epinephrine and norepinephrine has an antagonistic effect on insulin secretion and glucose uptake (Renstrom *et al.*, 1996; Porte, 1967). They also inhibit insulin -stimulated glycogenesis through inactivation of glycogen synthase and activation of phosphorylase with consequent accumulation of glucose-6-PO_4. In addition, it has been reported that epinephrine enhances glycolysis through an increased activation of phosphofructokinase.

The adrenergic receptors are seven-pass transmembrane receptors that are coupled to G-proteins. Adrenergic receptors are mainly classified into α_1, α_2 and β-adrenergic receptors. α_1 has three subclasses- α_{1A}, α_{1B}, α_{1C} (Price *et al* 1994) and α_2 has $\alpha_{2A,D}$, α_{2B} and α_{2C}. (Hamamdzic *et al.*, 1995). β-adrenergic receptors are subclassified into β_1, β_2 and β_3 (Dohlman *et al.*, 1991). EPI and NE bind to these receptors in a concentration dependant manner At low concentration EPI and NE can bind and activate β-adrenergic receptors which in turn stimulate the insulin secretion from pancreatic islets and at high concentration they can bind to α_{2A} receptors and inhibit insulin secretion (Lacey *et al*

1993). Previous studies had shown that in diabetic condition α_{2A} receptors are more activated which brought out the insulin inhibition and in turn hyperglycaemia (Lacey *et al.*, 1993). Rat islet cell membrane is equipped with α_{2A}-adrenoceptors (Filipponi *et al.*, 1986) which are linked to adenylate cyclase inhibits insulin secretion. β_3 adrenoceptors stimulation also results in enhanced insulin secretion (Alef *et al.*, 1996).

2.4.2. 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 & Sehlin, 1983). Dopamine D_3 receptors are implicated in the control of blood glucose levels (Alster & Hillegaart, 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.

2.4.3. Acetylcholine

Acetylcholine is a principle transmitter of the parasympathetic system. Acetylcholine, through vagal and non-vagal muscarinic pathways (Greenberg & Pokol, 1994) increases insulin secretion via muscarinic receptors on pancreatic islet cells (Tassava *et al.*, 1992).

2.4.4. γ-Aminobutyric acid (GABA)

Gamma aminobutyric acid (GABA) is the major inhibitory neurotransmitter in central nervous system. GABA is reported to be present in the endocrine pancreas at concentrations comparable with those found in central nervous system. The highest concentration of GABA within the pancreatic islet is confined to β-cells (Sorenson *et al* 1991). Glutamate decarboxylase, the primary enzyme that is involved in the synthesis of GABA, has been identified as an early target antigen of the T-lymphocyte mediated destruction of pancreatic β-cells causing insulin-dependent diabetes mellitus (Baekkeskov *et al* 1990). GABA through its receptors have 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 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 pancreatic islets is important in the pathogenesis of diabetes.

2.4.5. Serotonin

The brain 5-HT content is decreased during diabetes (Jackson & Paulose, 1999; Sandrini *et al* 1997; Sumiyoshi *et al* 1997; Thorre *et al.*, 1997; Shimizu, 1991, Chu *et al* 1986; Kulikov *et al* 1986). This decrease is reported to be due to a decrease in uptake of tryptophan through the BBB (Madras *et al.*, 1974, Fernstrom & Wurtman, 1972; Fernstrom & Wurtman, 1971) and a decrease in rate of 5-HT synthesis (Carndall *et al* 1981). The turnover rate of 5-HT to 5-HIAA in diabetic rats was also reported to be lower (Sandrini *et al.*, 1997; Kwok & Juorio, 1987). A decrease in brain 5-HT will lead to an up-regulation of $5\text{-}HT_{2A}$ receptors of cerebral cortex and brain stem which in turn can inhibit insulin secretion due to increased sympathetic activity (Jackson & Paulose, 1999).

2.5. CLASSIFICATION OF 5-HT RECEPTORS

Protein receptors that mediate the actions of 5-HT have existed in the membranes of a variety of animal cell types for millions of years. Their ancestry have been traced to be older than adrenoreceptors (Hen, 1992; Venter *et al.*, 1988). It is likely that during such a long period of time the older receptors must have undergone mutations and during evolution a number of its variants or subclasses must have been formed. This undoubtedly is the case with 5-HT and NE receptors. Of all the neurotransmitter receptors 5-HT receptors have the largest number of variants or subclasses.

The 5-HT receptors can be classified into seven main classes (Peroutka, 1993). They comprise the 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_5 and the recently cloned 5-HT_6 and 5-HT_7 but these receptors are yet to be fully characterised operationally and transductionally in intact tissues.

2.5.1. 5-HT_1 receptor family

5-HT_1 receptors were first identified as a high-affinity site for 5-HT in radioligand binding studies on brain homogenates using [^{3}H]5-HT (Peroutka & Synder, 1979). Several subtypes of 5-HT_1 receptor family have been characterised, they are 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D}. These subtypes can be labelled in brain under appropriate conditions with [^{3}H]8-OH-DPAT, [^{125}I]Cyanopindolol, [^{3}H]Mesclergide and [^{125}I]GTI respectively (Boulenguez *et al.*, 1992; Bruinvels *et al.*, 1991, Hoyer *et al* 1985a; Hoyer *et al.* 1985b Gozlan *et al.*, 1983).

2.5.1.1. 5-HT_{1A} receptors

5-HT_{1A} receptors are most widely distributed in the hippocampus and dorsal raphe (Radja *et al.*, 1991, Marcinkiewicz *et al.*, 1984). Many of these regions are components of pathways involved in the modulation of emotion and the limbic system. Their distribution is common to several mammals including humans (Pazos *et al* 1987a; Hoyer *et al* 1986a). The presence of high densities of 5-HT_{1A} receptors in raphe nuclei indicates that 5-HT can modulate the activity of serotonergic neurons. 5-HT_{1A} receptors are also present in the cerebral cortex, hypothalamus and the substantia gelatinosa of the spinal cord. The localisation of 5-HT_{1A} receptors in these areas also suggests that 5-HT_{1A} mechanisms could also be involved in the function of the hypothalamus and in the integrative function of the cerebral cortex.

The 5-HT_{1A} receptor was also reported to exist in two isoforms in rat brain regions, i.e., a high affinity 5-HT_{1A} receptor and a low affinity 5-HT_{1A} receptor They are two independent 5-HT_{1A} receptor proteins rather than two inter-convertible stages of a single protein. These two isoforms can be labelled by high and low concentrations of [^{3}H]8-OH-DPAT (Nenonene *et al* 1994).

A number of agonists show selectivity for 5-HT_{1A} receptors e.g., 8-OH-DPAT, 5-CT, buspirone, ipsapirone, gepirone, 5-methyl-urapidil, flesinoxan and MDL72832 (Richardson & Hoyer, 1990). The most significant antagonists for 5-HT_{1A} are NAN190 (Glennon *et al.* 1988), MDL73005 (Hibert & Moser, 1990), 5-F-8-OH-DPAT and (±)WAY100135, of these (±)WAY100135 has been described as a selective antagonist that is devoid of any partial agonist activity (Bill *et al.*, 1993). [^{3}H]8-OH-DPAT has been used as the radioligand for 5-HT_{1A} receptors (Gozlan *et al* 1983).

The 5-HT_{1A} receptor is coded by a single intron-less mRNA. The human 5-HT_{1A} receptor gene was first identified by screening a human library with probes for the β_2-adrenoreceptor isolated from clone G21 (Kobilka *et al.*, 1987). G21 is intron-less and the corresponding protein has 421 amino acids with 7 transmembrane domains. The rat 5-HT_{1A} receptor has also been cloned (Albert *et al.*, 1990) and the receptor has 99% sequence homology with the human equivalent in the putative trans-membrane domains. The G-protein coupling to 5-HT_{1A} receptor appears to mediate both stimulation and inhibition of adenylate cyclase activity (Shenker *et al.*, 1987, Shenker *et al.*, 1985, Shenker *et al* 1983). The 5-HT_{1A} may be coupling to at least two different G-proteins (G_s and G_i) in the same tissue or alternatively, inhibition and stimulation of adenylate cyclase are mediated by two closely related receptors, which are difficult to distinguish pharmacologically Transduction system other than adenylate cyclase has been described for 5-HT_{1A} receptors. Andrade *et al* (1986) reported the presence of pertussis toxin-sensitive G-protein that couples 5-HT_{1A} receptors in hippocampal pyramidal cells to a K^+ channel. Activation of the receptor leads to channel opening and hyperpolarisation.

In HeLa cells, it has been reported that 5-HT_{1A} receptors mediate sodium-dependent potassium transport and Na^+/K^+ ATPase activity (Raymond *et al* 1991, Middleton *et al.*, 1990; Fargin *et al* 1989; Raymond *et al* 1989). Eventhough there are reports of different second messenger coupling for 5-HT_{1A} receptor, at present it appears that this receptor like the other members of 5-HT_1 family negatively couples to adenylate cyclase via α_1 adrenergic receptor There are also reports that some isoforms of cyclase (type-II and IV) which are present in the brain can be activated by β/γ-subunits (Tang & Gillman, 1991). This has been demonstrated by injecting *Xenopus* oocytes with mRNAs

for the 5-HT_{1A} receptor in combination with adenylate cyclase type-II and cystic fibrosis transmembrane conductance regulator gene (Uezono *et al.*, 1993). Activation of 5-HT_{1A} receptor leads to cAMP production via protein kinase A, which stimulates the cystic fibrosis transmembrane conductance regulator leading to chloride channel activation.

2.5.1.2. 5-HT_{1B} receptors

High densities of 5-HT_{1B} receptors are found in the globus pallidus and pars reticulata of the substantia nigra (Pazos & Palacios, 1985). In addition, the terminal autoreceptors of rat cortex has also been identified as 5-HT_{1B} receptors (Middlemiss, 1986; Middlemiss, 1985; Middlemiss, 1984). Functionally the 5-HT_{1B} receptors in vena cava appear to mediate NE release (Gothert *et al.*, 1986b). The 5-HT_{1B} receptors are also associated with DNA synthesis in hamster fibroblast (Seuwen *et al* 1988). 5-HT_{1B} receptors mediate hyperlocomotor activity produced by the 5-HT_{1B} agonist RU 24969 (Lucki, 1992). Activation of 5-HT_{1B} receptors also mediate hypophagia (Kennet & Curzon, 1988a).

Some of the indol β-adrenoreceptor antagonists such as SDZ 21009 and cyanopindolol act as 5-HT_{1B} antagonists. 5-HT_{1B} receptor binding can be performed with [^{3}H]5-HT in the presence of blocking concentrations of 5-HT_{1A} and 5-HT_{2C} receptor ligands (Peroutka *et al.*, 1988) or with [^{125}I]Iodocyanopindolol in the presence of 30μM isoprenaline to avoid β-adrenoreceptor binding (Hoyer *et al.*, 1985a).

The rat 5-HT_{1B} receptor gene is intron-less, encoding a 386-amino acid protein, and has 96% homology in the TMR with the equivalent human clone (Adham *et al* 1992, Voigt *et al.* 1991). The 5-HT_{1B} receptors are negatively coupled to adenylate cyclase (Bauhelal *et al* 1988).

2.5.1.3. 5-HT_{1D} receptors

The 5-HT_{1D} receptors have been found to exist in brain of a wide range of non-rodent mammalian species including guinea pig, rabbit, dog, pig, calf and human (Maura *et al.*, 1993; Beer *et al.*, 1992). The 5-HT_{1B} sites appear to be absent in these species and

5-HT_{1D} receptor reflects the distribution and function of 5-HT_{1B} receptors found in rodents. Radioreceptor assay is done using [^{3}H]5-HT in presence of 100mM 8-OH DPAT and mesulergine to block 5-HT_{1A}/5-HT_{1C} binding, but these binding conditions are not homogenous and includes 5-HT_{1E} receptors (Beer *et al.*, 1992; Sumner & Humphrey, 1989; Hoyer & Neigt, 1988a). Activation of 5-HT_{1D} receptors leads to inhibition of forskolin-stimulated adenylate cyclase activity in calf and guinea pig substantia nigra (Waeber *et al.*, 1989; Hoyer & Shoeffeter, 1988b). In addition, most studies performed with cells transfected with 5-HT_{1D} receptors (both 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$) show that these receptors are negatively coupled to adenylate cyclase.

2.5.1.4. 5-HT_{1E} receptors

5-HT_{1E} is present in human frontal cortex and other brain regions similar to 5-HT_{1D} receptor in varying relative proportions (Beer *et al.*, 1992; Lowther *et al* 1992, Leonhardt *et al.*, 1989). The function of 5-HT_{1E} receptor is not clearly known, although it appears to be coupled negatively to adenylate cyclase. The receptor consists of a single protein of 365 amino acids.

2.5.1.5. 5-HT_{1F} receptors

The mRNA for 5-HT_{1F} is concentrated in the dorsal raphe, hippocampus and cortex (Adham *et al.*, 1993) but is not found in kidney, liver, spleen, heart and pancreas. In NIH3T3 cells, the transfected 5-HT_{1F} receptor clones show negative coupling to adenylate cyclase like other 5-HT_1 receptors. The intron-less gene for 5-HT_{1F} receptor has a long open reading frame encoding a protein of 366 (human and rat) or 367 (mouse) amino acids in length (Adham *et al.*, 1993, Lovenberg *et al.* 1993; Almaiky *et al.*, 1992).

2.5.1.6. 5-HT_1 like receptors

5-HT_1 like receptors are a group of related receptors that have not yet been positively equated with any of the 5-HT_1- binding site subtypes, identified in the CNS. These receptors mediate a number of functions like smooth muscle contraction and decreased EPI release from sympathetic nerves.

2.5.2. 5-HT_2 receptor family

The 5-HT_2 subclass have been further classified into 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors, each of these receptors have been cloned and consists of a G-protein coupled single protein with seven trans-membrane domains (458 to 471 amino acids). All these subtypes mediate their effects through activation of phosphoinositide metabolism.

2.5.2.1. 5-HT_{2A} receptor

5-HT_{2A} receptors are widely distributed in peripheral tissues and cortex (Pazos *et al.*, 1987b; Bardley *et al.*, 1986a; Hoyer *et al* 1986b; Pazos & Palacios, 1985). The effects mediated by these receptors include contractile response of vascular, bronchial, uterine and urinary smooth muscles. The 5-HT_{2A} receptors also mediate platelet aggregation and increased capillary permeability In the neocortex, these sites are mainly concentrated in laminae I and IV (rat) and III and V (human). In addition, 5-HT_{2A} receptor distribution is found in the claustrum, some components of limbic system, particularly the olfactory nuclei and parts of basal ganglia.

Leysen *et al* (1982) have reported that [^{3}H] ketanserin can be used as a selective ligand for the 5-HT_{2A} receptors. Two subtypes of 5-HT_{2A} receptors have been proposed and labelled by [^{3}H]DOB and [^{3}H] ketanserin (Peroutka *et al* 1988). The 5-HT_{2A} receptor polypeptide contains seven transmembrane regions and the amino acid sequence within the transmembrane regions is 80% identical with that of the 5-HT_{2C} receptor The 5-HT_{2A} receptors are linked to phosphatidylinositol turnover The receptors are coupled to phospholipase C, and inositol phospholipid hydrolysis and Ca^{2+} mobilisation are involved in the post-receptor events (Conn & Sanders-Bush, 1984a).

2.5.2.2. 5-HT_{2B} receptor

When functionally expressed in COS cells, the 5-HT_{2B} receptors display high affinity for [^{3}H]5-HT and [^{125}I]DOI. The receptor is coupled to phospholipase C. The human receptor protein is 80% homologous to the rat receptor and the intron/exon distribution in the gene is conserved in both species (Foguet *et al.*, 1992).

2.5.2.3. 5-HT_{2C} receptor

The 5-HT_{2C} receptors are distributed throughout the choroid plexus of all mammals (Yagaloff & Hartig, 1985). The 5-HT_{2C} transcripts are also found in significant densities in the olfactory nucleus, cingulate cortex and subthalamic nucleus (Mengod *et al.*, 1990). The gene for 5-HT_{2C} has introns and it is possible that different gene products can occur due to alternate splicing. The protein sequence consists of 460 amino acids. The mouse and human homologues have been cloned and show 98% homology in the transmembrane regions (Yu *et al.*, 1991).

2.5.3. 5-HT_3 receptor

5-HT_3 receptors are found exclusively associated with neurons of both central and peripheral origin and in a variety of neuronally derived cell lines such as NIE-115, NCB-20 and N18 cells (Peters *et al* 1991). In the brain, the highest densities of 5-HT_3 receptors are found in discrete nuclei of the lower brain stem and the substantia gelatinosa at all levels of the spinal cord (Pratt *et al.*, 1990). 5-HT_3 receptor is a ligand-gated ion channel receptor The activation of this receptor triggers a rapid depolarisation and a rapid influx of Ca^{2+} into the cytosol from the extracellular environment (Peters *et al* 1991).

2.5.4. Other 5-HT receptors

The 5-HT_4 receptors are located on nerve cells where they mediate inhibition of voltage-activated potassium channels via stimulation of a cAMP-dependent protein kinase (Fagni *et al.*, 1992). The 5-HT_5 receptors are further classified into 5-HT_{5A} and 5-HT_{5B} receptors. Both these receptors show pharmacological properties similar to 5-HT_1 receptors (Matthes *et al.*, 1993). The 5-HT_6 receptor has been cloned and belongs to a G-protein coupled receptor family. The receptor consists of 436 amino acids and has 36% homology in the transmembrane region with that of various 5-HT_1 and 5-HT_2 receptors (Rout *et al.*, 1993a). The 5-HT_7 receptor is positively linked to adenylate cyclase and is predominantly expressed in rat hypothalamus and to a lesser extent in other brain regions (Lovenberg *et al* 1993, Rout *et al.*, 1993b).

2.6. EFFECT OF 5-HT ON BLOOD GLUCOSE LEVEL

There are conflicting reports about the effect of 5-HT on blood glucose level. It has been reported that 5-HT can induce both hypoglycaemia and hyperglycaemia (Sugimoto *et al.*, 1990; Itaya & Itoh, 1979). In normal mice, 5-HT induced a dose-dependent hypoglycaemia and an increase in serum insulin level. 5-HT also inhibited glucose-induced hyperglycaemia and increased glucose-stimulated insulin release. But in STZ-induced diabetic mice, 5-HT changed neither the glucose nor insulin levels (Sugimoto *et al.*, 1990). Itaya & Itoh, (1979) reported an increase in plasma cAMP and glucose after i.p. administration of 5-HT

2.7. 5-HT IS PRESENT WITHIN THE PANCREATIC ISLETS

Islet monoamines are located in the insulin storage granules (Bird *et al* 1980; Jain-Etcheverry & Zieher, 1968; Falck & Hellmann, 1963). Consequently the role of intracellular islet monoamines in the regulation of insulin secretion has been the subject of many investigations. Pharmacological manipulations of pancreatic islet serotonin and dopamine content in *in vitro* and *in vivo* systems has resulted in evidence for monoaminergic inhibition (Feldman *et al.*, 1972a; Feldman & Leboritz, 1972b) and stimulation of insulin secretion (Telib *et al.*, 1968). Several amino acids are able to stimulate insulin release in the presence of glucose. 5-HTP is readily taken up into the islet in the presence of glucose and stimulates insulin secretion. But the enzyme 5-hydroxytryptophan decarboxylase readily converts it into 5-HT that inhibits insulin secretion (Sundler *et al.*, 1990; Lindstrom & Sehlin, 1983). When 5-HTP was tested in conjunction with a decarboxylase inhibitor, the glucose stimulated insulin release from rabbit pancreas was significantly enhanced (Gylfe *et al* 1973). Tryptophan, a precursor of 5-HTP, has a stimulating effect on insulin release from hamster pancreas. The presence of monoamine oxidase enzyme that catabolises 5-HT within the β-cells shows an effective 5-HT metabolism within the islets (Pizzinat *et al* 1999; Feldman & Chapman, 1975). 5-HT can also act as a marker for insulin secretion. 5-HT is taken up into insulin granules and co-released with insulin on stimulation of pancreatic β-cells by glucose (Zhou & Misler, 1996). All these evidences show the presence of 5-HT within the pancreatic islets

and has a role in the regulation of insulin secretion from the β-cells. An increase in the level of EPI was also noted in diabetic islets. However, in total pancreas the NE and EPI contents were same in diabetic and non-diabetic rats (Ostenson *et al* 1993).

2.8. ROLE OF TRYPTOPHAN IN THE PHYSIOLOGICAL REGULATION OF BRAIN SEROTONIN

Administration of L-tryptophan which is a precursor of 5-HT can increase the brain 5-HT content during diabetes. The rate limiting enzyme, tryptophan hydroxylase is usually not saturated with tryptophan. Any process that increases the brain tryptophan leads to an increase in brain 5-HT content (Curzon & Mursden, 1975, Friedman *et al* 1972; Ecclestron *et al* 1965). Administration of an amino acid mixture, containing all essential amino acids but not tryptophan caused a parallel depletion of total and free serum tryptophan and thereby decrease in brain tryptophan and serotonin (Biggo *et al* 1975). An intraperitoneal administration of 50-100mg/kg L-tryptophan brought an increase in hypothalamic tryptophan (286%), 5-HT (23%) and 5-HIAA (20%) after 30min. Rest of the brain also showed an increase in tryptophan (256%), 5-HT (29%) and 5-HIAA (12%) after 30min. Administration of 100mg/kg i.p p-chlorophenylalanine (p-CPA) which is a tryptophan hydroxylase inhibitor did not bring any increase in brain 5-HT after administration of L-tryptophan. Hutson *et al.*, (1985) have reported a similar increase in 5-HT and 5-HIAA in the cerebrospinal fluid (CSF) as seen in brain after i.p. administration of tryptophan.

2.9. INSULIN IS A MAJOR DETERMINANT FOR THE TRANSPORT OF TRYPTOPHAN ACROSS THE BLOOD-BRAIN-BARRIER

The major determinant of brain tryptophan concentration is insulin as this can result in decreased plasma concentration of large neutral amino acids (valine, leucine, and isoleucine). These amino acids compete with tryptophan for uptake into the brain across the BBB (Madras *et al.*, 1974; Fernstrom & Wurtman, 1972; Fernstrom & Wurtman, 1971). Trulson & Mackenzie, (1978) have reported that after 4 weeks of administration of streptozotocin the brain tryptophan content was decreased by 27%. Insulin administration

was able to bring back the brain tryptophan and 5-HIAA levels to normal. Tryptophan uptake across the BBB is increased in the presence of insulin. Insulin enhances the uptake of branched chain amino acids thereby decreasing their plasma concentration. Since these amino acids compete with tryptophan for transport into brain, there is a resultant increase in brain tryptophan (Curzon & Mursden, 1975).

There are also contradicting reports that during diabetes there is an increase in brain tryptophan uptake (Demontis *et al* 1977). They state that during diabetes there is an increase in lipolysis. The free fatty acids will tend to bind to plasma albumin. This will increase the free plasma tryptophan and thereby increases the chances for tryptophan uptake into brain. From the above reports we can conclude that the effect of insulin on brain tryptophan is not direct, but mediated via insulin induced changes in serum tryptophan to other competing amino acids ratio.

2.10. DIET CAN INFLUENCE BRAIN SEROTONIN SYNTHESIS

Tryptophan is transported into the brain by a competitive carrier system that is shared by large neutral amino acids such as tyrosine, phenylalanine, leucine, isoleucine and valine. Physiological variations in the plasma neutral amino acid pattern, either as a change in plasma tryptophan or in the plasma concentration of one or more of its competitors directly alters this competitive process. This variation in tryptophan uptake influences brain tryptophan level and thus serotonin synthesis (Fernstrom & Fernstrom, 1995; Fernstrom, 1991, Fernstrom, 1979; Biggio *et al* 1974). It is not only tryptophan that is influenced by the diet but other amino acids such as tyrosine, which is the precursor for DA and NE, is also influenced by diet. The same process is applicable for the uptake of choline, the precursor of acetylcholine (Fernstrom, 1994, Fernstrom, 1977; Wurtman & Fernstrom, 1975). A similar observation was reported by DeMarte & Enesco, (1985). They maintained a group of mice for 78 weeks on tryptophan restricted, protein restricted and control diet. They found that brain 5-HT levels were significantly reduced only in mice on the tryptophan-restricted diet, but not in mice on the protein restricted diet.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. BIOCHEMICALS AND THEIR SOURCES

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

The following are the chemicals used for this study

i) Biochemicals: (Sigma Chemical Co., USA.)

(±)Norepinephrine, (±)epinephrine, normetanephrine, 5-hydroxytyramine, 5-hydroxytryptophan, 5-hydroxy indole acetic acid, homovanillic acid, sodium octyl sulfonic acid, ethylene glycol bis (β-aminoethyl ether)-EGTA, ethylene diamine tetra acetic acid-EDTA, HEPES - [n' (2-hydroxy ethyl)] piperazine-n'-[2-ethanesulfonic acid], ascorbic acid, streptozotocin, pargyline, Tris buffer, foetal calf serum (heat inactivated), collagenase type V, D-glucose, calcium chloride, 8-hydroxydipropylaminotetraline (8-OH-DPAT), collagenase type XI and bovine serum albumin fraction V

ii) Radiochemicals

5-Hydroxy[G-^{3}H]tryptamine creatine sulphate ([^{3}H]5-HT, 39 0Ci/mmol) and [^{3}H](±)2,3-dimethoxyphenyl-1-[2-(4-piperidine)-methanol] ([^{3}H]MDL100907, 82.0Ci/mmol) were purchased from Amersham Life Science, UK.

[Propyl-2,3-ring-1,2,3-^{3}H]8-hydroxydipropylaminotetraline ([^{3}H]8-OH-DPAT, 127.0 Ci/mmol) was from NEN Life Sciences products, Inc., Boston, USA.

Radioimmunoassay kit for insulin was from Bhabha Atomic Research Centre, Mumbai, India.

iii) Molecular biology chemicals

Restriction enzymes were purchased from Bangalore Genei, India. Titan™ one tube RT-PCR system were purchased from Roche Diagnostics, Germany PCR primers used in this study were synthesised by Genemed Synthesis Inc., San Francisco, USA.

3.2. ANIMAL EXPERIMENTS

Adult male Wistar rats of 200-240g body weight were purchased from Central Institute of Fisheries Technology, Cochin and used for all experiments. They were housed in separate cages under 12 hour light and 12 hour dark periods and were maintained on standard food pellets and water *ad libitum*.

Animals were randomly divided into the following groups. Each group consisted of 4-6 animals.

i) Control [C]
ii) Diabetic [D]
iii) Diabetic rats treated with insulin [D+I]
iv) Diabetic rats treated with insulin and L-tryptophan [D+I+T]
v) Diabetic rats treated with L-tryptophan alone [D+T]

Diabetes was induced by a single intrafemoral dose (65 mg/kg body weight) of STZ prepared in citrate buffer, pH 4.5 (Hohenegger & Rudas, 1971, Arison *et al* 1967). The insulin treated diabetic group received a daily dose (1Unit/kg body weight) of Lente and Plain insulin (Boots India). The dose was increased daily according to the blood glucose level (Sasaki & Bunag, 1983). 100mg L-tryptophan was orally administered through drinking water to D+I+T and D+T groups.

3.2.1. Tissue preparation

Rats were sacrificed by decapitation on the 14^{th} day of the experiment. The cerebral cortex, brain stem and hypothalamus were dissected out quickly over ice according to the procedure of Glowinski & Iversen, (1966). The tissues were stored at -70^0 C until assay

3.2.2. Estimation of blood glucose

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

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

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

The hydrogen peroxide formed in this reaction reacts with 4-aminoantipyrine and 4-hydroxybenzoic acid in the presence of peroxidase (POD) to form N-(-4-antipyryl)-p-benzo quinoneimine. The addition of mutarotase accelerates the reactions. The amount of dye formed is proportional to the glucose concentration. The absorbance was read at 505nm. in (Milton Roy Genesys 5 Spectronic) spectrophotometer

3.3. QUANTIFICATION OF BRAIN MONOAMINES AND THEIR METABOLITES

The monoamines were assayed according to Paulose *et al* (1988) The tissues from brain regions were homogenised in 0.4N perchloric acid. The homogenate was centrifuged at 5000xg for 10 minutes at 4°C (Kubota refrigerated centrifuge) and the clear supernatant was filtered through 0.45 μm HPLC grade filters and used for HPLC analysis

Norepinephrine (NE), epinephrine (EPI), dopamine (DA), 5-hydroxy tryptamine (5-HT), 5-hydroxyindole acetic acid (5-HIAA), 5-hydroxytryptophan (5-HTP), normetanephrine (NMN) and homovanillic acid (HVA) were determined in high performance liquid chromatography (HPLC) with electrochemical detector (HPLC-ECD) (Shimadzu, Japan) fitted with CLC-ODS reverse phase columns of 5 μm particle size. The mobile phase consisted of 75 mM sodium dihydrogen orthophosphate, 1mM sodium octyl sulfonate, 50mM EDTA and 7% acetonitrile. The pH was adjusted to 3.25 with orthophosphoric acid, filtered through 0.45 μm filter (Millipore) and degassed. A Shimadzu (model 10 AS) pump was used to deliver the solvent at a rate of 1 ml/min. The catecholamines were identified by an amperometric detection using an electrochemical detector (Model 6A, Shimadzu, Japan) with a reduction potential of + 0.8 V, with the range set at 16 and a time constant of 1.5 seconds. Twenty microlitre aliquots of the acidified supernatant were injected into the system. The peaks were identified by relative

retention times compared with external standards and quantitatively estimated using an integrator (Shimadzu, C-R6A -Chromatopac) interfaced with the detector Data from different brain regions of the experimental and control rats were statistically analysed and tabulated.

3.4. PROTEIN DETERMINATION

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

3.5. SEROTONIN RECEPTOR BINDING STUDIES USING [^{3}H] RADIOLIGANDS

3.5.1. 5-HT$_{1A}$ receptor binding studies using [^{3}H]8-OH-DPAT

5-HT$_{1A}$ receptor binding assay was done according to the modified procedure of Nenonene *et al.*, (1994). Brain tissues were homogenised in a polytron homogeniser with 50 volumes of cold 50mM Tris-HCl buffer, pH.7 4 After first centrifugation at 40,000xg for 15 min, the pellets were resuspended in buffer and incubated at 37^{0}C for 20 min, to remove endogenous 5-HT After incubation the homogenates were centrifuged and washed twice by centrifugation at 40,000xg for 15 min and resuspended in appropriate volume of the buffer

Binding assays were done using different concentrations i.e., 0.20nM-100nM of [^{3}H]8-OH-DPAT in 50mM Tris buffer, pH 7.4 in a total incubation volume of 250µl Specific binding was determined using 100µM unlabelled 5-HT Competition studies were carried out with 1.0nM [^{3}H]8-OH-DPAT in each tube with unlabelled ligand concentrations varying from 10^{-12} 10^{-4}M of 5-HT The effect of guanine nucleotide on 5-HT$_{1A}$ receptor binding was studied by incubating tubes with 1 0nM [^{3}H]8-OH-DPAT, varying concentration of 5-HT (10^{-12} - 10^{-4}M) and in the presence and absence of 100µM Gpp[NH]p.

Tubes were incubated at 25^{0}C for 60 min. and filtered rapidly through GF/C filters

(Whatman). The filters were washed quickly by three successive washing with 3.0 ml of ice cold 50mM Tris buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter

3.5.2. 5-HT_{2A} receptor binding studies using [^{3}H]MDL100907

5-HT_{2A} receptor binding assay was done according to the modified procedure of Green *et al.*, (1990). The cerebral cortex and brain stem were homogenised in 10 volumes of ice cold 0.32M sucrose in a Potter-Elvejhem homogenizer The homogenate was centrifuged at 900xg for 10 min and the supernatant again centrifuged at 17,000xg for 1hour. The pellet was resuspended in 50 volumes of 50mM Tris HCl, pH 7.5 and recentrifuged at 17,000xg for another 1hour The final pellet was resuspended in a minimum volume of 50mM Tris HCl, pH 7 7 containing 4mM $CaCl_2$

Binding assays were done using different concentrations i.e., 0.25nM-2.5nM of [^{3}H]MDL100907 in 50mM Tris buffer, pH 7 7 containing $CaCl_2$ (4mM), ascorbate (0 2%), and pargyline (10µM) in a total incubation volume of 250µl. Specific binding was determined using 100µM unlabelled ketanserin. Competition studies were carried out with 0 5nM [^{3}H]MDL100907 in each tube with unlabelled ligand concentrations varying from 10^{-9} 10^{-4}M of ketanserin.

Tubes were incubated at 37^{0}C for 30 min. and filtered rapidly through GF/B filters (Whatman). The filters were washed quickly by three successive washing with 3 0 ml of ice cold 50mM Tris buffer, pH 7 7 Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter

3.6. ANALYSIS OF THE RECEPTOR BINDING DATA

3.6.1. Linear regression analysis for Scatchard plots

The data was analysed according to Scatchard, (1949). The specific binding was determined by subtracting non-specific binding from the total. The binding parameters, maximal binding (B_{max}) and equilibrium dissociation constant (K_d), were derived by linear

regression analysis by plotting the specific binding of the radioligand on X-axis and bound/free on Y-axis. The maximal binding is a measure of the total number of receptors present in the tissue and the equilibrium dissociation constant is the measure of the affinity of the receptors for the radioligand. The K_d is inversely related to receptor affinity

3.6.2. Nonlinear regression analysis for displacement curve

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

3.7. ISOLATION OF PANCREATIC ISLETS

Pancreatic islets were isolated from male Wistar rats by standard collagenase digestion procedures using aseptic techniques (Howell & Taylor, 1968). The islet cells were isolated in HEPES-buffered sodium free Hanks Balanced Salt Solution (HBSS) (Pipeleers *et al* 1985) with the following composition: 137mM choline chloride, 5 4mM KCl, 1.8mM $CaCl_2$, 0.8mM $MgSO_4$, 1mM KH_2PO_4, 14.3mM $KHCO_3$, 10mM HEPES with 0.2% (w/v) BSA (Fraction V), equilibrated with 5% CO_2 and pH 7 3 at room temperature. Autoclaved triple distilled water was used for making up the medium and the medium was filtered through 0.22 μm filters (Millipore).

Splenic portion of 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 20 minutes at 37°C in an environmental shaker with vigorous shaking (300rpm/min). The tissue digest was filtered through 500 μm nylon screen and the filtrate was washed with three successive centrifugations and resuspensions in cold HBSS medium.

The washed filtrate was transferred to a sterile petridish with a black base and examined under a dissection microscope. 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 was used for all other experiments.

3.7.1. 5-HT uptake studies by pancreatic islets in vitro

The islets isolated by the above mentioned method were resuspended in HEPES buffered HBSS with 4mM glucose and pre-incubated for 1hour at 37^0C (Howell & Taylor, 1968). The islet suspension was centrifuged at 4^0C at 500xg to remove inherent insulin. The pre-incubated islets were then washed thrice with cold 10mM Tris HCl buffer, pH 7 4 and finally resuspended in HBSS without glucose. 200µl of islet suspension was transferred to tubes containing 1nM, 5nM and 10nM concentrations of [^{3}H]5-HT Each of the [^{3}H]5-HT concentration had three glucose concentrations i.e., (i) without glucose, (ii) 4mM glucose and (iii) 20mM glucose. The final incubation volume was made up to 0.5ml. The tubes were incubated for 2hours at 37^0C in a shaking water bath.

At the end of incubation period the tubes were centrifuged at 1,500xg for 10min at 4^0C. The supernatant was aspirated out and pellet washed superficially with 0.2ml of HBSS twice to remove free [^{3}H]5-HT The pellet was digested with 100µl of 1M KOH overnight and counted in a liquid scintillation counter with Cocktail-T to measure the [^{3}H]5-HT uptake.

3.7.2. In vitro insulin secretion in the presence of different concentrations of 5-HT in vitro

The isolated islets were incubated for 2hours at 37^0C with 1nM, 5nM and 10 nM of 5-HT and two different concentrations of glucose i.e., (i) without glucose, (ii) 4mM glucose and (iii) 20mM glucose. After incubation and centrifugation at 1,500xg for 10 min at 4^0C, the supernatant was transferred to fresh tubes for insulin assay by radioimmunoassay

3.8. RADIOIMMUNO ASSAY OF INSULIN

Principle of the assay

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

Assay Protocol

50µl of standards (ranging from 0 to 200 µU/ml), 50µl of insulin free serum and 50µl insulin antiserum were added together and the volume was made up to 250µl with assay buffer. They were incubated overnight at 2°C To this mixture 50µl of [^{125}I]insulin was added and again incubated at room temperature for 3hours. This was followed by addition of 50µl of second antibody and 500µl of PEG. The tubes were vortexed and incubated for 20minutes at the end of which they were centrifuged at 1500xg for 20minutes and the supernatant was aspirated out and the radioactivity in the pellet was determined in a gamma counter The unknown samples were assayed in the same way except that insulin free serum was avoided and the mixture contained the same volume of buffer

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

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

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

3.9. 5-HT UPTAKE STUDIES IN FRACTIONATED PANCREATIC ISLETS INCUBATED WITH [^{3}H]5-HT

The isolated islets were incubated with 5nM 5-HT and two different concentrations of glucose (i) 4mM and (ii) 20mM glucose. The total incubation volume was made up to 0.5ml with HBSS. The tubes were incubated at 37^{0}C for 30min, 1hour and 3hours. Incubations were arrested at different time intervals by centrifuging the cells at 1,500xg for 10min at 4^{0}C. The pellet was washed once with 500µl of HBSS and resuspended in 100µl HBSS.

The cells were broken by homogenisation in a glass homogeniser Tubes were then centrifuged at 800xg for 10min at 4^{0}C to separate the nuclear fraction (Boam, 1996) The supernatant was transferred to fresh tubes and centrifuged at 30,000xg for 20min at 4^{0}C to separate the mitochondrial + plasma membrane fraction. The supernatant that consisted of cytosolic fraction was transferred to fresh tubes. The pellets were digested by incubating at 37^{0}C overnight with 100µl of 1M KOH. The bound radioactivity in nuclear fraction, mitochondrial + plasma membrane fraction and cytosolic fraction were counted in a liquid scintillation counter with cocktail-T The DPM obtained were analysed to determine the amount of [^{3}H]5-HT bound to each subcellular fraction in the presence of different concentrations of glucose.

3.10. PANCREATIC ISLET NUCLEAR 5-HT BINDING PROTEIN STUDIES

Nuclear fraction was isolated as mentioned above (Boam, 1996). The nuclear fraction was resuspended in HBSS buffer of pH 7.4 and used for Scatchard and displacement assays. Binding assays were done using different concentrations [^{3}H]5-HT i.e., 10nM-200nM in HBSS, pH 7 4 in a total incubation volume of 500µl. Specific binding was determined using 100µM unlabelled 5-HT Tubes were incubated at 37^{0}C for 30minutes. Incubations were arrested by centrifugation at 10,000xg for 10 min at 4^{0}C. The pellet was digested by incubating at 37^{0}C overnight with 100µl of 1M KOH. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter

3.11. DETERMINATION OF MOLECULAR WEIGHT OF PANCREATIC ISLET NUCLEAR 5-HT BINDING PROTEIN BY LIGAND BLOTTING TECHNIQUE

3.11.1. Tissue solubilisation

The pancreatic islets were isolated by collagenase digestion method as described earlier The islets were homogenised in HBSS. The tubes were centrifuged at 800xg for 10min at 4^0C to separate the nuclear fraction. The pellet was solubilised in 200µl solubilisation buffer by sucking up and down through a 12 gauge needle and syringe. The solubilising solution consisted of Tris HCl, pH 7 4, 1.6% w/v Triton X-100 and 1mM PMSF Tubes were incubated over ice for 20min and then centrifuged at 30,000xg for 30min at 4^0C (Soutar & Wade, 1997).

3.11.2. SDS-PAGE

Proteins were resolved by discontinuous 10% SDS-PAGE according to Laemmili, (1970). 200µg protein was mixed with gel loading buffer, heated for 2min in a boiling water bath and applied to each well. Standard molecular weight markers ranging from 205-kDa to 29-kDa (Sigma Chemical Co USA) were used to determine the molecular weight of the 5-HT binding protein.

3.12. PROTEIN BLOTTING TO PVDF MEMBRANE AND MOLECULAR WEIGHT DETERMINATION

After electrophoresis the gel was transferred to renaturation buffer (50mM Tris, 20% Glycerol, pH 7 4) for 20min and then changed to transfer buffer (25mM Tris-HCl, 192mM Glycine, pH 8.3) for 1.5hours to remove SDS and allow the gel to swell. One lane each with mol. wt. marker and sample was cut out and stained with coomassie blue. The protein in the remaining gel was transferred to a polyvinylidene fluoride (PVDF) membrane by electroblotting at 200mA current for 16hours in transfer buffer without methanol (Soutar & Wade, 1997). The membrane was incubated for 1hour in 10ml blocking solution (HBSS, pH 7 4 containing 1% BSA) at 37^0C. The membrane was then transferred to 10ml fresh blocking solution containing 0.15µCi/ml [^{3}H]5-HT and

incubated for 1hour at 37^0C with mild agitation and then washed three times in fresh HBSS to remove unbound [^{3}H]5-HT The membrane was dried and cut into 3mm slices and counted in a liquid scintillation counter with cocktail-T (Hames, 1981). The molecular weight of the slice with maximum DPM was determined by comparing with the molecular wt. of the stained band. Unlabelled 5-HT was used to confirm specificity of the binding.

3.13. AMINO ACID ANALYSIS OF PANCREATIC ISLET NUCLEAR 5-HT BINDING PROTEIN

The protein was resolved in a 10% discontinuous SDS-PAGE as described above. Two lanes one with marker and another with sample were cut out from both ends of the gel and were processed as follows (Schagger, 1994).

1. Fixed in 50% methanol/10% acetic acid for 15 minutes
2. Staining in 10% acetic acid/0.025% coomassie blue for 30 minutes.
3. Destaining was done in 10% acetic acid for 2 x 15 minutes.

The unstained portion of the gel was also kept in 10% acetic acid but without coomassie blue stain to get uniform swelling of stained and unstained portion of the gel. The band of interest was cut out from the unstained portion of the gel by keeping it along with the stained portion. The cut out portion of the gel was homogenised with 1ml of 50mM Tris HCl. pH 7.4 containing 0.1% SDS. The homogenate was stored at 4^0C overnight and centrifuged at 10,000xg for 15minutes. The supernatant was used for amino acid analysis.

Amino acid analysis

Amino acid analysis of the 14-kDa 5-HT binding protein was done by hydrolysing the protein in 6N HCl for 24 hours at 110^OC under vacuum. The amino acid composition was analysed HPLC LC-10A (Shimadzu).

3.14. EXPRESSION STUDIES OF 5-HT_{2A}, RECEPTOR IN DIFFERENT BRAIN REGIONS AND PANCREATIC ISLETS

3.14.1. Isolation of RNA

RNA was isolated using Tri Reagent kit (Sigma Chemical Co., USA) and the following protocol was adopted.

25-50mg tissue was homogenised in 0.5ml Tri Reagent.

⇩

The homogenate was centrifuged at 12,000xg for 10minutes at 4^0C.

⇩

The clear supernatant was transferred to a fresh tube and it was allowed to stand at room temperature for 5minutes.

⇩

100µl of chloroform was added to it, shaken vigorously for 15minutes and allowed to stand at room temperature for 15minutes.

⇩

The tube was centrifuged at 12,000xg for 15minutes at 4^0C. Three distinct phases appeared after centrifugation. The bottom red organic phase contained protein, interphase contained DNA and the colourless upper aqueous phase contained RNA.

⇩

The upper aqueous phase was transferred to a fresh tube and 200µl of isopropanol was added and the tubes were allowed to stand at room temperature for 10minutes.

⇩

The tubes were centrifuged at 12,000xg for 10min at 4^0C. RNA precipitate formed 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 12,000xg for 5minutes at 4^0C.

⇩

The pellet was briefly air dried and dissolved in minimum volume of DEPC-treated water and incubated at 60^0C for 10-15 minutes.

10µl of RNA was made up to 1ml and absorbance was measured at 260nm and 280nm. For pure RNA preparation the ratio of absorbance at 260/280 was ≥ 1 7 The concentration of RNA was calculated as 1 Absorbance $_{260}$ = 42µg.

RT-PCR (Reverse Transcription Polymerase Chain Reaction)

RT-PCR was carried out using Titan™ one tube RT-PCR system (Roche Diagnostics, Germany). cDNA synthesis was performed with AMV (Avian Melanoma Virus) reverse transcriptase enzyme. The PCR step was carried out with a high fidelity enzyme blend consisting of Taq DNA Polymerase and Pwo DNA Polymerase. Enzyme was stored in storage buffer (20mM Tris HCl, 100mM KCl, 0.1mM EDTA, 1mM Dithiothrietol (DTT), 0.5% Tween-20 (v/v), 0.5% Nonidet P40 (v/v), 50% Glycerol (v/v). pH 7.5 (25^{0}C).

Preparation of RNA

RNA was isolated from brain regions of control, diabetic, diabetic + insulin, diabetic + insulin + tryptophan and diabetic + tryptophan treated groups using the Tri reagent kit as mentioned above.

RT PCR Primers

The following primers were used for 5-HT$_{2A}$ receptor mRNA expression studies.

5'-CAACTCCAGAGATGCTAACACTTCG-3' Forward Primer 5'-GGGTTCTGGATGGCGACATAG-3' Reverse Primer	Rat 5-HT$_{2A}$ primer

RT-PCR of 5-HT$_{2A}$ receptors

RT-PCR was carried out according to the procedure of Titan™ one tube RT-PCR system from Roche Diagnostics with modifications. The reaction was carried out in a total volume of 40µl reaction mixture in 0.2ml tubes. RT-PCR was performed on an Eppendorf Personal thermocycler. Three separate master mixes were made (Eason & Liggett, 1993). Mix# 1 consisted of 50ng RNA template, 50ng Random hexamers and RNase inhibitor (10units). The tube was incubated at 42^{0}C for 10min. After incubation mix # 2 was

added. Mix# 2 consisted of reaction buffer containing 1.5mM $MgCl_2$ (5x stock containing 7.5mM $MgCl_2$ and DMSO), DTT and 5mM dNTPs (10mM stock containing mixture of dATP, dCTP, dGTP and dTTP). The tube was again incubated at 42^0C for 2min.

The reaction was started after adding mix# 3 containing 0.3µM 5-HT_{2A} forward primer (14.52µM stock), 0.3µM 5-HT_{2A} reverse primer (16.87 µM stock), enzyme mix and DEPC treated water to make up the volume to 40µl.

Mix # 1

Component	Volume	Final concentration in the RT-PCR tube	
RNA template	2.0µl	50ng	Incubate at 42^0C for 10min.
Random hexamers	1.0µl	50ng	
RNase inhibitor	2.0µl	10U	
D H_2O	5.0µl		
Total	10.0µl		

Mix # 2

5 x buffer	8.0µl	1.5mM $MgCl_2$	Incubate at 42^0C for 2min.
DTT	2.0µl	5mM	
dNTPs	0.8µl	0.2mM	
Total	10.80µl		

Mix # 3

5-HT_{2A} forward primer	0.826µl	0.3µM	Touch down RT-PCR according to thermocycling profile
5-HT_{2A} reverse primer	0.712µl	0.3µM	
Enzyme mix	0.8µl	AMV and Expand™ high fidelity enzyme blend	
D H_2O	16.35µl		
Total	19.20µl		
Grand Total	**40µl**		

3.14.3. Thermocycling profile for touch down RT-PCR

For obtaining higher stringency conditions a touch down RT-PCR profile was adopted (Kidd & Ruano, 1995). The strategy of touch down PCR involves starting the cycle with a very high annealing temperature and then lowering the annealing temperature with successive cycles. RT-PCR was performed for 35 cycles. For first two cycles the annealing temperature was set at 55^0C accompanied with the lowering of the annealing temperature at the rate of 1^0C for every two cycles.

Following is the thermocycling profile used for 5-HT_{2A} receptor RT-PCR.

I.	42^0C	1 hour	} RT step	
II.	50^0C	20 min		
III.	94^0C	3 min	Denaturation	
IV	94^0C	40 sec	Denaturation	} 10 cycles, with reduction in annealing temperature by 1^0C every second cycle.
V	55^0C	1.0 min ---	Annealing	
VI.	68^0C	1 0 min ---	Extention	
VII.	94^0C	40 sec	Denaturation	} 25 cycles
VIII.	50^0C	1 0 min ---	Annealing	
IX.	68^0C	1.0 min ---	Extention	
X.	68^0C	10 min	Final extention	

3.14.4. Analysis of RT-PCR product

After completion of RT-PCR reaction 10µl of Bromophenol blue gel-loading buffer was added to 40µl reaction mixture and the total volume was applied to a 2% agarose gel containing ethidium bromide. The gel was run at constant 40V with 0.5x Tris borate EDTA buffer. The image of the bands was captured using an Imagemaster gel documentation system (Pharmacia Biotech) and densitometrically analysed using Imagemaster ID software to quantitate the 5-HT_{2A} receptor mRNA expression in control, diabetic and diabetic rats treated with insulin, tryptophan and a combination of insulin and tryptophan. The product size of 5-HT_{2A} receptor is 443 base pairs. The forward primer is

located at position 155 of the mRNA sequence and the reverse primer at position 598 of the mRNA. Therefore the expected RT-PCR product size is 598 - 155 = 443 bp.

3.15. STATISTICS

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

RESULTS

4. RESULTS

4.1. BLOOD GLUCOSE LEVEL AND BODY WEIGHT OF EXPERIMENTAL RATS

Streptozotocin administration led to a significant increase ($p<0.001$) in blood glucose of diabetic group. Insulin treatment was able to significantly reduce ($p<0.001$) the increased blood glucose level to normal when compared to diabetic group. Tryptophan treatment alone was also able to significantly reduce ($p<0.001$) the blood glucose when compared to diabetic, but the reduction was not as much as compared to insulin and insulin + tryptophan treatment (Table-1).

Treatment of diabetic rats with a combination of insulin and L-tryptophan brought about a significant decrease ($p<0.001$) in blood glucose level. There was no significant difference in body weight between different groups compared to the control (Table-1).

4.1.1. Effect of 8-OH-DPAT on blood glucose level

The blood glucose level was significantly increased after 30min of administration (i.p) of 8-OH-DPAT, which is a specific agonist of 5-HT_{1A} receptor The increase in blood glucose was immediate, starting after 5min of 8-OH-DPAT administration (Fig.-1)

4.2. BRAIN 5-HT AND OTHER MONOAMINE CONTENTS OF EXPERIMENTAL RATS

4.2.1. Cerebral Cortex

There was a significant decrease ($p<0.01$) in 5-HT content of cerebral cortex in 14-day diabetic rats (Table-2). The 5-HT content was significantly reversed ($p<0.01$) to control level by insulin, tryptophan and insulin + tryptophan treatment compared to diabetic group. The turnover rate of 5-HIAA/5-HT was significantly increased ($p<0.01$) in diabetic group when compared to control. There was a significant decrease ($p<0.05$) in 5-HTP content. This increased turnover of 5-HIAA/5-HT was significantly reversed

($p<0.001$) by insulin, tryptophan and insulin + tryptophan treatment. The turnover of 5-HTP/5-HT did not show any significant change in diabetic group. The NE and 5-HIAA content did not show any significant change in diabetic and treated groups.

4.2.2. Brain Stem

In brain stem also there was a significant decrease ($p<0.05$) in 5-HT content of diabetic rats compared to controls (Table-3). This decrease was significantly reversed ($p<0.05$) to control by insulin, tryptophan and insulin + tryptophan treatment. The turnover of 5-HTP/5-HT and 5-HIAA/5-HT was significantly increased ($p<0.001$ and $p<0.05$ respectively) in diabetic group compared to control. Insulin and tryptophan treatment significantly reversed the 5-HTP/5-HT and 5-HIAA/5-HT turnover ($p<0.001$ and $p<0.01$ respectively) when compared to diabetic group. Combination of insulin and tryptophan treatment also significantly reversed the 5-HTP/5-HT and 5-HIAA/5-HT turnover rate ($p<0.001$ and $p<0.05$ respectively) The NE content was significantly increased ($p<0.001$) in diabetic group compared to control. This increase was effectively reversed ($p<0.001$) by insulin, tryptophan and insulin + tryptophan treatment. 5-HTP and 5-HIAA content did not show any significant change.

4.2.3. Hypothalamus

There was no significant change in 5-HT content of diabetic and treated groups, but the turnover rate of 5-HTP/5-HT was significantly decreased ($p<0.001$) in diabetic group compared to control (Table-4). This decrease in turnover of 5-HTP/5-HT was significantly reversed ($p<0.001$) by insulin, tryptophan and insulin + tryptophan treatment. The 5-HTP content showed a significant decrease ($p<0.001$) in diabetic group compared to control. Treatment with insulin, tryptophan and insulin + tryptophan significantly reversed ($p<0.001$, $p<0.01$ and $p<0.001$ respectively) the decreased 5-HTP content. There was no significant change in EPI, 5-HIAA contents and turnover of 5-HIAA/5-HT But the NE content showed a significant increase ($p<0.001$) in diabetic group compared to control. This increase was significantly reversed ($p<0.001$) by insulin, tryptophan and insulin + tryptophan treatment.

4.3. PANCREATIC 5-HT AND OTHER MONOAMINE CONTENTS OF EXPERIMENTAL RATS

There was a significant increase ($p<0.05$) in 5-HT content of pancreas in diabetic group compared to control (Table-5). Treatment with insulin, tryptophan and insulin + tryptophan reversed to near control level ($p<0.05$). The 5-HTP content was significantly increased ($p<0.001$) in diabetic group compared to control. Insulin, tryptophan and insulin + tryptophan treatment significantly reversed ($p<0.001$, $p<0.001$ and $p<0.01$ respectively) the increased 5-HTP content. The turnover rate of 5-HTP/5-HT in diabetic group was also significantly increased ($p<0.001$) compared to control. Insulin treatment alone significantly reversed ($p<0.001$) the turnover of 5-HTP/5-HT to control compared to diabetic group. EPI content in diabetic group was significantly increased ($p<0.05$) compared to control but was not reversed by treatment with insulin, tryptophan and insulin + tryptophan. NE, 5-HIAA and turnover of 5-HIAA/5-HT did not show any significant change.

4.4. PLASMA 5-HT AND OTHER MONOAMINE CONCENTRATIONS OF EXPERIMENTAL RATS

The plasma 5-HT concentration in diabetic group was significantly increased ($p<0.001$) compared to control (Table-6). Insulin, tryptophan and insulin + tryptophan treatment significantly reversed ($p<0.001$ and $p<0.01$ respectively) the altered 5-HT content. The turnover of 5-HTP/5-HT was significantly decreased ($p<0.001$) in diabetic group compared to control. The 5-HTP/5-HT turnover was significantly reversed ($p<0.001$) to control by insulin and insulin + tryptophan treatment. The 5-HIAA/5-HT turnover in diabetic group was significantly decreased ($p<0.01$) compared to control, which was significantly reversed ($p<0.01$) to control value by tryptophan treatment alone. Insulin and insulin + tryptophan treatment did not show any significant change. The NE and EPI content in diabetic group also showed a significant increase ($p<0.05$) compared to control. Insulin, tryptophan and insulin + tryptophan treatment significantly reversed ($p<0.01$, $p<0.05$ and $p<0.01$ respectively) the increased EPI content compared to diabetic

group. The 5-HTP and 5-HIAA content of diabetic group did not show any significant change.

4.5. ALTRERED BRAIN 5-HT_{2A} RECEPTOR BINDING PARAMETERS IN STREPTOZOTOCIN INDUCED DIABETIC RATS

4.5.1. Cerebral Cortex

Scatchard analysis in cerebral cortex of diabetic rats showed no significant change in B_{max} when compared to control (Table-7, Fig.- 2a-2c). But the K_d was significantly decreased ($p<0.05$) in diabetic group. Insulin treatment significantly reversed ($p<0.05$) the K_d to normal when compared to diabetic group. Treatment with tryptophan and insulin + tryptophan also reversed the K_d to control ($p<0.05$) when compared to diabetic rats. Tryptophan and insulin + tryptophan combination were more effective in reversing the K_d to control than insulin treatment alone. A decrease in K_d without any significant change in B_{max} shows an increase in affinity of 5-HT_{2A} receptors without any change in its number during diabetes.

4.5.1.1. Displacement analysis of [^{3}H]MDL100907 against ketanserin in cerebral cortex of experimental rats

In displacement analysis we have used different concentrations of unlabelled ketanserin against [^{3}H]MDL100907, since ketanserin has a higher potency than MDL100907 In diabetic group competition binding showed the appearance of a low affinity site in addition to the high affinity site thus fitting the equation to a two-site model instead of the one-site model observed in control group (Table-8, Fig.- 3a-3c). The Hill slope value was away from unity (0.669) in diabetic group compared to control (1.098) confirming the two-site model. Treatment with insulin, tryptophan and insulin + tryptophan were able to reverse the two-site model back to one-site model when compared to diabetic group. The Hill slope value for all the treated groups were above unity These results confirm an increase in affinity of the 5-HT_{2A} receptors for 5-HT without any alteration in their number during diabetes.

4.5.2. Brain Stem

Scatchard analysis of [^{3}H]MDL100907 against ketanserin in brain stem of diabetic rats showed a significant increase ($p<0.05$) in B_{max} and K_d ($p<0.05$) compared to control (Table-9, Fig.- 4a-4c). An increase in B_{max} and K_d signifies an up-regulation of 5-HT_{2A} accompanied with a decrease in its affinity Insulin treatment to diabetic rats significantly reversed ($p<0.05$) the B_{max} and K_d to control value. Tryptophan and insulin + tryptophan treatment significantly reversed ($p<0.05$) the altered parameters to control value.

4.5.2.1. Displacement analysis of [^{3}H]MDL100907 against ketanserin in brain stem of experimental rats

Diabetic brain stem also showed an additional low affinity site along with the high affinity site thereby fitting the equation to a two-site model instead of the one-site model seen in control (Table-10, Fig.- 5a-5c). The two-site model is further confirmed by the Hill slope value that is away from unity (0.546) in diabetic group. The two-site model is reversed to a one-site model by treatment with insulin, tryptophan and combination of insulin and tryptophan.

4.5.3. Hypothalamus

Scatchard analysis in hypothalamus of diabetic rats did not show any significant change in B_{max} when compared to control (Table-11, Fig.- 6a-6c). Diabetic rats treated with insulin, tryptophan and combination of insulin and tryptophan also did not show any change in B_{max}. The K_d of diabetic rats showed a significant decrease ($p<0.05$) when compared to control. This decrease in K_d without any change in B_{max} shows an increase in affinity of the 5-HT_{2A} receptors for 5-HT without any change in its number Insulin, tryptophan and insulin + tryptophan treatment was able to significantly reverse ($p<0.05$) back the decreased K_d to control compared to diabetic group.

4.5.3.1. Displacement analysis of [^{3}H]MDL100907 against ketanserin in hypothalamus of experimental rats

Displacement analysis in hypothalamus of diabetic rats showed the appearance of

an additional low affinity site thus fitting the equation to a two-site model (Table-12, Fig.-7a-7c). The Hill slope value was away from unity (0.654) in diabetic group compared to control (1.362) confirming the two-site model. Treatment with insulin reversed the two-site model to a one-site model similar to control. Tryptophan treatment and a combination of tryptophan and insulin treatment also reversed the two-site model to a one-site model.

4.6. 5-HT_{2A} RECEPTOR GENE EXPRESSION IN DIFFERENT BRAIN REGIONS OF EXPERIMENTAL RATS

Quantitation of 5-HT_{2A} receptor mRNA by RT-PCR showed no significant change in its expression in cerebral cortex and hypothalamus of experimental animals (Plate-1 & 3, Fig.-8 & 10). These results correlate with our receptor data, which showed no significant difference in number of receptors but an increase in its affinity for 5-HT in cerebral cortex and hypothalamus of diabetic rats. The 5-HT_{2A} receptor expression was significantly increased in brain stem of diabetic rats which was reversed by insulin, tryptophan and insulin + tryptophan treated rats which is confirmatory to our receptor binding studies (Plate-2, Fig.-9). There was a significant decrease in expression of 5-HT_{2A} receptors in corpus striatum, cerebellum and pancreatic islets of diabetic rats and diabetic rats treated with insulin, tryptophan and insulin + tryptophan (Plate-4 - 6, Fig.- 11-13).

4.7. ALTERED BRAIN 5-HT_{1A} RECEPTOR BINDING PARAMETERS IN STREPTOZOTOCIN INDUCED DIABETIC RATS

4.7.1. Cerebral Cortex

Scatchard analysis for 5-HT_{1A} receptors was done using [^{3}H]8-OH-DPAT, which is a specific 5-HT_{1A} receptor agonist. Scatchard analysis of high affinity receptors in diabetic group showed a significant decrease ($p<0.01$) in B_{max} without any significant change in K_d compared to control (Table- 13, Fig.- 19a-19e). The B_{max} and K_d were significantly decreased ($p<0.001$) by insulin, tryptophan and insulin + tryptophan treatment compared to control.

In the case of low affinity 5-HT_{1A} receptors the B_{max} and K_d were significantly

increased ($p<0.05$) in diabetic group compared to control. Insulin treatment did not reverse the altered binding parameters. Insulin + tryptophan and tryptophan alone treatment significantly reversed ($p<0.05$ and $p<0.01$ respectively) the K_d to control when compared to diabetic group. These results show that during diabetes there is an up-regulation of low affinity 5-HT_{1A} receptors which is not controlled by insulin and insulin + tryptophan treatments. However, tryptophan treatment alone showed a complete reversal. In addition to this there is a simultaneous down-regulation of the high affinity receptors, which are not reversed to control by insulin, tryptophan and insulin + tryptophan treatments.

4.7.1.1. Displacement analysis of [^{3}H]8-OH-DPAT against 5-HT in cerebral cortex of experimental rats

In displacement analysis we have used different concentrations of unlabelled 5-HT against [^{3}H]8-OH-DPAT All the experimental groups fitted best to a two-site equation (Table-14, Fig.- 15a-15c). This was confirmed by the Hill slope value that was above unity in all the groups. In diabetic group the $Ki_{(H)}$ value showed a shift in affinity towards a higher affinity state. There was no significant change in the $Ki_{(L)}$ of diabetic rats. Treatment with insulin and insulin + tryptophan was able to reverse back the shift in affinity when compared to diabetic group.

4.7.1.2. Displacement analysis of [^{3}H]8-OH-DPAT against 5-HT in cerebral cortex of experimental rats in presence of Gpp[NH]p

In control rats the presence of Gpp[NH]p led to a shift in the $Ki_{(H)}$ value of the receptors towards a higher affinity state, but in the case of diabetic rats the $Ki_{(H)}$ showed a shift of the 5-HT_{1A} receptors towards a low affinity state in presence of Gpp[NH]p (Table-15, Fig.- 16a-16c). Treatment with insulin, tryptophan and insulin + tryptophan reversed the shift in the affinity of the receptors from a low affinity state to a high affinity state in presence of Gpp[NH]p.

4.7.2. Brain stem

Scatchard analysis of high affinity receptors in diabetic rats showed a significant

increase ($p<0.05$) in B_{max} without any significant change in K_d compared to control (Table-16, Fig.- 17a-17e). The B_{max} was significantly reversed ($p<0.001$) to control by insulin, tryptophan and insulin + tryptophan treatment.

There was no significant change in B_{max} of low affinity 5-HT_{1A} receptors of different experimental groups. But the K_d of diabetic rats showed a significant decrease ($p<0.05$) compared to control. Insulin, tryptophan and insulin + tryptophan treatments significantly reversed ($p<0.05$) the decreased K_d of low affinity receptors.

4.7.2.1. Displacement analysis of [^{3}H]8-OH-DPAT against 5-HT in brain stem of experimental rats

In case of brain stem also all the experimental groups fitted best to a two-site equation (Table-17, Fig.- 18a-18c). This was confirmed by the Hill slope value that was above unity in all the groups. In diabetic group the $Ki_{(H)}$ value showed a shift in affinity towards a higher affinity state without any significant change in the $Ki_{(L)}$ value. Treatment of diabetic rats with insulin, tryptophan and insulin + tryptophan reversed the altered $Ki_{(L)}$ value.

4.7.2.2. Displacement analysis of [^{3}H]8-OH-DPAT against 5-HT in brain stem of experimental rats in presence of Gpp[NH]p

In the brain stem of control rats there was no change in the $Ki_{(H)}$ value in the presence of Gpp[NH]p. But in case of diabetic group a shift in affinity of the 5-HT_{1A} receptors towards a low affinity state was observed in the presence of Gpp[NH]p. A similar shift in affinity of the 5-HT_{1A} receptors towards a low affinity state was observed in cerebral cortex. Rats treated with insulin, tryptophan and insulin + tryptophan reversed the affinity of the receptors from a low affinity state to a high affinity state in the presence of Gpp[NH]p. All the experimental groups fitted to a two-site model confirmed by the Hill slope value which is above unity

4.7.3. Hypothalamus

Scatchard analysis of high affinity receptors in diabetic rats showed a significant decrease ($p<0.001$) in B_{max} accompanied any significant decrease ($p<0.001$) in K_d compared to control (Table-19, Fig.- 20a-20e). The B_{max} and K_d of low affinity 5-HT_{1A} receptors of diabetic group showed a significant increase ($p<0.001$) compared to control. Insulin, tryptophan and insulin + tryptophan treatment showed no significant change in B_{max} and K_d of high affinity receptors.

4.7.3.1. Displacement analysis of [^{3}H]8-OH-DPAT against 5-HT in hypothalamus of experimental rats

In hypothalamus the competition binding data fitted to a two-site model for all the experimental groups (Table-20, Fig.- 21a-21c). This was confirmed by the Hill slope value above unity for all the groups. In diabetic group the $Ki_{(H)}$ value showed a shift in affinity towards a low affinity region. This was not reversed back to control by treatment with insulin, tryptophan and insulin + tryptophan.

4.8. T_3 AND T_4 CONCENTRATIONS IN PLASMA OF EXPERIMENTAL RATS

The T_3 and T_4 concentrations in plasma of diabetic group were significantly decreased ($p<0.01$ and $p<0.001$ respectively) compared to control (Table-21). Insulin, tryptophan and insulin + tryptophan treatment did not reverse the altered T_3 and T_4 levels.

4.9. [^{3}H] 5-HT UPTAKE BY ISOLATED PANCREATIC ISLETS

Our results showed a significant increase ($p<0.01$) in [^{3}H]5-HT uptake by pancreatic islets in the presence of 1nM, 5nM [^{3}H]5-HT and 20mM glucose when compared to cells incubated with 1nM [^{3}H]5-HT but without glucose (Fig.-22). 4mM and 20mM glucose in the incubation medium can be considered equivalent to normal and diabetic states respectively. In cells incubated with 10nM [^{3}H]5-HT and different concentrations of glucose there was no significant increase in [^{3}H]5-HT uptake in the presence of glucose. These results show that there is a rapid uptake of 5-HT into the

pancreatic islets only in the presence of high glucose concentration as seen in a diabetic state showing an inhibition at 10nM [^{3}H]5-HT

4.10. EFFECT OF SEROTONIN ON GLUCOSE INDUCED INSULIN SECRETION

The pancreatic islets that were incubated with 1nM, 5nM and 10nM 5-HT but without glucose showed a significant increase ($p<0.01$, $p<0.05$ and $p<0.05$ respectively) in insulin secretion even though there was no glucose stimulation for insulin secretion (Fig.-23). But 5-HT stimulation of insulin secretion decreased significantly with increasing glucose concentration. The above results show that 5-HT actsas a stimulant for insulin secretion in the absence of glucose but inhibits glucose induced insulin secretion at high glucose concentration. The same mechanism can also take place *in vivo*. Figure-24 shows an increase in % of insulin inhibition with increased % uptake of 5-HT in the presence of 20mM glucose - for 5% of 10nM 5-HT uptake there is 40% inhibition of insulin secretion and for 15% uptake of 1nM 5-HT there is 80% inhibition of insulin secretion.

4.11. SCATCHARD ANALYSIS OF [^{3}H]5-HT TO NOVEL NUCLEAR 5-HT BINDING SITES IN PANCREATIC ISLETS

Scatchard analysis of nuclear binding sites in islet nuclear membrane was done using [^{3}H]5-HT The islets were fractionated to membrane + mitochondrial and nuclear fractions by differential centrifugation. Both fractions were analysed for [^{3}H]5-HT binding and the nuclear fraction only showed positive binding with [^{3}H]5-HT (Table-22). The B_{max} and K_d of nuclear 5-HT binding protein in diabetic rats were significantly decreased ($p<0.01$, $p<0.05$) compared to control. Insulin, tryptophan and tryptophan + insulin treatment did not reverse the altered B_{max} and K_d (Table-23, Fig.- 25a-25c).

4.12. [^{3}H]5-HT UPTAKE AND BINDING TO WHOLE CELL AND SUBCELLULAR FRACTIONS OF PANCREATIC ISLETS AT DIFFERENT TIME INTERVALS

The results of [^{3}H]5-HT binding to the whole cell and subcellular fractions of pancreatic islets at different concentrationsof glucose i.e., 4mM and 20mM for different time intervals are shown as follows:

4.12.1. [^{3}H]5-HT uptake into whole cell

There was a significant increase ($p<0.01$) in [^{3}H]5-HT uptake into islets incubated with 4mM and 20mM glucose for 1hour compared to islets incubated for 30min (Fig.-26a). But after 3hours of incubation there was a significant increase ($p<0.001$) in [^{3}H]5-HT uptake into islets incubated with 20mM glucose compared to 30min incubation. In the case of islets incubated with 4mM glucose there was no significant increase in [^{3}H]5-HT uptake after 3hours of incubation.

4.12.2. [^{3}H]5-HT uptake to nuclear fraction

Incubation of [^{3}H]5-HT with 4mM and 20mM glucose for 30min did not show any significant change in [^{3}H]5-HT binding to nuclear membrane (Fig.-26b). One hour and 3hour incubations with 20mM glucose showed a significant increase ($p<0.05$) in [^{3}H]5-HT binding compared to 4mM glucose and 30min. incubation. Incubations with 4mM glucose did not show any change in [^{3}H]5-HT binding pattern.

4.12.3. [^{3}H]5-HT uptake to mitochondrial and membrane fractions

Mitochondrial and membrane fractions did not show any significant change in binding pattern of [^{3}H]5-HT when incubated with 4mM and 20mM glucose for 30min, 1hour and 3hours (Fig.-26c).

4.12.4. [^{3}H]5-HT uptake to cytosolic fraction

The cytosolic fraction after 1hour incubation showed a significant increase in the presence of [^{3}H]5-HT, 4mM and 20mM glucose ($p<0.05$ and $p<0.01$ respectively),

compared to 30min incubation (Fig.-26d). Incubations for 3 hours also showed a significant increase in [^{3}H]5-HT at both glucose concentrations ($p<0.05$ and $p<0.001$ respectively) compared to 30min incubation. The [^{3}H]5-HT content was significantly high ($p<0.01$) in the cytosol of cells incubated with 20mM glucose for 3 hours compared to the same group at 1hour incubation. However, there was no significant change observed in cells incubated with 4mM glucose for 3hours compared to 1hour incubation.

4.13. MOLECULAR WEIGHT DETERMINATION OF NUCLEAR 5-HT BINDING PROTEIN

PVDF membrane slice No. 42 (Plate-7, Fig.-27) showed maximum DPM after incubation with [^{3}H]5-HT The band corresponding to this slice was compared with known markers stained with coomassie blue and the molecular weight was calculated from the standard curve. The mol. wt. of the 5-HT binding protein was determined as 14,000D (14-kDa).

4.14. AMINO ACID COMPOSITION OF 14-kDa 5-HT BINDING PROTEIN

The 14-kDa 5-HT binding protein was mainly composed of Histidine (79.08%) followed by glycine (6.18%) and glutamine (1.12%). All the other amino acids were present at concentrations below 1% (Table-24, Fig.-28)

4.15. 14-kDa 5-HT BINDING PROTEIN SHOWED MAXIMUM HOMOLOGY TO THE RAT BASIC HELIX-LOOP-HELIX PROTEIN 2 (bHLH2)

The Swiss-Prot online protein composition homology search showed maximum homology to a basic Helix-Loop-Helix protein. This protein is a DNA binding protein and is involved in the control of cell type determination and can act as a transcription factor It belongs to the basic Helix-Loop-Helix family of transcription factors.

Table-1

Blood glucose level and body weight of experimental rats

Animal status	Blood glucose level (mg/dl)	Body weight (g)
Control	83.41 ± 13.39	211 ± 19
Diabetic	376.50 ± 27.28***	198 ± 14
Diabetic + Insulin	124 81 ± 15 72ttt	200 ± 20
Diabetic + Insulin + Tryptophan	180.58 ± 20.80ttt	200 ± 24
Diabetic + Tryptophan	220.25 ± 18.82ttt	160 ± 10

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

***p< 0.001 compared to control.

tttp<0.001 compared to diabetic.

Table-2

Norepinephrine, Epinephrine and Serotonin & metabolites in the cerebral cortex of 14-day experimental rats

Animal status	NE	EPI	5-HTP	5-HIAA	5-HT	5-HTP/5-HT	5-HIAA/5-HT
Control	1.31 ± 0.43	ND	0.22 ± 0 01	1 14 ± 0.11	1.36 ± 0.06	0.18 ± 0.04	0.84 ± 0.07
Diabetic	2.30 ± 0.32	ND	0.09 ± 0.03*	0.96 ± 0.1	0.35 ± 0.01**	0.28 ± 0.02	2.65 ± 0.07**
Diabetic + insulin	1.43 ± 0.62	ND	0.56 ± 0.11†	1 17 ± 0.14	1.01 ± 0.02††	0.55 ± 0.07	1 15 ± 0.10†††
Diabetic + insulin + tryptophan	1.64 ± 0.67	ND	0 46 ± 0 07†	1 47 ± 0.05	1.46 ± 0.05††	0.38 ± 0.08	1.05 ± 0.06†††
Diabetic + tryptophan	1.46 ± 0.52	ND	0.35 ± 0.02†	1.59 ± 0.02	1.66 ± 0.04††	0.24 ± 0.05	0.99 ± 0.03†††

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

*p<0.05 when compared to control, **p<0.01 when compared to control

†††p<0.001 when compared to diabetic. ††p<0.01 when compared to diabetic

†p<0.05 when compared to diabetic, ND- Not detected

NE - Norepinephrine, EPI - Epinephrine, 5-HT - Serotonin, 5-HTP - 5-hydroxytryptophan, 5-HIAA - 5-hydroxyindole acetic acid

Table-3

Norepinephrine, Epinephrine and Serotonin & metabolites in the brainstem of 14-day experimental rats

Animal status	NE	EPI	5-HTP	5-HIAA	5-HT	5-HTP/5-HT	5-HIAA/5-HT
Control	4.75 ± 0.52	ND	0.19 ± 0.04	2.11 ± 0.08	1.36 ± 0.10	0.15 ± 0.08	1.65 ± 0.19
Diabetic	13.62 ± 0.75***	ND	1.51 ± 0.95	1.74 ± 0.10	0.55 ± 0.24*	2.76 ± 0.48***	3.18 ± 0.28*
Diabetic + insulin	7.60 ± 0.58†††	ND	0.45 ± 0.05	2.22 ± 0.25	2.07 ± 0.79†	0.22 ± 0.42†††	1.16 ± 0.50††
Diabetic + insulin + tryptophan	7.90 ± 0.87†††	ND	0.76 ± 0.15	2.55 ± 0.21	2.47 ± 0.36†	0.31 ± 0.25†††	1.05 ± 0.25††
Diabetic + tryptophan	6.58 ± 0.77†††	ND	0.43 ± 0.08	2.26 ± 0.12	1.39 ± 0.27†	0.32 ± 0.19†††	1.54 ± 0.21†

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

*** $p<0.001$ when compared to control, * $p<0.05$ when compared to control

††† $p<0.001$ when compared to diabetic, †† $p<0.01$ when compared to diabetic

† $p<0.05$ when compared to diabetic, ND- Not detected

NE - Norepinephrine, EPI - Epinephrine, 5-HT - Serotonin, 5-HTP - 5-hydroxytryptophan, 5-HIAA - 5-hydroxyindole acetic acid

Table-4

Norepinephrine, Epinephrine and Serotonin & metabolites in the hypothalamus of 14-day experimental rats

Animal status	NE	EPI	5-HTP	5-HIAA	5-HT	5-HTP/5-HT	5-HIAA/5-HT
Control	2.29 ± 0.17	0.55 ± 0.11	0.81 ± 0.04	2.67 ± 0.56	0.37 ± 0.05	2.02 ± 0.05	7.00 ± 0.31
Diabetic	28.15 ±2.39***	0.78 ± 0.14	0 12 ± 0 04***	1 94 ± 0.42	0 23 ± 0.02	0.54 ± 0.03***	8.16 ± 0.21
Diabetic + insulin	3.55 ± 1 00†††	0.65 ± 0.25	0.95 ± 0.13†††	4 05 ± 0.51	0.65 ± 0.14	1 44 ± 0.10†††	6.11 ± 0.30
Diabetic + insulin + tryptophan	5.03 ± 1.47†††	0.53 ± 0.17	0.61 ± 0.08††	4.00 ± 0.60	0.49 ± 0.03	1.24 ± 0.06†††	8.09 ± 0.29
Diabetic + tryptophan	9.45 ± 0.85†††	0.68 ± 0.21	0 99 ± 0.07†††	2.79 ± 0.14	0 49 ± 0.30	2.15 ± 0.16†††	6.06 ± 0.21

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

***$p<0.05$ when compared to control

†††$p<0.01$ when compared to diabetic

††$p<0.01$ when compared to diabetic

NE - Norepinephrine, EPI - Epinephrine, 5-HT - Serotonin, 5-HTP - 5-hydroxytryptophan, 5-HIAA - 5-hydroxyindole acetic acid

Table-5

Norepinephrine, Epinephrine and Serotonin & metabolites in the pancreas of 14-day experimental rats

Animal status	NE	EPI	5-HTP	5-HIAA	5-HT	5-HTP/5-HT	5-HIAA/5-HT
Control	3.04 ± 0.35	0.64 ± 0.34	0.25 ± 0 04	0.20 ± 0.03	0.18 ± 0.07	1.38 ± 0.08	0.99 ± 0.07
Diabetic	4.27 ± 0.34*	1.54 ± 0.48*	0.97 ± 0.07***	0.26 ± 0 13	0.42 ± 0.06*	2.40 ± 0.06***	0.62 ± 0.11
Diabetic + insulin	3.36 ± 0.67	2.22 ± 0.54	0.27 ± 0.07†††	0.22 ± 0.11	0.26 ± 0.04†	1.12 ± 0.06†††	0.83 ± 0.08
Diabetic + insulin + tryptophan	2.90 ± 0.78	0.91 ± 0.64	0.43 ± 0.05†††	0 18 ± 0.10	0.20 ± 0.04†	2.58 ± 0.04	0.94 ± 0.07
Diabetic + tryptophan	3.41 ± 0.68	1 15 ± 0.72	0 65 ± 0.08††	0.20 ± 0.15	0.21 ± 0.05†	2.86 ± 0.06	0.94 ± 0.10

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

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

†††p<0.001 when compared to diabetic, ††p<0.01 when compared to diabetic

†p<0.05 when compared to diabetic

NE - Norepinephrine, EPI - Epinephrine, 5-HT - Serotonin, 5-HTP - 5-hydroxytryptophan, 5-HIAA - 5-hydroxyindole acetic acid

Table-6

Norepinephrine, Epinephrine and Serotonin & metabolites in the plasma of 14-day experimental rats

Animal status	NE	EPI	5-HTP	5-HIAA	5-HT	5-HTP/5-HT	5-HIAA/5-HT
Control	46.53 ± 14.63	45.51 ± 3.99	4.65 ± 0.74	0.39 ± .02	0.34 ± 0.22	13.04 ± 0.45	1 15 ± 0.12
Diabetic	69.67 ± 17 91	73.82 ± 5.53*	8.07 ± 0.65*	0 43 ± 0.02	1.82 ± 0.30***	4.52 ± 0.69***	0.24 ± 0.16**
Diabetic + insulin	58.73 ± 9 72	34 07 ± 6.33††	5.02 ± 0.50†	0.27 ± 0 10	0.37 ± 0.18†††	13.33 ± 0.35†††	0.73 ± 0.15
Diabetic + insulin + tryptophan	47 17 ± 18.37	47.39 ± 9 00†	3.80 ± 0.97†	0.42 ± 0.19	0.64 ± 0.13††	6.20 ± 0.58***	0.65 ± 0.15
Diabetic + tryptophan	41.38 ± 9.02	33.84 ± 6.66††	5 15 ± 0.65†	0 48 + 0 06	0 45 ± 0.09††	11.68 ± 0.40†††	1.07 ± 0.08††

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

***p<0.001 when compared to control, **p<0.01 when compared to control

*p<0.05 when compared to control, †††p<0.001 when compared to diabetic

††p<0.01 when compared to diabetic, †p<0.05 when compared to diabetic.

NE - Norepinephrine, EPI - Epinephrine, 5-HT - Serotonin, 5-HTP - 5-hydroxytryptophan, 5-HIAA - 5-hydroxyindole acetic acid

Table-7

[*m-methoxy*- ^{3}H] MDL100907 (5-HT_{2A} receptor) binding parameters in cerebral cortex of experimental rats

Animal Status	[*m-methoxy*- ^{3}H] MDL100907 binding	
	B_{max} (fmoles/mg protein)	K_d (nM)
Control	230.00 ± 45 70	1 08 ± 0 11
Diabetic	208.66 ± 59 84	0.60 ± 0.09*
Diabetic + Insulin	212.00 ± 39 58	0.95 ± 0.15†
Diabetic + Insulin + Tryptophan	226.34 ± 43.21	1 13 ± 0.20†
Diabetic + Tryptophan	246.26 ± 40.26	1.20 ± 0 18†

Values are mean ± S.E.M. of 4-6 separate experiments.
*$p < 0.05$ compared to control.
†$p<0.05$ compared to diabetic.
B_{max} - Binding maximum, (fmoles/mg protein).
K_d - Dissociation constant (nM).

Table-8

Binding parameters of [^{3}H]MDL100907 against ketanserin in cerebral cortex of experimental rats

Animal status	Best-fit model	log(EC_{50})-1	log(EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control	One-site	-7.45		$2.63x10^{-8}$		1.09
Diabetic	Two-site	-7 98	-5.99	$8.15x10^{-9}$	$8.05x10^{-7}$	0.66
Diabetic + Insulin	One-site	-7.58		$2.15x10^{-8}$		1 42
Diabetic + Insulin + Tryptophan	One-site	-7 91		$6.45x10^{-9}$		1 25
Diabetic + Tryptophan	One-site	-7 74		$1.28x10^{-8}$		1.12

Data are from displacement curves as determined by non-linear regression analysis using the computer program PRISM and a one-site Vs two-site model. 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 and it is same as IC_{50}. The equation built into the programme is defined in terms of the log(EC_{50}).

Table-9

[*m-methoxy*- ^{3}H] MDL100907 (5-HT_{2A} receptor) binding parameters in brain stem of experimental rats

Animal Status	[*m-methoxy*- ^{3}H] MDL100907 binding	
	B_{max} (fmoles/mg protein)	K_d (nM)
Control	34 77 ± 5.27	0.77 ± 0.30
Diabetic	80.60 ± 7 40*	2.62 ± 0.53*
Diabetic + Insulin	47.22 ± 7.50†	0.90 ± 0.35†
Diabetic + Insulin + Tryptophan	63.32 ± 6.35	1.08 ± 0 42
Diabetic + Tryptophan	46.52 ± 6.83†	0.77 ± 0.39†

Values are mean ± S.E.M. of 4-6 separate experiments.
*$p < 0.05$ compared to control.
†$p<0.05$ compared to diabetic.
B_{max} - Binding maximum, (fmoles/mg protein).
K_d - Dissociation constant (nM).

Table-10

Binding parameters of [^{3}H]MDL100907 against ketanserin in brain stem of experimental rats

Animal status	Best-fit model	log(EC_{50})-1	log(EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control	One-site	-9 47	-	$2.29x10^{-10}$	-	2.54
Diabetic	Two-site	-9.30	-7 14	$4 46x10^{-10}$	$6.475x10^{-8}$	0 54
Diabetic + Insulin	One-site	-9.37		$3.04x10^{-10}$		2.24
Diabetic + Insulin + Tryptophan	One-site	-9 46		$2.35x10^{-10}$		3 33
Diabetic + Tryptophan	One-site	-9.62		$1 44x10^{-10}$		0 99

Data are from displacement curves as determined by non-linear regression analysis using the computer program PRISM and a one-site Vs two-site model. 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 and it is same as IC_{50}. The equation built into the programme is defined in terms of the log(EC_{50}).

Table-11

[*m-methoxy*- ^{3}H[MDL100907 (5-HT_{2A} receptor) binding parameters in hypothalamus of experimental rats

Animal status	[*m-methoxy*-^{3}H]MDL100907	
	B_{max} (fmoles/mg protein)	K_d (nM)
Control	34.50 ± 4.52	0.23 ± 0 09
Diabetic	35.00 ± 6.34	0 15 ± 0.07*
Diabetic + insulin	52.00 ± 6.50	0.38 ± 0 11†
Diabetic + insulin + tryptophan	40.50 ± 6.50	0.25 ± 0 10†
Diabetic + tryptophan	32.00 ± 5.84	0.22 ± 0.10†

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

*$p<0.05$ when compared to control

†$p<0.05$ when compared to diabetic

B_{max} - Binding maximum, (fmoles/mg protein); K_d - Dissociation constant (nM).

Table-12

Binding parameters of [*m-methoxy*-^{3}H]MDL100907 against ketanserin in hypothalamus of experimental rats

Animal status	Best fit model	Log(EC_{50})-1	Log(EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control	One-site	-6.76		$8.24x10^{-8}$		1.36
Diabetic	Two-site	-9.22	-5.53	$2.83x10^{-10}$	$1.39x10^{-6}$	0 65
Diabetic + insulin	One-site	-6 70		$9\ 41x10^{-8}$		1 75
Diabetic + insulin + tryptophan	One-site	-6 77		$7\ 98x10^{-8}$		1 46
Diabetic + tryptophan	One-site	-6.44		$1\ 72x10^{-7}$		1.26

Data are from displacement curves as determined by non-linear regression analysis using the computer program PRISM and a one-site Vs two-site model. 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 and it is same as IC_{50}. The equation built into the programme is defined in terms of the log(EC_{50}).

Table-13

[^{3}H]8-OH-DPAT (5-HT_{1A} receptor) binding parameters in cerebral cortex of experimental rats

Animal status	[^{3}H]8-OH-DPAT binding			
	High affinity		Low affinity	
	B_{max} (fmoles/mg protein)	K_d (nM)	B_{max} (fmoles/mg protein)	K_d (nM)
Control	153.50 ± 15.00	0.98 ± 0.08	718 55 ± 73 15	11 17 ± 2.11
Diabetic	126.50 ± 13.50**	0.97 ± 0.22	956.15 ± 96 42*	32.44 ±6 77*
Diabetic + Insulin	86.86 ± 3.82***	0.43 ± 0.11***	889 10 ± 32.30	22.07 ± 1 92
Diabetic + Insulin + Tryptophan	79.50 ± 3.20***	0.26 ± 0.05***	925.56 ± 68.75	10.39 ± 5 61†
Diabetic + Tryptophan	71.50 ± 2.12***	0.32 ± 0.08***	652.50 ± 87.50	6.85 ± 2.29††

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

$^{*}p< 0.05$ compared to control, $^{**}p< 0.01$ compared to control,

$^{***}p< 0.001$ compared to control, $^{\dagger}p<0.05$ compared to diabetic.

$^{\dagger\dagger}p<0.01$ compared to diabetic.

B_{max} - Binding maximum, (fmoles/mg protein)

K_d - Dissociation constant (nM)

Table-14

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

Animal status	Best-fit model	log(EC_{50})-1	log(EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control	Two-site	-11.68	-6.22	1.42×10^{-12}	4.05×10^{-7}	1.32
Diabetic	Two-site	-12.21	-5.93	4.18×10^{-13}	7.99×10^{-7}	1.21
Diabetic + Insulin	Two-site	-10.30	-5.60	3.39×10^{-11}	1.73×10^{-6}	1.55
Diabetic + Insulin + Tryptophan	Two-site	-12.59	-5.55	1.76×10^{-13}	1.91×10^{-6}	1.56
Diabetic + Tryptophan	Two-site	-9.13	-5.99	5.08×10^{-10}	7.05×10^{-7}	1.23

Data are from displacement curves as determined by non-linear regression analysis using the computer program PRISM and a one-site Vs two-site model. 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 and it is same as IC_{50}. The equation built into the programme is defined in terms of the log(EC_{50}).

Table-15

Binding parameters of [^{3}H]8-OH-DPAT against 5-HT in the cerebral cortex of experimental rats with and without Gpp[NH]p

Animal status	Best fit model	Log(EC_{50})-1	Log(EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control	Two-site	-11.68	-6.22	$1.41x10^{-12}$	$4.05x10^{-7}$	1.32
Control + Gpp[NH]p	Two-site	-12.41	-5.93	$2.64x10^{-13}$	$7.95x10^{-7}$	1 18
Diabetic	Two-site	-12.21	-5 93	$4.16x10^{-13}$	$7.96x10^{-7}$	1.21
Diabetic + Gpp[NH]p	Two-site	-12.42	-5.74	$2.55x10^{-13}$	$1.23x10^{-6}$	1 17
Diabetic + insulin	Two-site	-10.30	-5 60	$3.37x10^{-11}$	$1 72x10^{-6}$	1 55
Diabetic + insulin + Gpp[NH]p	Two-site	-12.46	-5.86	$2.34x10^{-13}$	$9.46x10^{-7}$	1 18
Diabetic + insulin + tryptophan	Two-site	-12.59	-5.55	$1 78x10^{-13}$	$1.89x10^{-6}$	1.56
Diabetic + insulin + tryptophan + Gpp[NH]p	Two-site	-12.38	-5.69	$2.81x10^{-13}$	$1.37x10^{-6}$	1 19
Diabetic + tryptophan	Two-site	-9 13	-5 99	$5 05x10^{-10}$	$7.01x10^{-7}$	1.23
Diabetic + tryptophan + Gpp[NH]p	Two-site	-12.39	-5 79	$2.79x10^{-13}$	$1 11x10^{-6}$	1 18

Data are from displacement curves as determined by non-linear regression analysis using the computer program PRISM and a one-site Vs two-site model. 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 and it is same as IC_{50}. The equation built into the programme is defined in terms of the log(EC_{50}).

Table-16

[^{3}H]8-OH-DPAT (5-HT$_{1A}$ receptor) binding parameters in brain stem of experimental rats

Animal status	[^{3}H]8-OH-DPAT binding			
	High affinity		Low affinity	
	B_{max} (fmoles/mg protein)	K_d (nM)	B_{max} (fmoles/mg protein)	K_d (nM)
Control	54.00 ± 6 00	2.77 ± 0.64	290.00 ± 30 00	24.18 ± 6.22
Diabetic	70 00 ± 10.00*	3.11 ± 1.39	351.00 ± 36.00	9 42 ± 6.40*
Diabetic + Insulin	34.00 ± 11.00†††	1.38 ± 0.56	280.10 ±25.23	18.16 ± 5.34†
Diabetic + Insulin + Tryptophan	22.65 ± 4 63†††	0.80 ± 0.20	296.54 ± 42.71	21.63 ± 10 99†
Diabetic + Tryptophan	32.75 ± 4.75†††	1.16 ± 0.21	200.52 ± 47.56	14.25 ± 8.94†

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

$^{*}p<0.05$ compared to control, $^{**}p<0.01$ compared to control.

$^{\dagger}p<0.05$ compared to diabetic, $^{\dagger\dagger\dagger}p<0.001$ compared to diabetic

B_{max} - Binding maximum, (fmoles/mg protein); K_d - Dissociation constant (nM).

Table-17

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

Animal status	Best fit model	Log(EC_{50})-1	Log(EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control	Two-site	-12.35	-6.53	$2.65x10^{-13}$	$1.79x10^{-7}$	1.23
Diabetic	Two-site	-12.26	-6.30	$3.26x10^{-13}$	$3.00x10^{-7}$	1.20
Diabetic + insulin	Two-site	-12.46	-6.12	$2.09x10^{-13}$	$4.55x10^{-7}$	1.25
Diabetic + insulin + tryptophan	Two-site	-9.85	-6.17	$8.47x10^{-11}$	$4.58x10^{-7}$	1.32
Diabetic + tryptophan	Two-site	-9.78	-6.25	$9.79x10^{-11}$	$3.30x10^{-7}$	1.30

Data are from displacement curves as determined by non-linear regression analysis using the computer program PRISM and a one-site Vs two-site model. 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 and it is same as IC_{50}. The equation built into the programme is defined in terms of the log(EC_{50}).

Table-18

Binding parameters of [^{3}H]8-OH-DPAT against 5-HT in the brain stem of experimental rats with and without Gpp[NH]p

Animal status	Best fit model	Log(EC_{50})-1	Log(EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control	Two-site	-12.35	-6.53	$2.65x10^{-13}$	$1.79x10^{-7}$	1.23
Control + Gpp[NH]p	Two-site	-12.43	-6.22	$2.05x10^{-13}$	$3.61x10^{-7}$	1.15
Diabetic	Two-site	-12.26	-6.30	$3.26x10^{-13}$	$3.00x10^{-7}$	1.20
Diabetic + Gpp[NH]p	Two-site	-12.42	-6.07	$2.29x10^{-13}$	$5.10x10^{-7}$	1.06
Diabetic + insulin	Two-site	-12.46	-6.12	$2.09x10^{-13}$	$4.55x10^{-7}$	1.25
Diabetic + insulin + Gpp[NH]p	Two-site	-12.38	-5.84	$2.47x10^{-13}$	$8.66x10^{-7}$	1.13
Diabetic + insulin + tryptophan	Two-site	-9.85	-6.17	$8.47x10^{-11}$	$4.58x10^{-7}$	1.32
Diabetic + insulin + tryptophan + Gpp[NH]p	Two-site	-12.32	-5.76	$2.84x10^{-13}$	$1.03x10^{-6}$	1.26
Diabetic + tryptophan	Two-site	-9.78	-6.25	$9.70x10^{-11}$	$3.30x10^{-7}$	1.30
Diabetic + tryptophan + Gpp[NH]p	Two-site	-12.23	-6.23	$3.54x10^{-13}$	$3.49x10^{-7}$	1.21

Data are from displacement curves as determined by non-linear regression analysis using the computer program PRISM and a one-site Vs two-site model. 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 and it is same as IC_{50}. The equation built-into the programme is defined in terms of the log(EC_{50}).

Table-19

[^{3}H]8-OH-DPAT (5-HT$_{1A}$ receptor) binding parameters in hypothalamus of experimental rats

Animal status	[3H]8-OH-DPAT binding			
	High affinity		Low affinity	
	B_{max} (fmoles/mg protein)	K_d (nM)	B_{max} (fmoles/mg protein)	K_d (nM)
Control	500.00 ± 12.70	2.97 ± 0.16	1550.00 ± 75 60	13.36 ± 1.36
Diabetic	210.00 ± 18 64***	0.95 ± 0.25***	3400.00 ± 80.80***	46.90 ± 1.87***
Diabetic + insulin	310.00 ± 15 03***	1 91 ± 0.13**	2750.00 ± 79.30**	30.33 ± 2.30***
Diabetic + insulin + tryptophan	175.30 ± 20.50***	0 90 ± 0.11***	2650.00 ± 81.30**	40.76 ± 1 54***
Diabetic + tryptophan	171 00 ± 19 63***	1 06 ± 0.13**	2750.00 ± 77 60**	45 83 ± 2.54***

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

*** $p<0.001$ when compared to control.

** $p<0.01$ when compared to control.

B_{max} - Binding maximum, (fmoles/mg protein); K_d - Dissociation constant (nM).

Table-20

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

Animal status	Best fit model	Log(EC_{50})-1	Log(EC_{50})-2	$Ki_{(H)}$	$Ki_{(L)}$	Hill slope
Control	Two-site	-13 11	-5.77	$6.77x10^{-14}$	$1\ 47x10^{-6}$	1.21
Diabetic	Two-site	-12.57	-5.51	$2.34x10^{-13}$	$2.71x10^{-6}$	1 32
Diabetic + insulin	Two-site	-12.12	-5.91	$6.61x10^{-13}$	$1.08x10^{-6}$	1 45
Diabetic + insulin + tryptophan	Two-site	-9 43	-6.11	$3.30x10^{-10}$	$6.80x10^{-7}$	1 02
Diabetic + tryptophan	Two-site	-10.41	-5.99	$3.34x10^{-11}$	$9.09x10^{-7}$	1.22

Data are from displacement curves as determined by non-linear regression analysis using the computer program PRISM and a one-site Vs two-site model. 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 and it is same as IC_{50}. The equation built into the programme is defined in terms of the log(EC_{50}).

Table-21

Tri-iodothyronine (T_3) and Thyroxine (T_4) levels in the plasma of experimental rats

Animal status	T_3 (ng/dl)	T_4 (μg/dl)
Control	96.00 ± 6.20	3 40 ± 0.24
Diabetic	50.50 ± 7.50**	1 05 ± 0 14***
Diabetic – Insulin	54.60 ± 8.60	1 60 ± 0.17
Diabetic + Insulin + tryptophan	46 00 ± 8 10	1 40 ± 0.10
Diabetic + tryptophan	49 00 ± 4.50	1.30 ± 0 18

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

*** $p<0.001$ when compared to the controls

** $p<0.01$ when compared to the controls

Table-22

[^{3}H]5-HT binding status to different fractions of isolated pancreatic islets of experimental rats

Animal status	Membrane + Mitochondrial fraction	Nuclear fraction
Control		+
Diabetic		+
Diabetic + Insulin		+
Diabetic + Insulin + Tryptophan		-
Diabetic + Tryptophan		+

- No [^{3}H]5-HT binding
+ [^{3}H]5-HT binding

Table-23

Binding parameters of [^{3}H]5-HT to nuclear binding sites in isolated pancreatic Islets of experimental rats

Animal Status	[^{3}H]5-HT binding	
	B_{max} (fmoles/mg protein)	K_d (nM)
Control	9750 ± 884	94 05 ± 36.07
Diabetic	1637 ± 488**	36.38 ± 21.04*
Diabetic + insulin	2238 ± 568**	42.25 ± 20.25*
Diabetic + insulin + tryptophan	2450 ± 603**	53.33 ± 19 48*
Diabetic + tryptophan	3588 ± 588**	46.75 ± 23.56*

Values are mean ± S.E.M. of 4-6 separate experiments.
** $p< 0.01$ compared to control, * $p<0.05$ compared to control.
B_{max} - Binding maximum, (fmoles/mg protein); K_d - Dissociation constant (nM)

Table-24

Amino acid composition of 14-kDa 5-HT binding nuclear protein of pancreatic islets

AMINO ACID COMPOSITION IN PERCENTAGE	
ASPARTATE	0.77
GLUTAMATE	1 12
SERINE	0.70
HISTIDINE	**79.08**
GLYCINE	6.18
THREONINE	0.42
ALANINE	0.54
PROLINE	0.36
TYROSINE	0.37
ARGININE	0.45
VALINE	0.55
METHIOININE	0.66
ISOLEUCINE	0.42
LEUCINE	0.80
PHENYL ALANINE	0.40
LYSINE	0.58

Figure-1

Effect of 8-OH-DPAT on blood glucose level

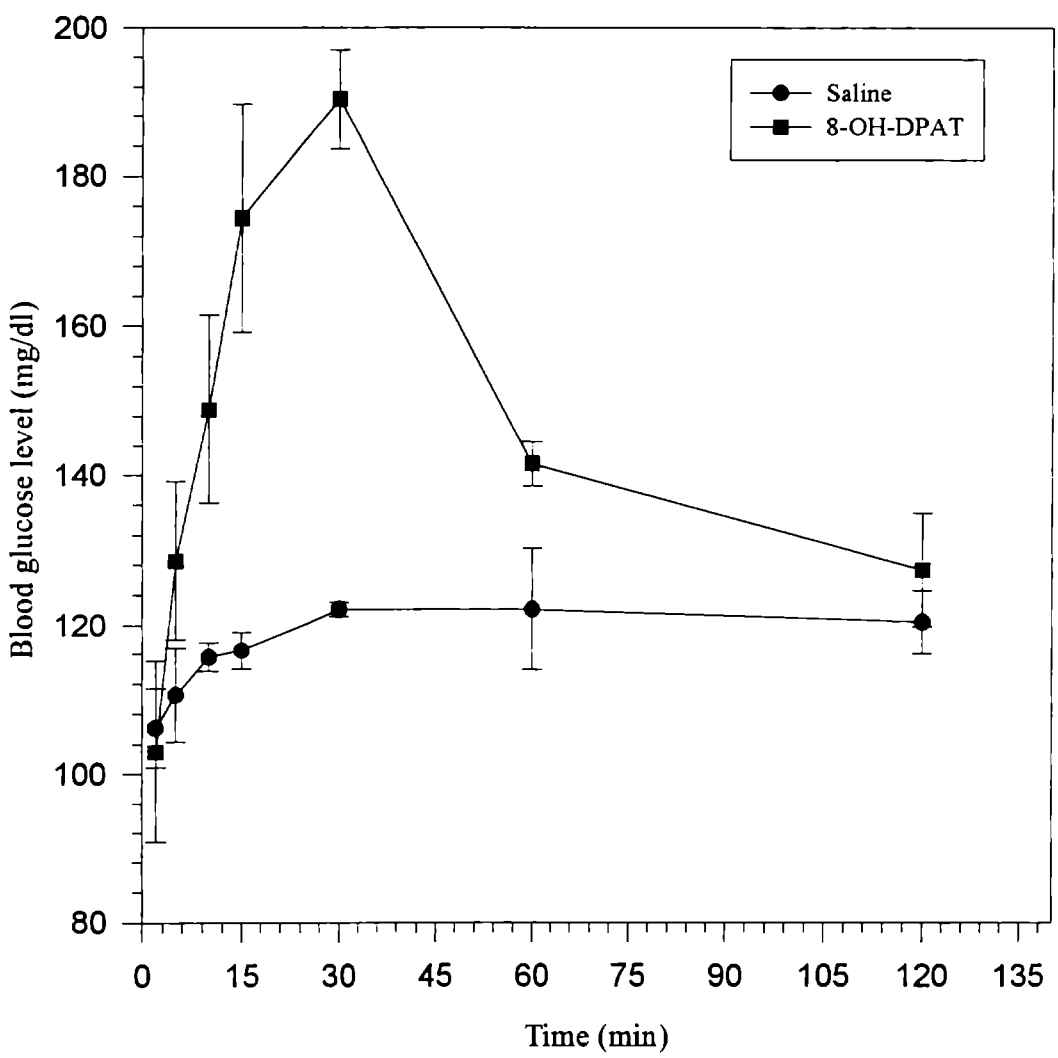


Figure-2a

Scatchard analysis of [^{3}H]MDL100907 binding against ketanserin in cerebral cortex of control and experimental rats

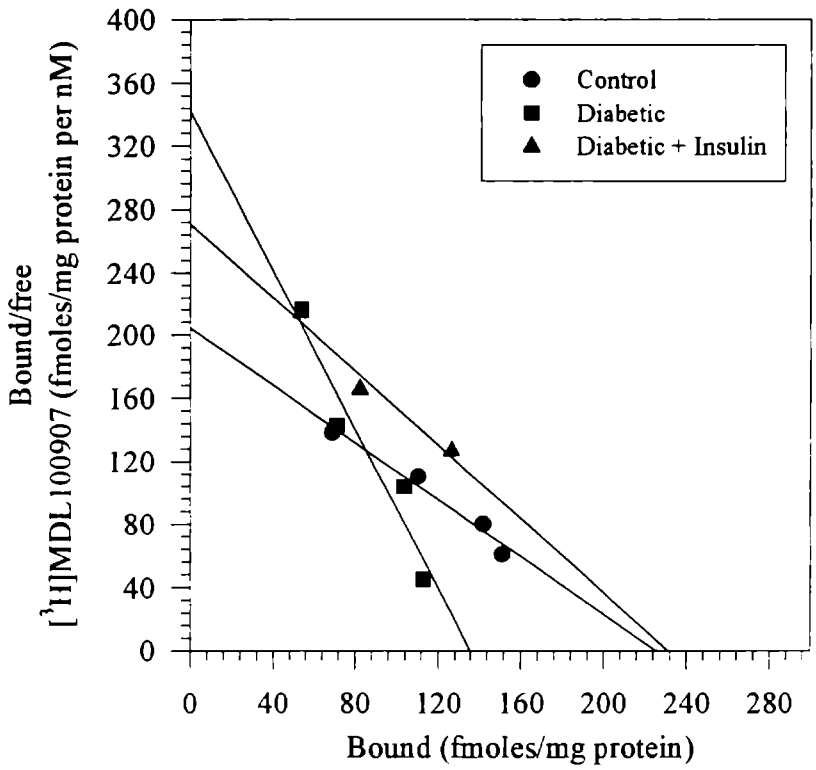


Figure-2b

Scatchard analysis of [^{3}H]MDL100907 binding against ketanserin in cerebral cortex of control and experimental rats

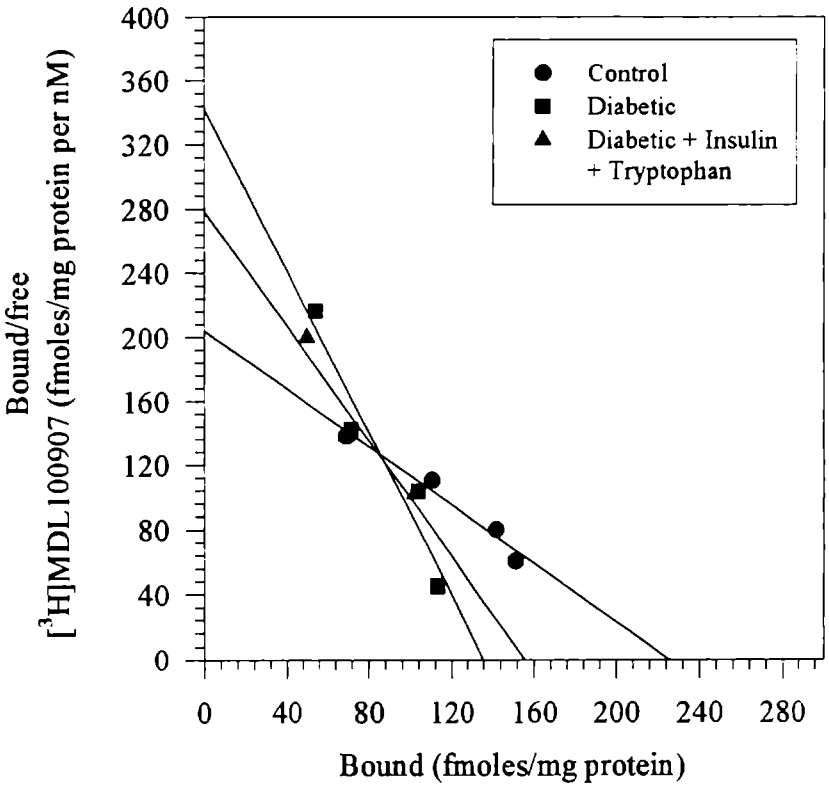
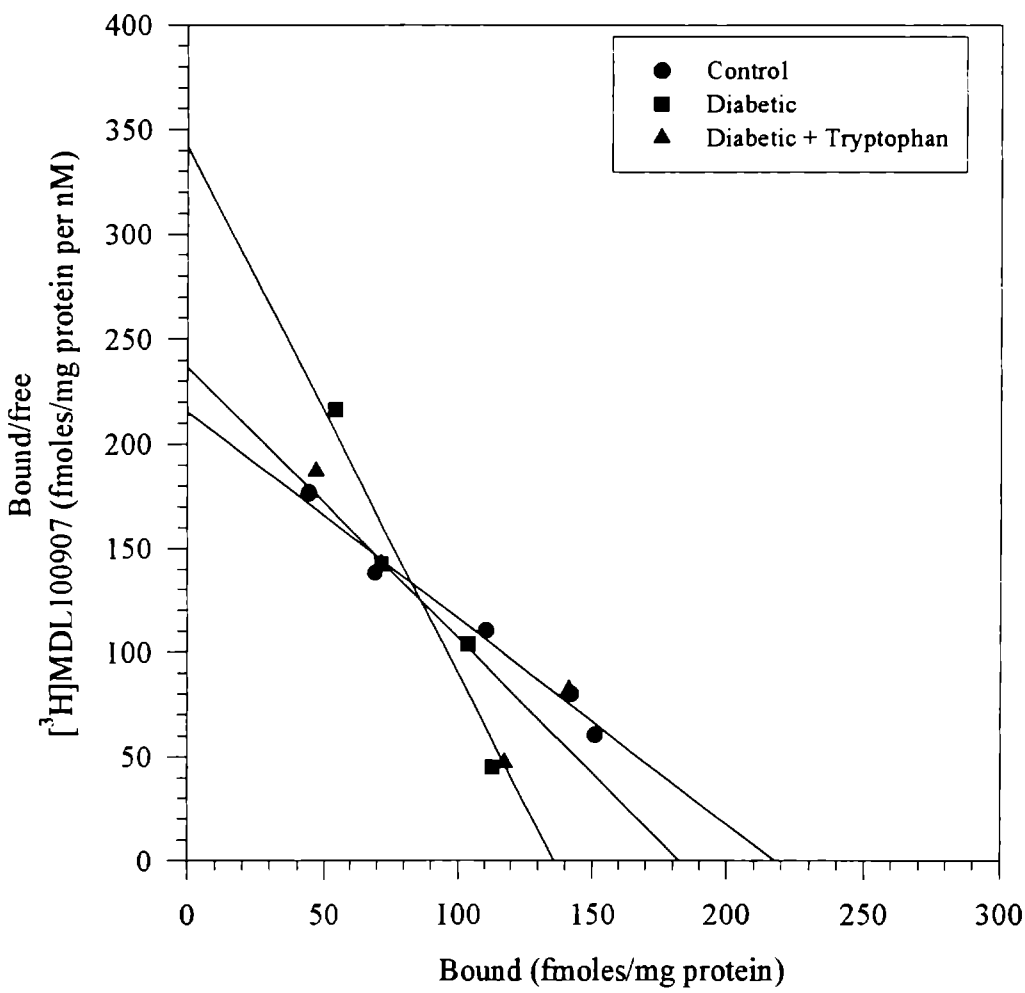


Figure-2c

Scatchard analysis of [^{3}H]MDL100907 binding against ketanserin in cerebral cortex of control and experimental rats



Displacement analysis using ketanserin against [^{3}H]MDL100907 in cerebral cortex of experimental rats

Figure-3a

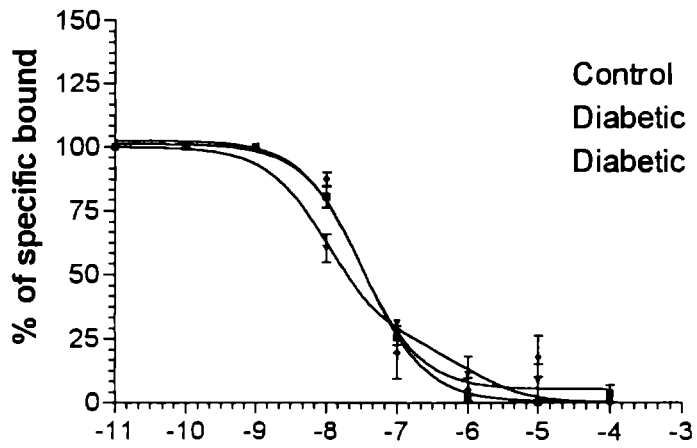


Figure-3b

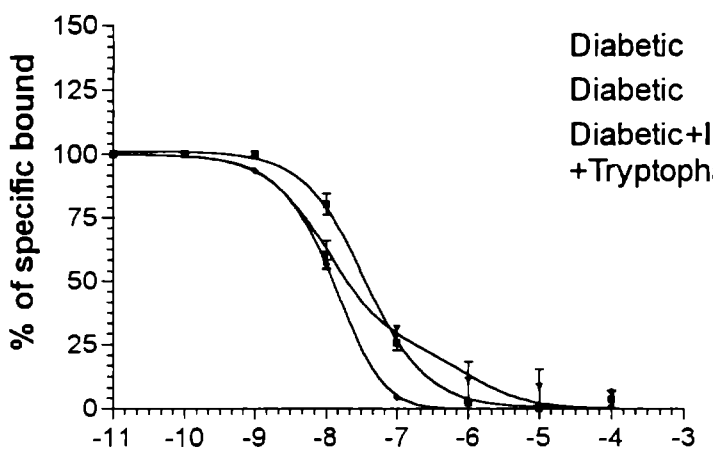


Figure-3c

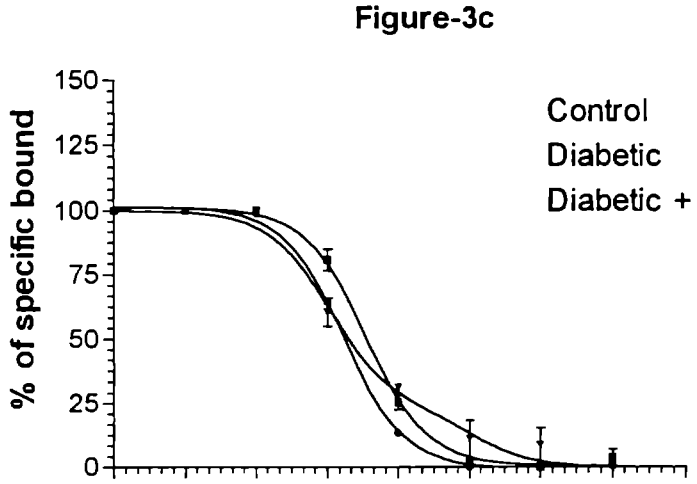


Figure-4a

Scatchard analysis of [^{3}H]MDL100907 binding against ketanserin in brain stem of control and experimental rats

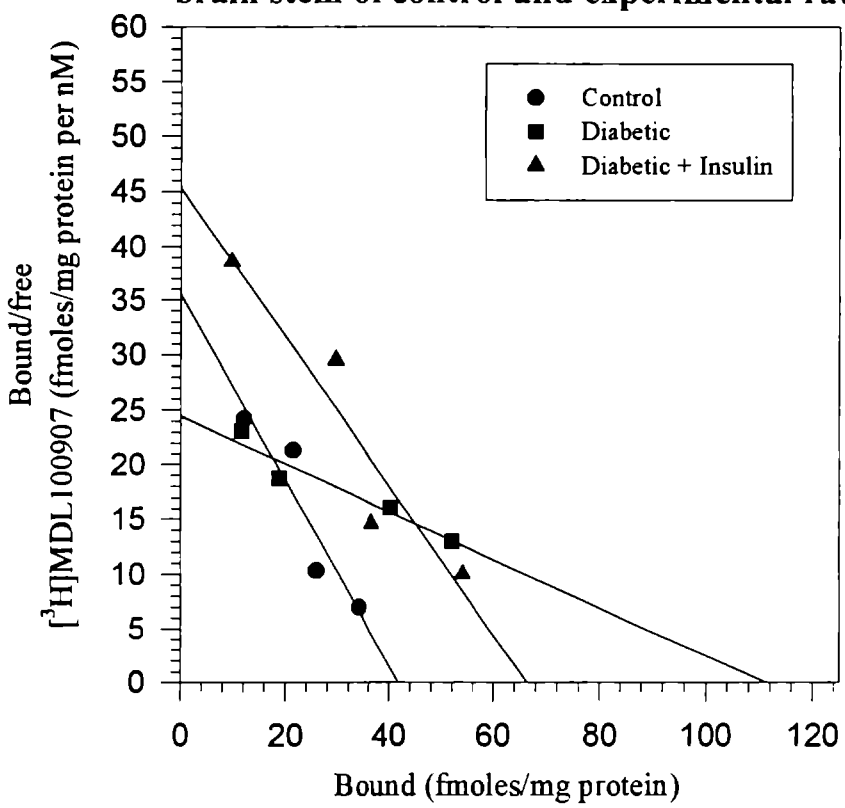


Figure-4b

Scatchard analysis of [^{3}H]MDL100907 binding against ketanserin in brain stem of control and experimental rats

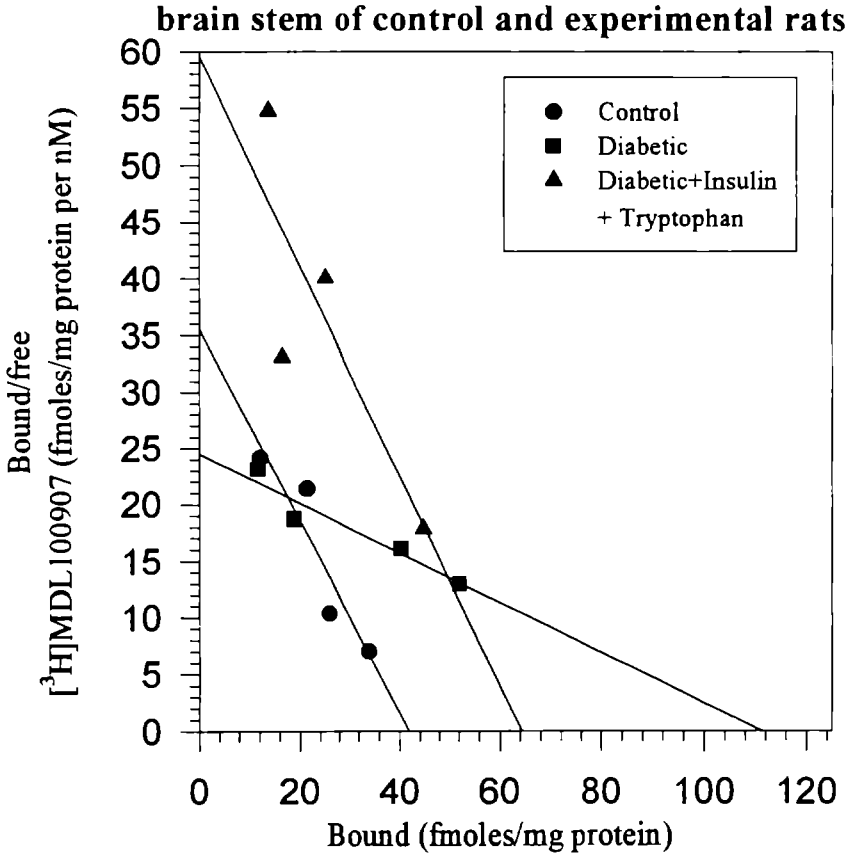
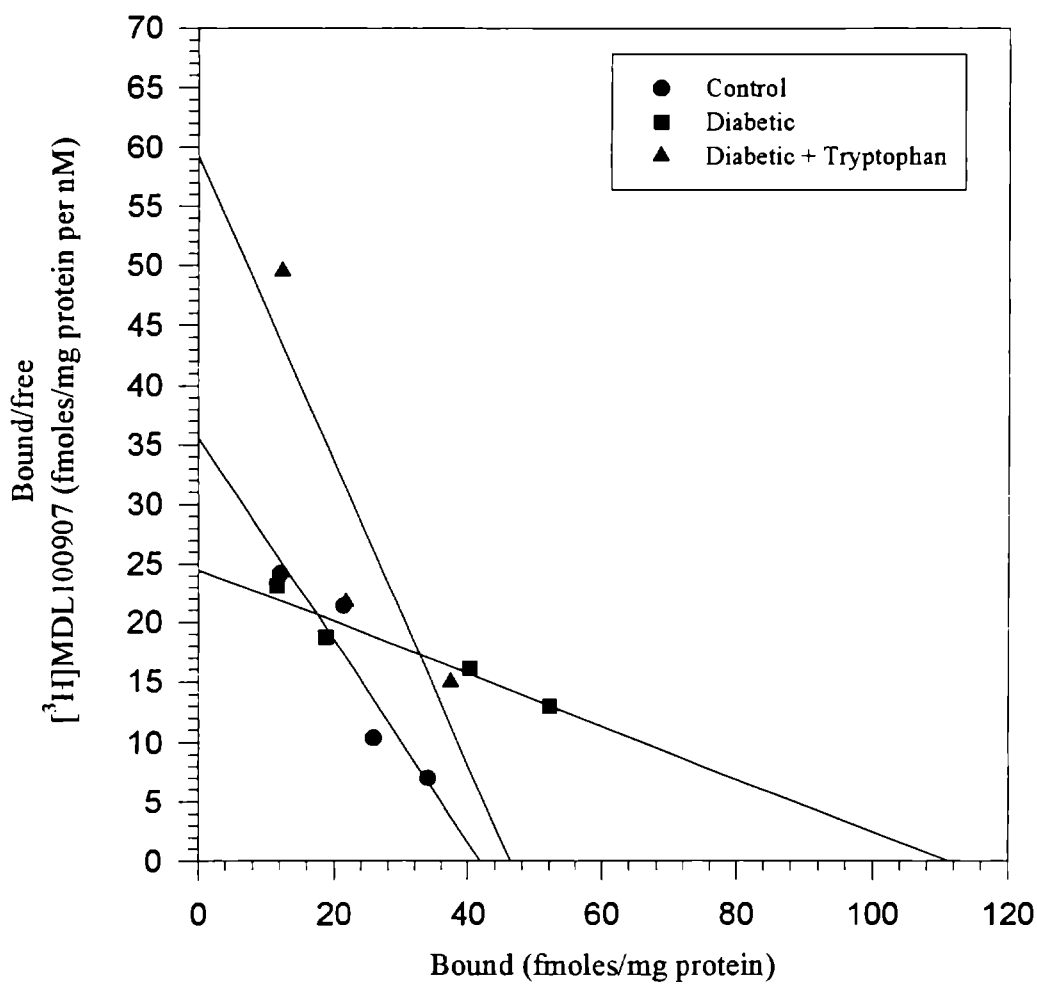


Figure-4c

Scatchard analysis of [^{3}H]MDL100907 binding against ketanserin in brain stem of control and experimental rats



Displacement analysis using ketanserin against [^{3}H]MDL100907 in brain stem of experimental rats

Figure-5a

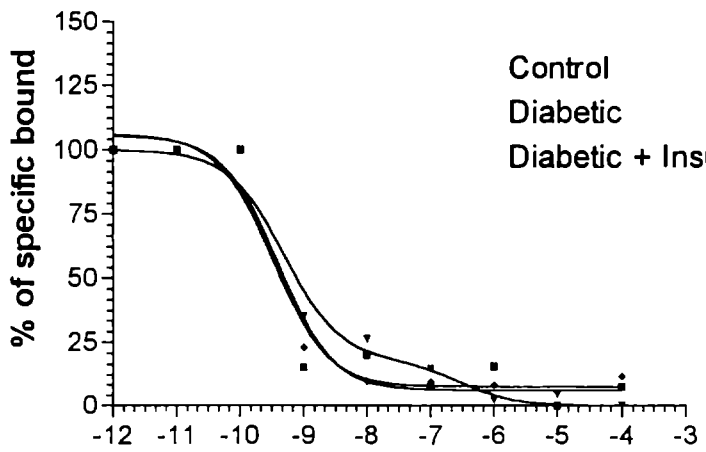


Figure-5b

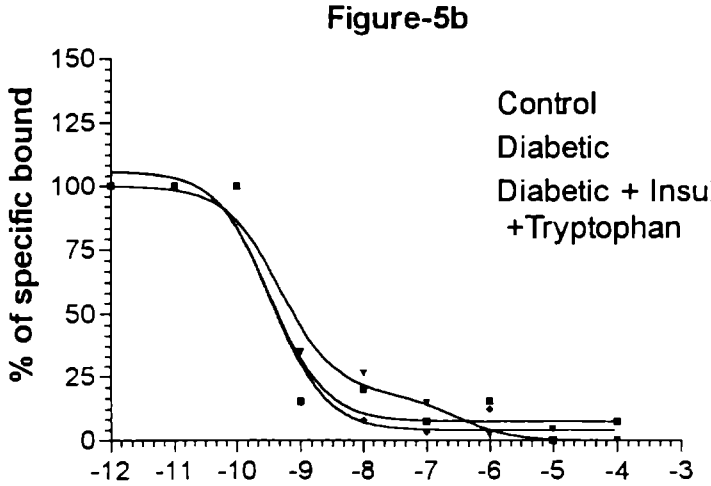


Figure-5c

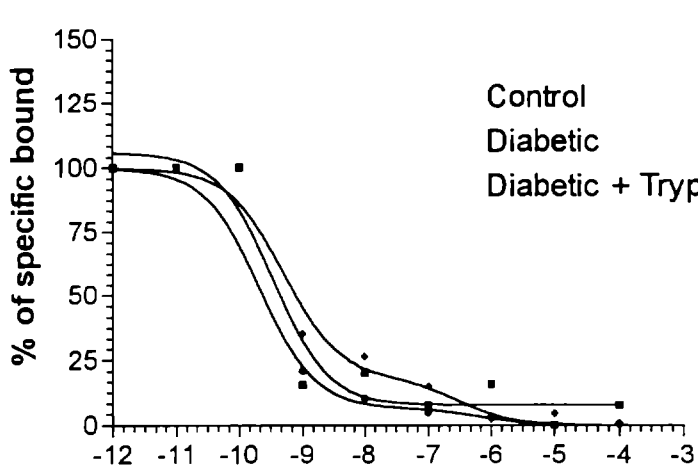


Figure-6a

Scatchard analysis of [^{3}H]MDL100907 binding against ketanserin in hypothalamus of control and experimental rats

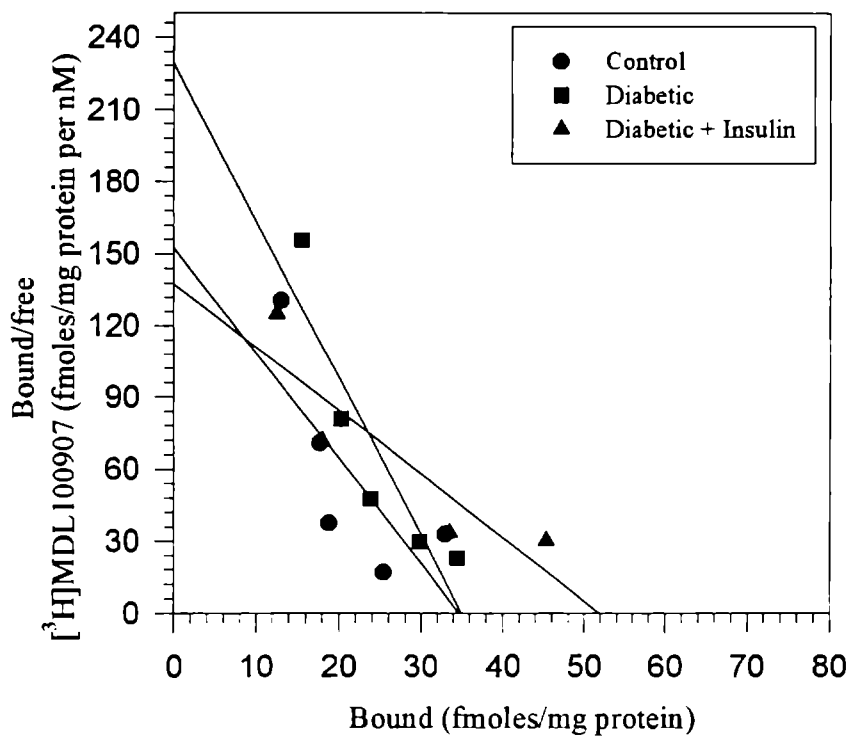


Figure-6b

Scatchard analysis of [^{3}H]MDL100907 binding against ketanserin in hypothalamus of control and experimental rats

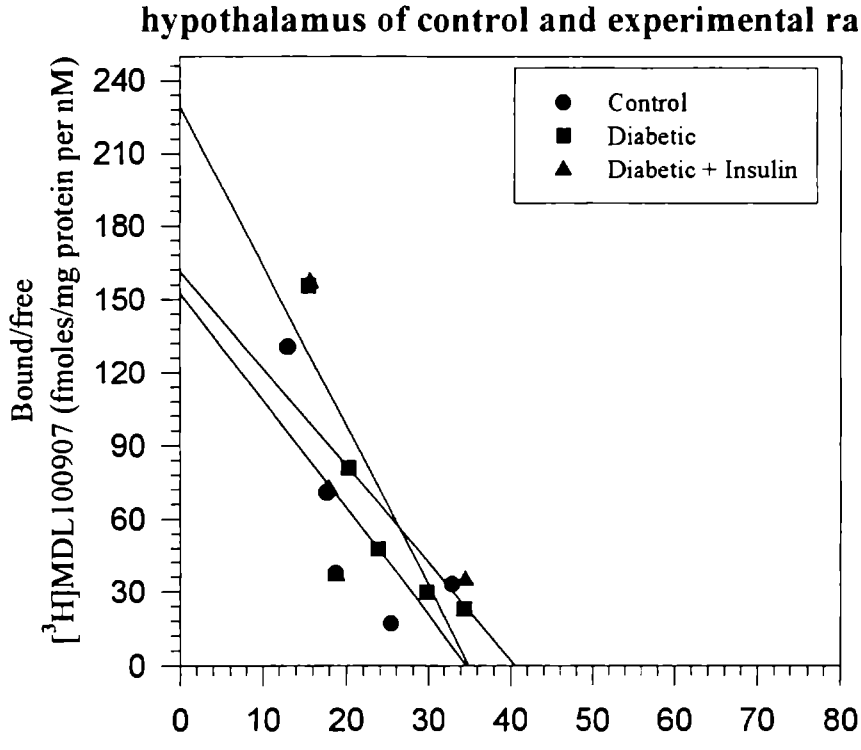
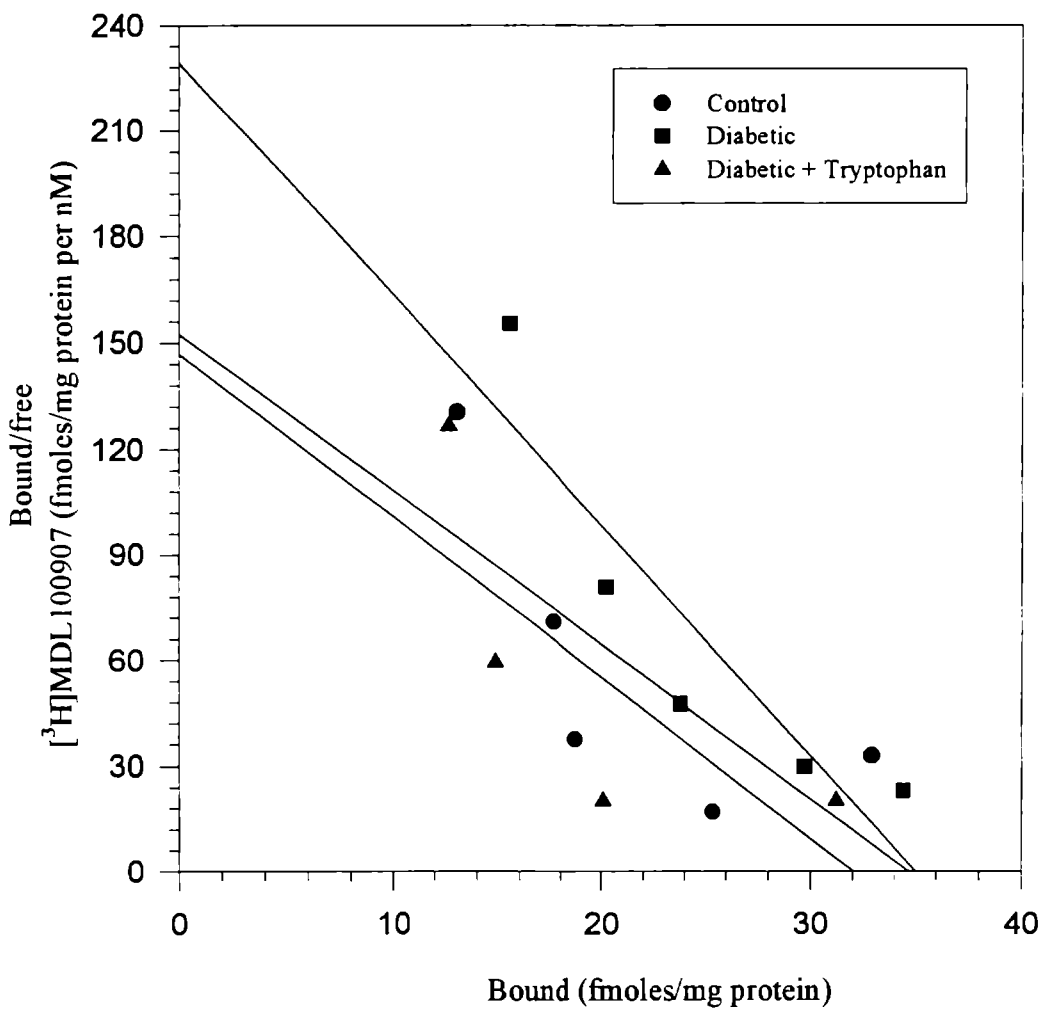


Figure-6c

Scatchard analysis of [^{3}H]MDL100907 binding against ketanserin in hypothalamus of control and experimental rats



Displacement analysis using ketanserin against [^{3}H]MDL100907 in hypothalamus of experimental rats

Figure-7a

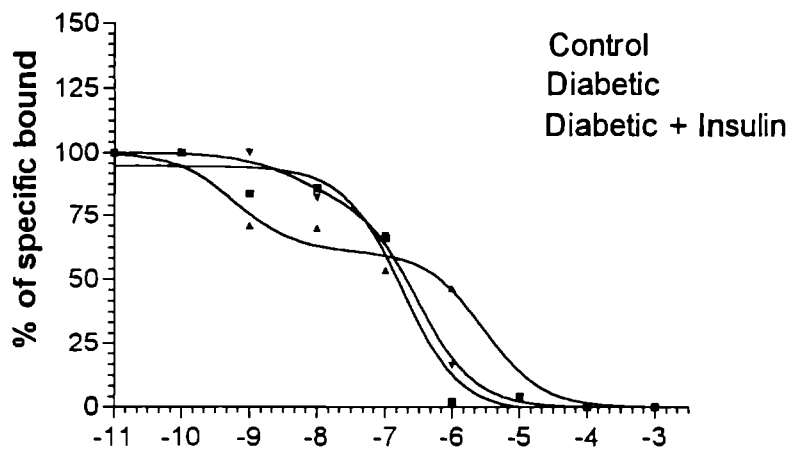


Figure-7b

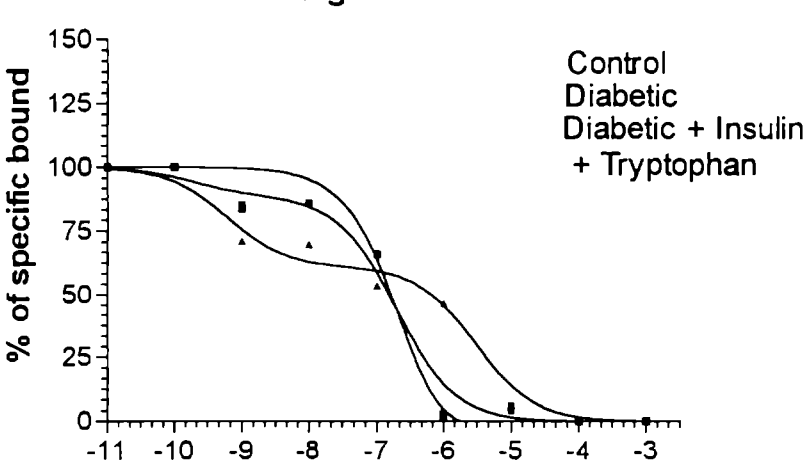


Figure-7c

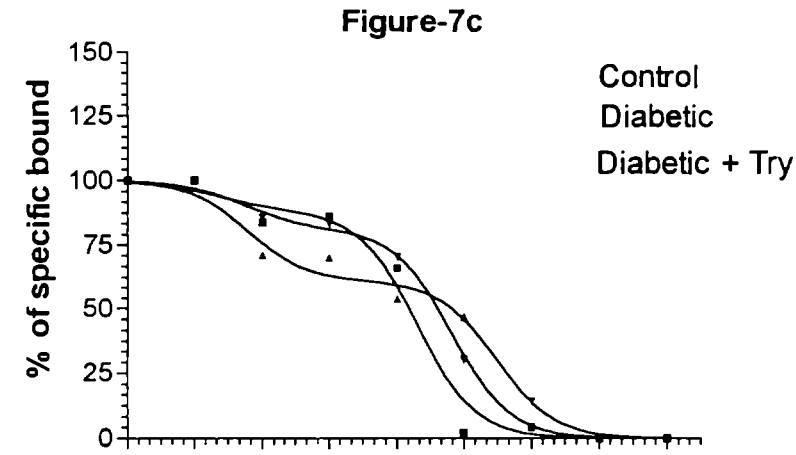
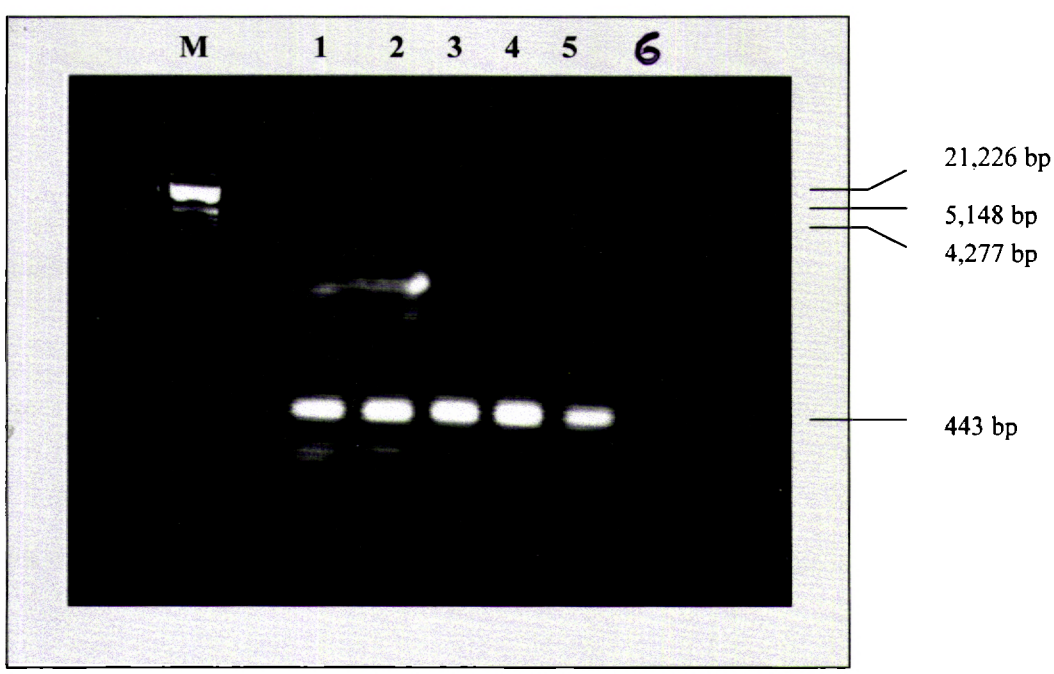


PLATE-1

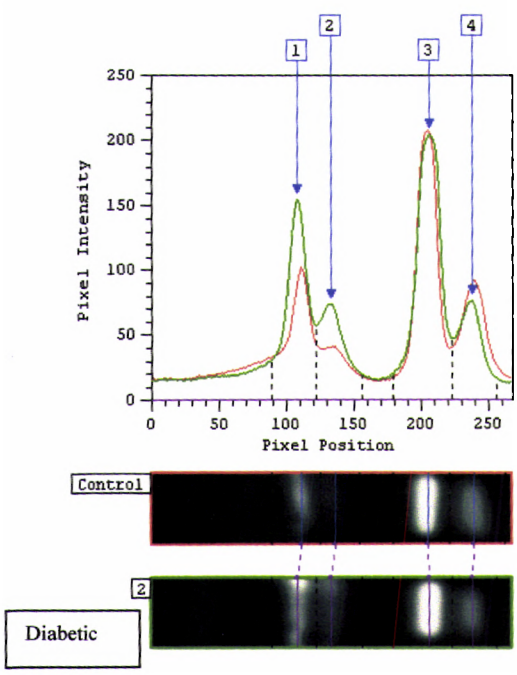


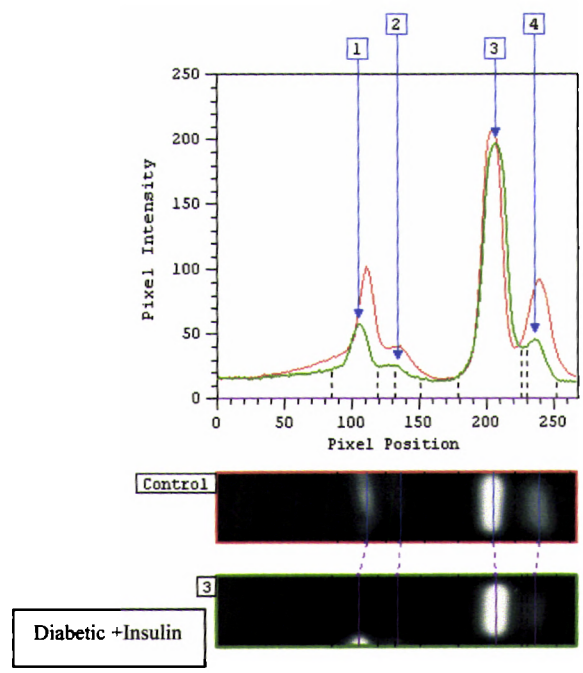
RT-PCR amplification product of 5-HT_{2A} receptor mRNA from cerebral cortex of experimental rats.

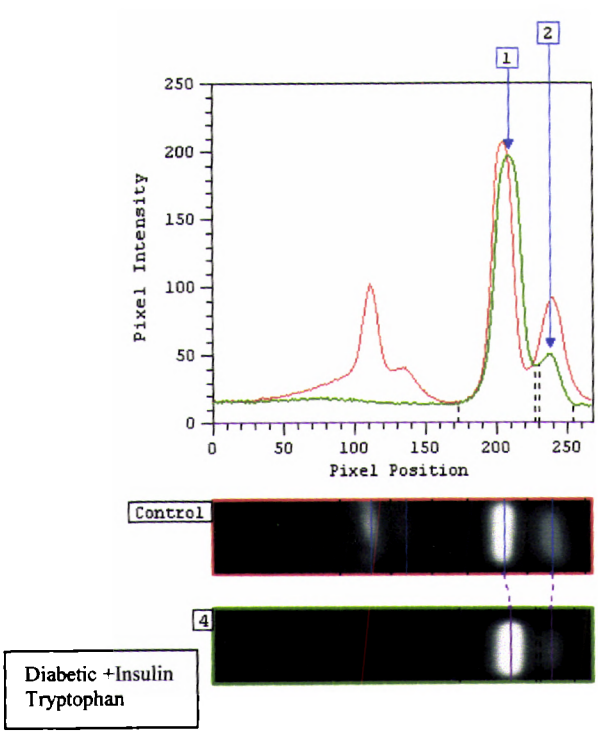
Lane-M—λ Hind III/Eco RI double digest DNA molecular weight marker
Lane-1—Control
Lane-2—Diabetic
Lane-3—Diabetic + Insulin
Lane-4—Diabetic + Insulin + Tryptophan
Lane-5—Diabetic + Tryptophan
Lane-6—Negative control without 5-HT_{2A} primer

Figure-8

Densitometric analysis of 5-HT_{2A} receptor gene expression in cerebral cortex of experimental rats







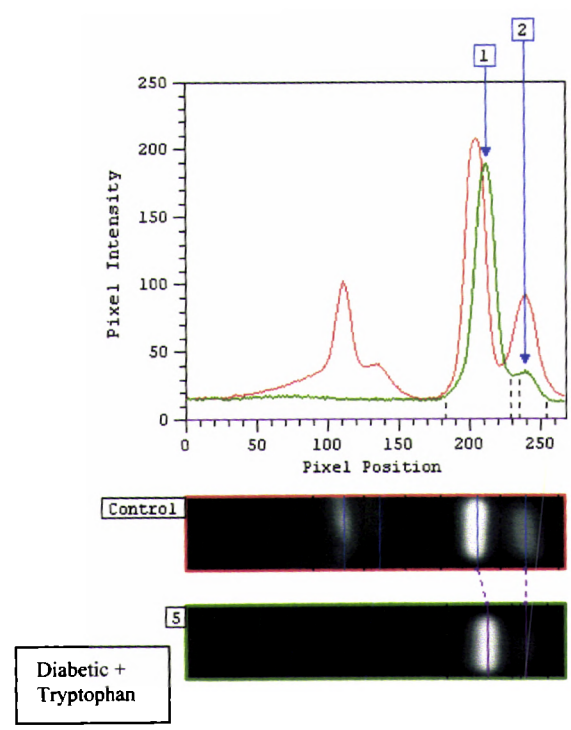
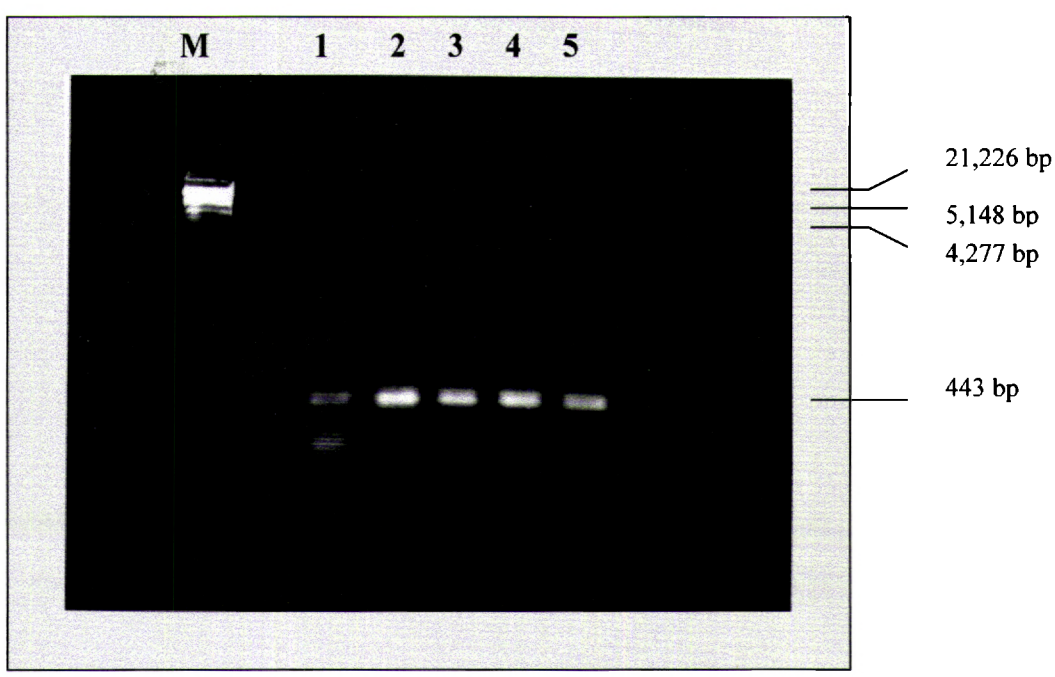


PLATE-2



RT-PCR amplification product of 5-HT_{2A} receptor mRNA from brain stem of experimental rats.

Lane-M—λ Hind III/Eco RI double digest DNA molecular weight marker
Lane-1—Control
Lane-2—Diabetic
Lane-3—Diabetic + Insulin
Lane-4—Diabetic + Insulin + Tryptophan
Lane-5—Diabetic + Tryptophan

Figure-9

Densitometric analysis of 5-HT_{2A} receptor gene expression in brain stem of experimental rats

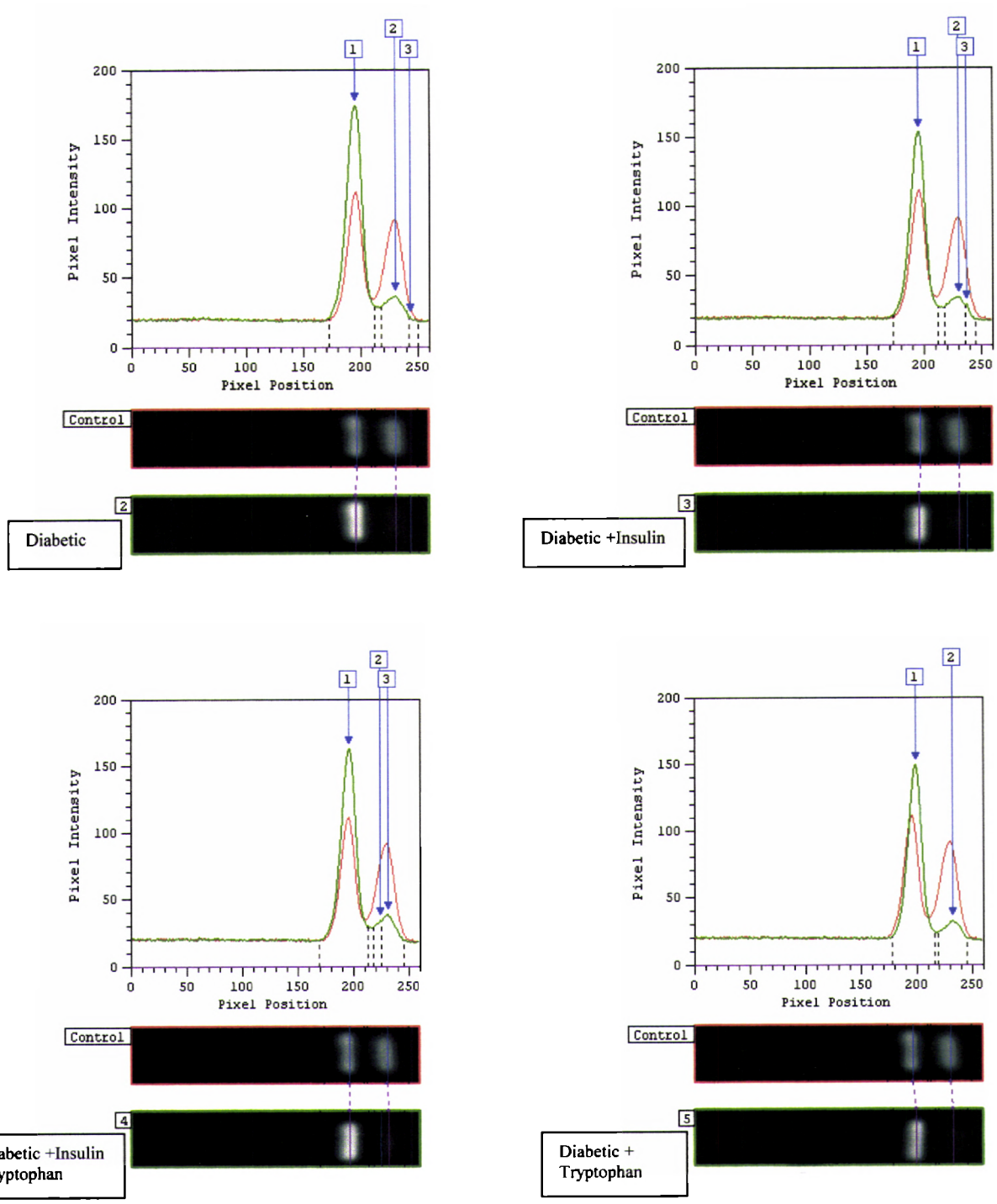
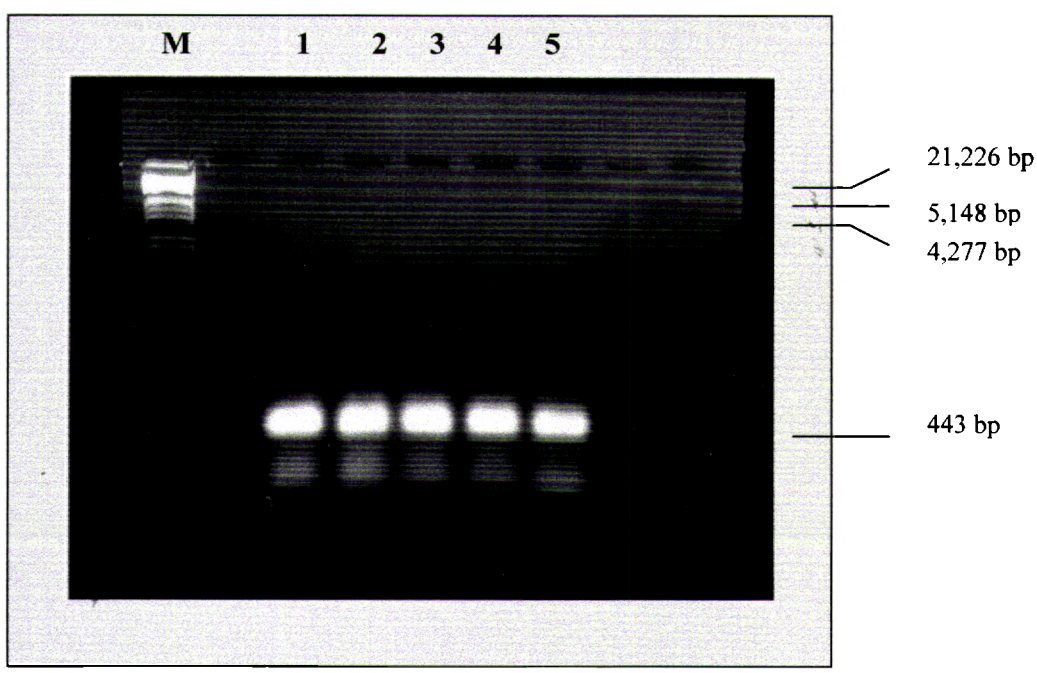


PLATE-3

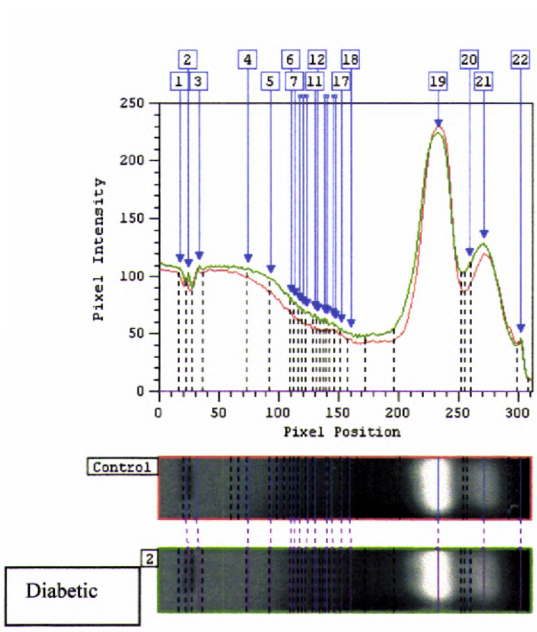


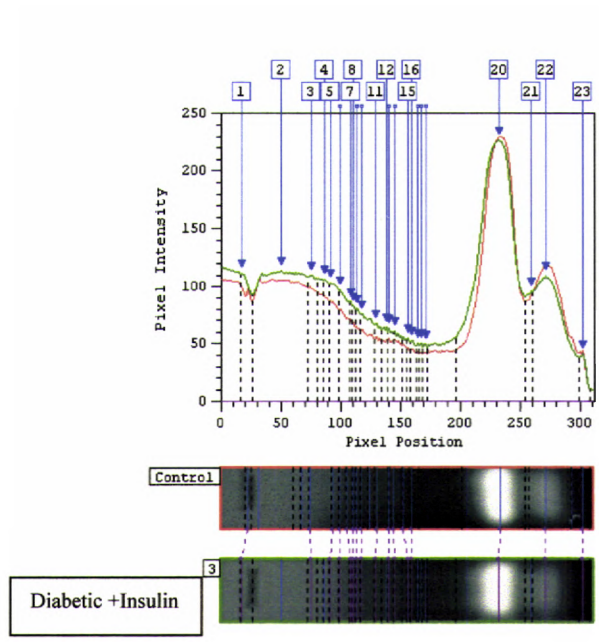
RT-PCR amplification product of 5-HT_{2A} receptor mRNA from hypothalamus of experimental rats.

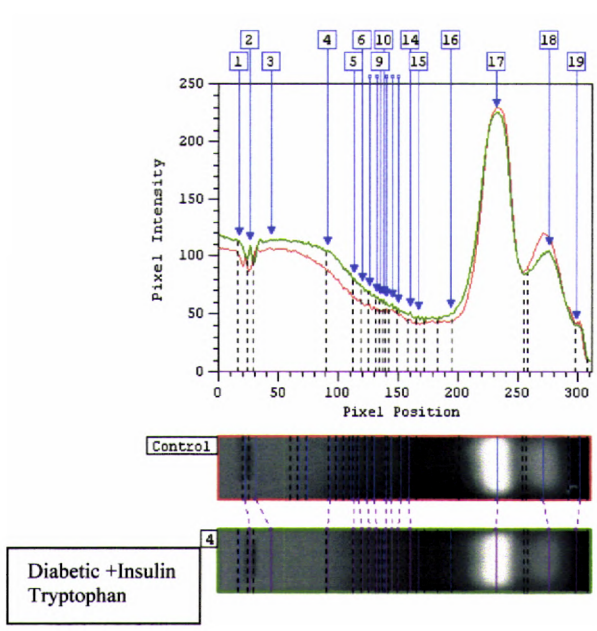
Lane-M—λ Hind III/Eco RI double digest DNA molecular weight marker
Lane-1—Control
Lane-2—Diabetic
Lane-3—Diabetic + Insulin
Lane-4—Diabetic + Insulin + Tryptophan
Lane-5—Diabetic + Tryptophan

Figure-10

Densitometric analysis of 5-HT_{2A} receptor gene expression in hypothalamus of experimental rats







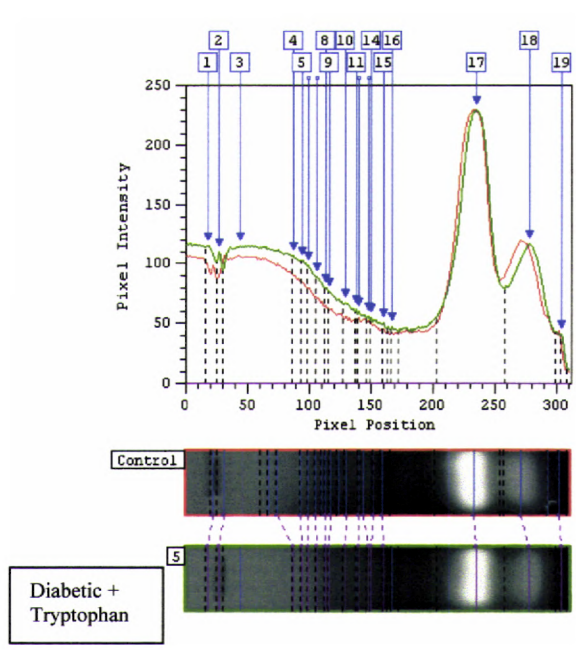
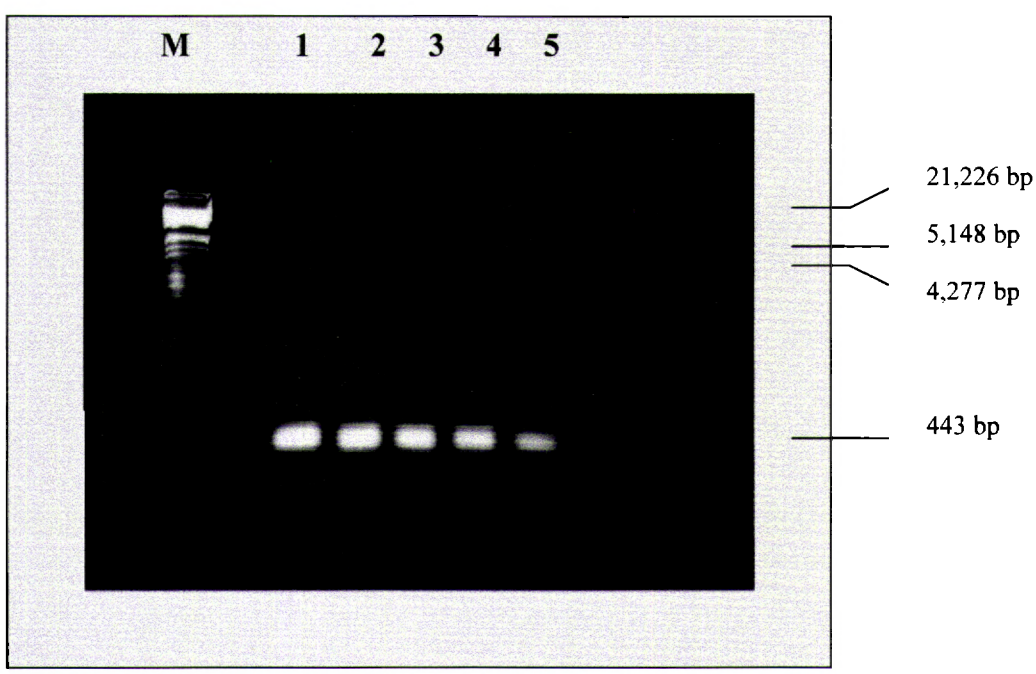


PLATE-4

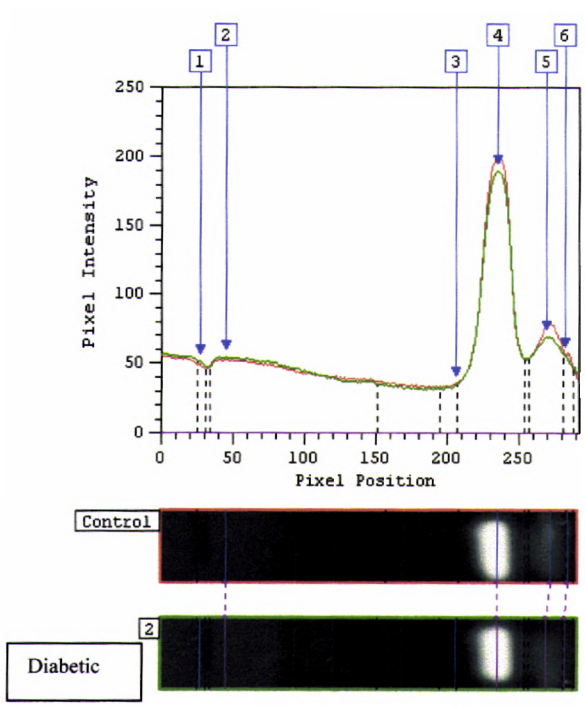


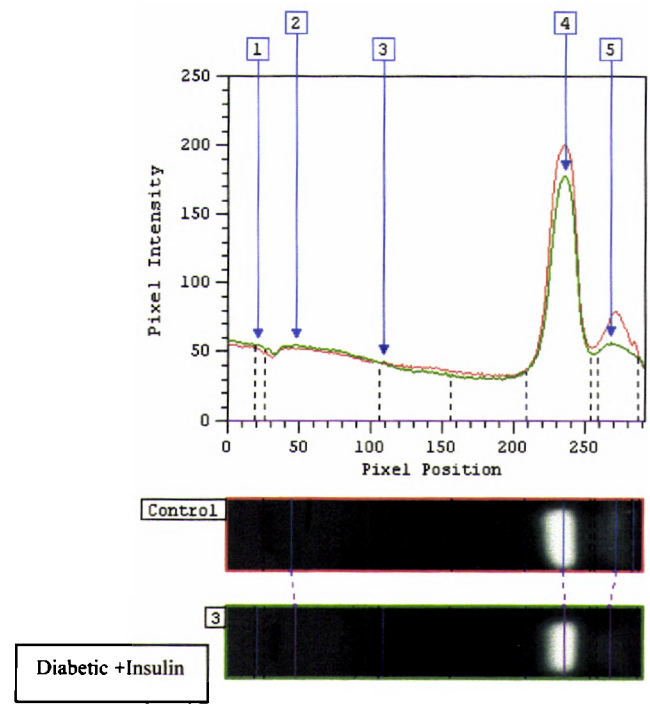
RT-PCR amplification product of 5-HT_{2A} receptor mRNA from corpus striatum of experimental rats.

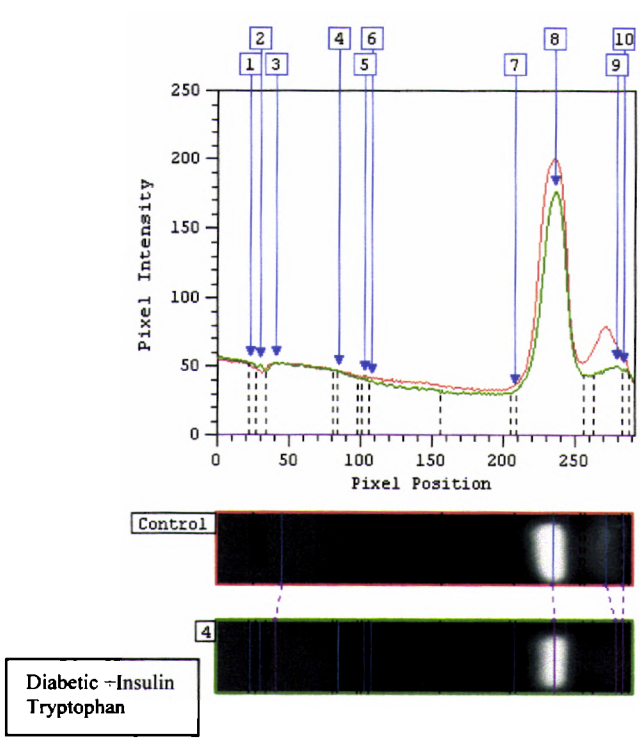
Lane-M—λ Hind III/Eco RI double digest DNA molecular weight marker
Lane-1—Control
Lane-2—Diabetic
Lane-3—Diabetic + Insulin
Lane-4—Diabetic + Insulin + Tryptophan
Lane-5—Diabetic + Tryptophan

Figure-11

Densitometric analysis of 5-HT_{2A} receptor gene expression in corpus striatum of experimental rats







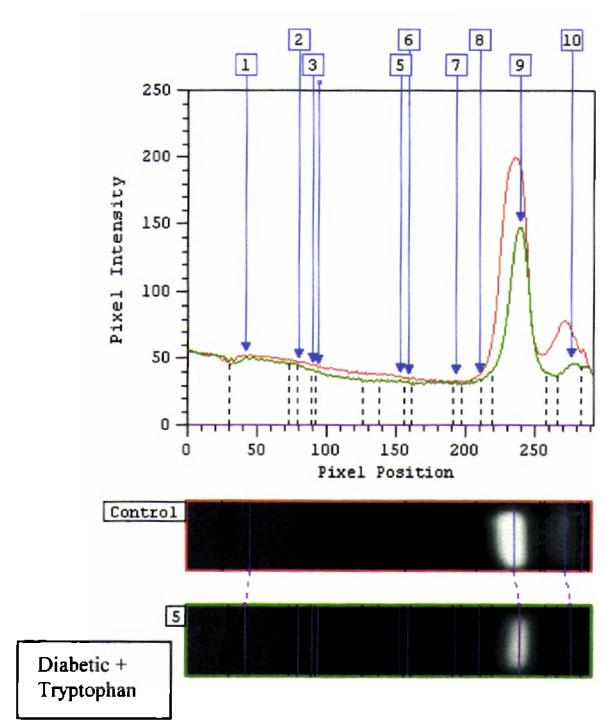
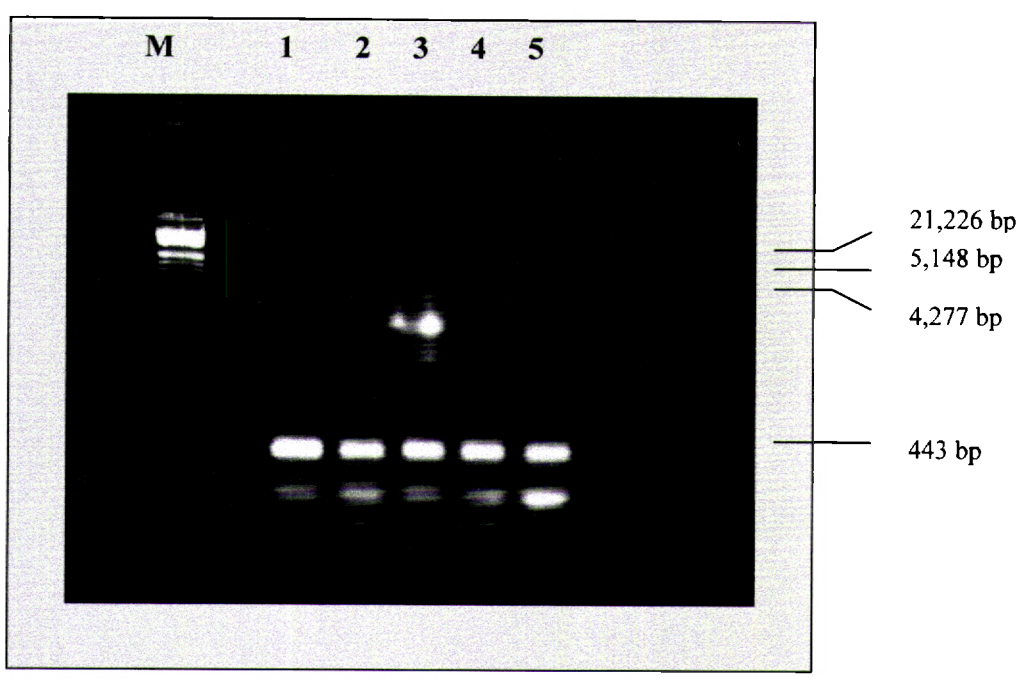


PLATE-5

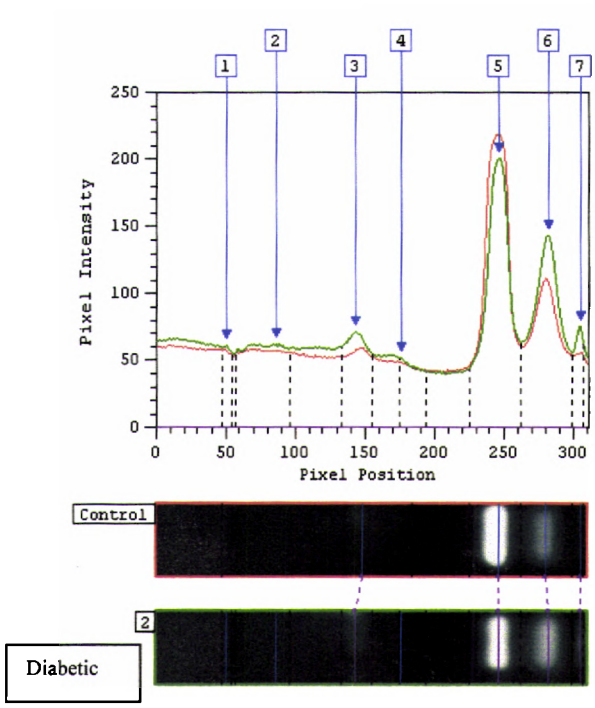


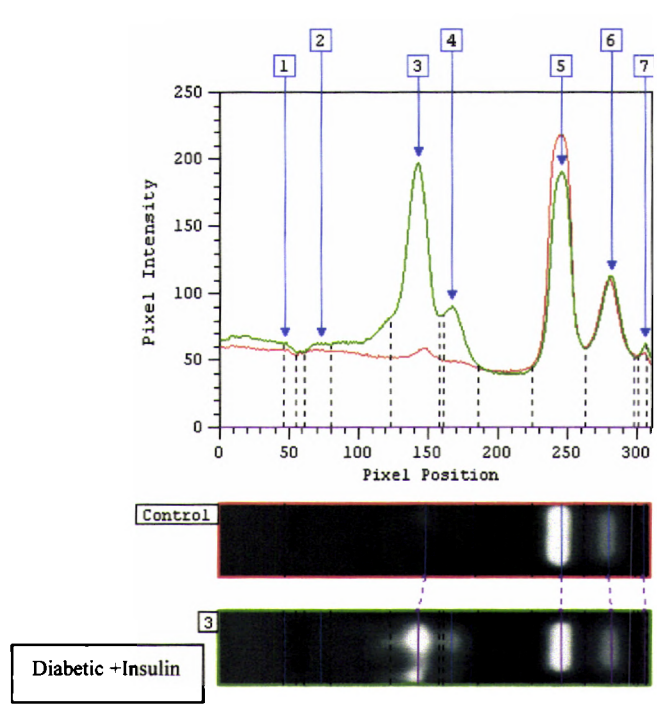
RT-PCR amplification product of 5-HT_{2A} receptor mRNA from cerebellum of experimental rats.

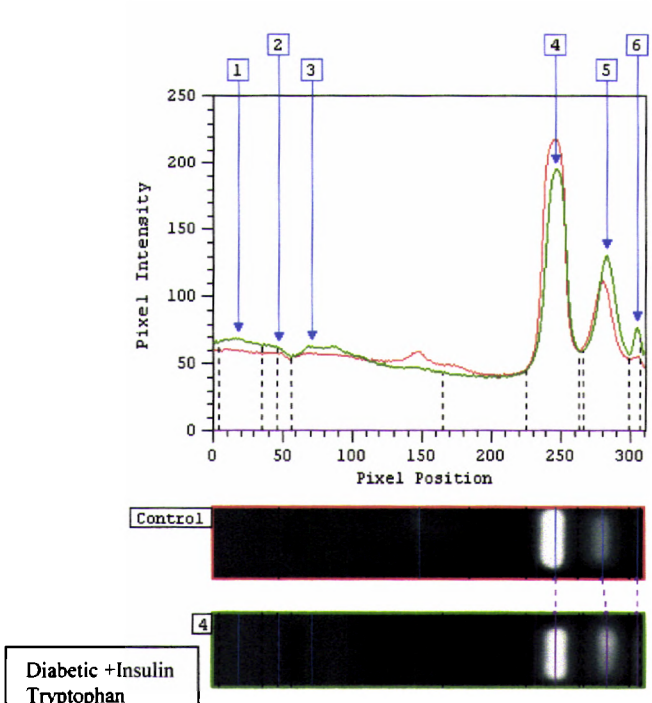
Lane-M—λ Hind III/Eco RI double digest DNA molecular weight marker
Lane-1—Control
Lane-2—Diabetic
Lane-3—Diabetic + Insulin
Lane-4—Diabetic + Insulin + Tryptophan
Lane-5—Diabetic + Tryptophan

Figure-12

Densitometric analysis of 5-HT_{2A} receptor gene expression in cerebellum of experimental rats







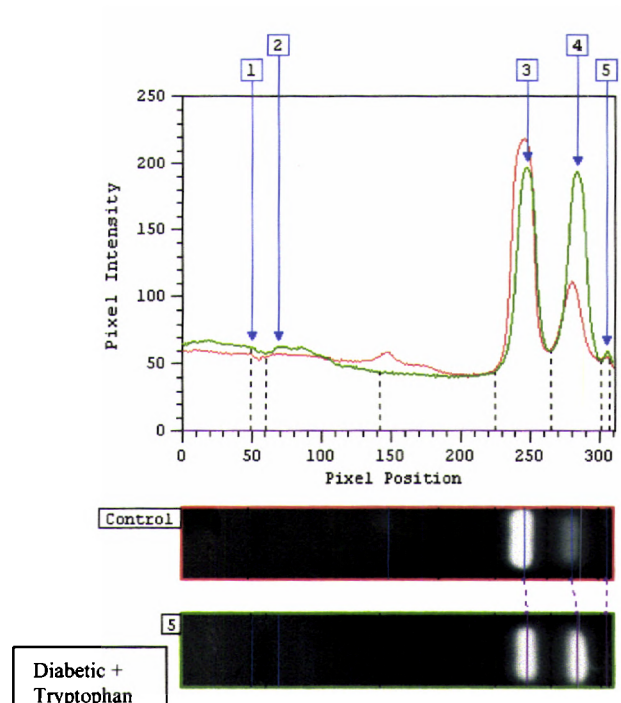
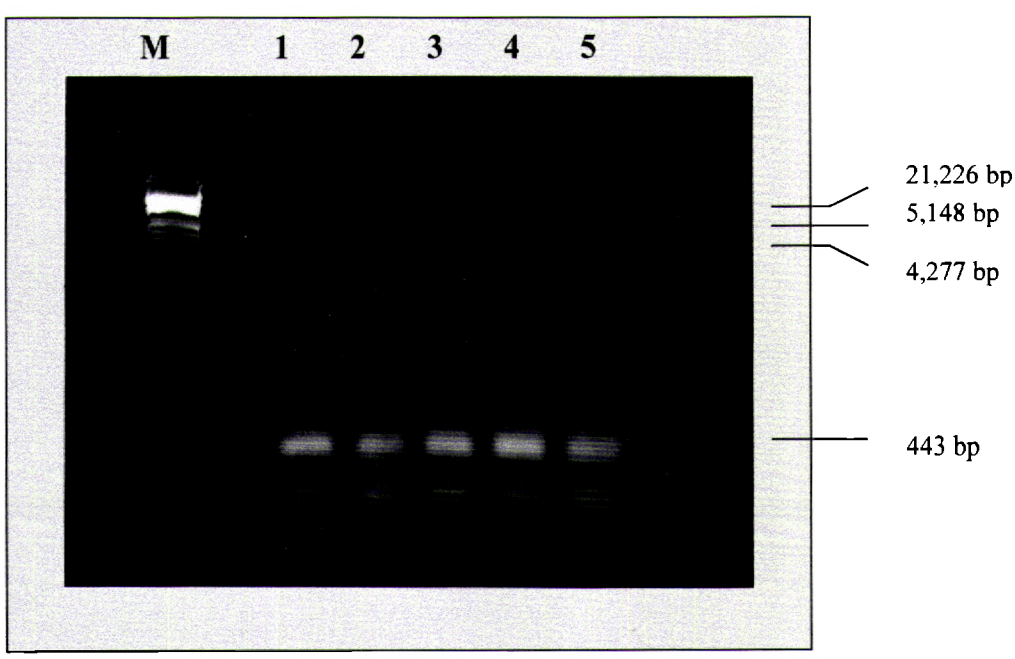


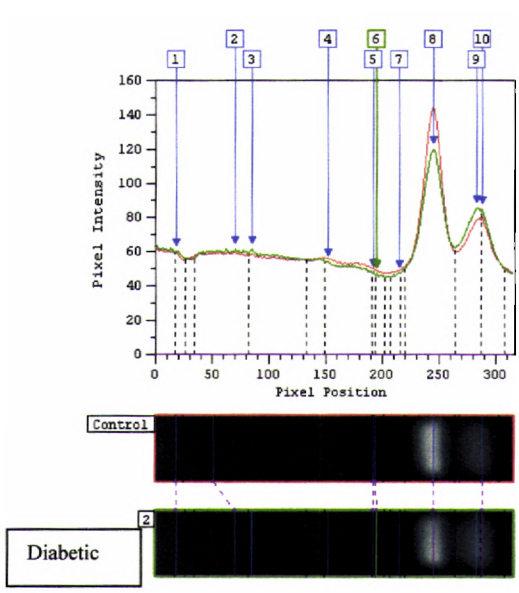
PLATE-6

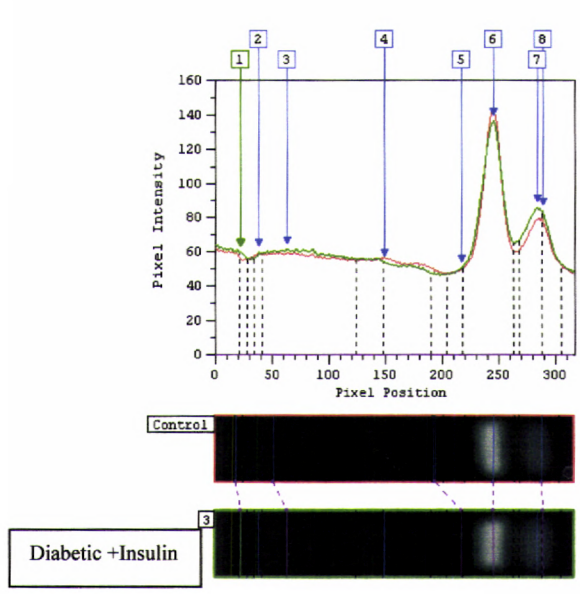


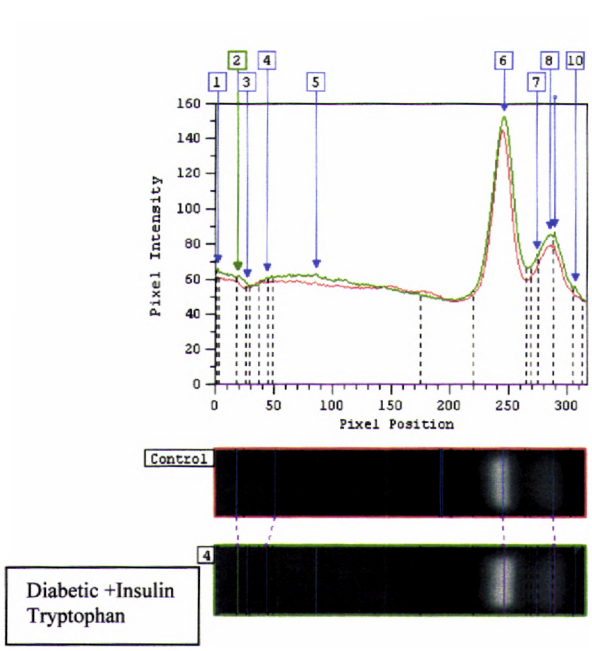
RT-PCR amplification product of 5-HT_{2A} receptor mRNA from pancreas of experimental rats.
Lane-M—λ Hind III/Eco RI double digest DNA molecular weight marker
Lane-1—Control
Lane-2—Diabetic
Lane-3—Diabetic + Insulin
Lane-4—Diabetic + Insulin + Tryptophan
Lane-5—Diabetic + Tryptophan

Figure-13

Densitometric analysis of 5-HT_{2A} receptor gene expression in pancreas of experimental animals







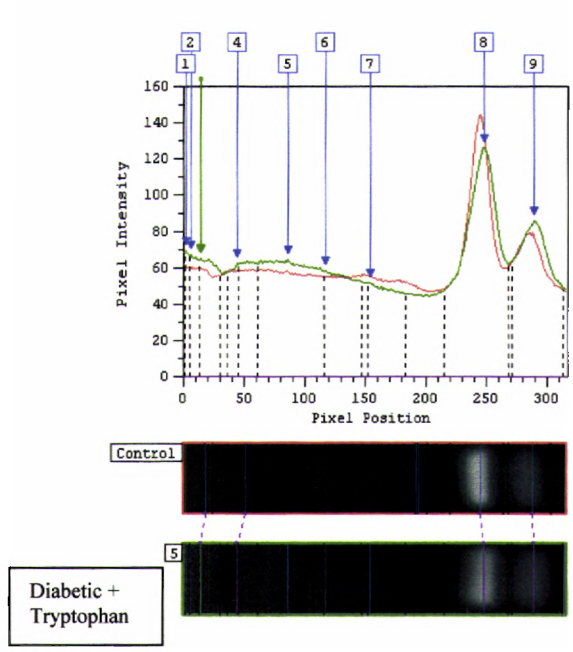


Figure-14a

Scatchard analysis of 5-HT_{1A} receptors with [^{3}H]8-OH-DPAT against 5-HT in cerebral cortex of control rats

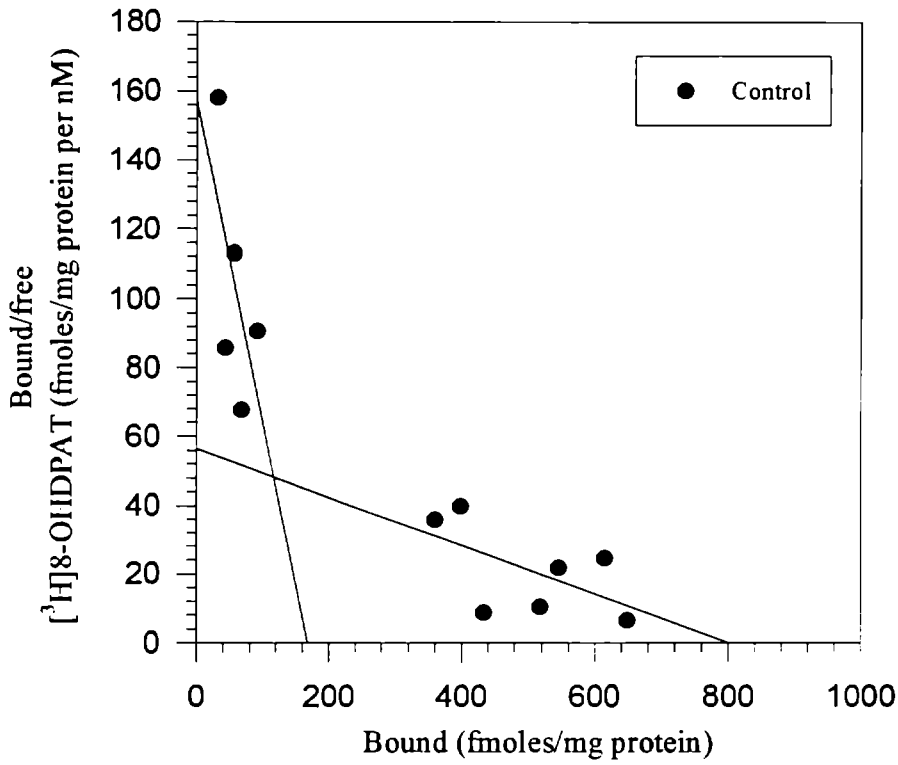


Figure-14b

Scatchard analysis of 5-HT_{1A} receptors with [^{3}H[8-OH-DPAT against 5-HT in cerebral cortex of diabetic rats

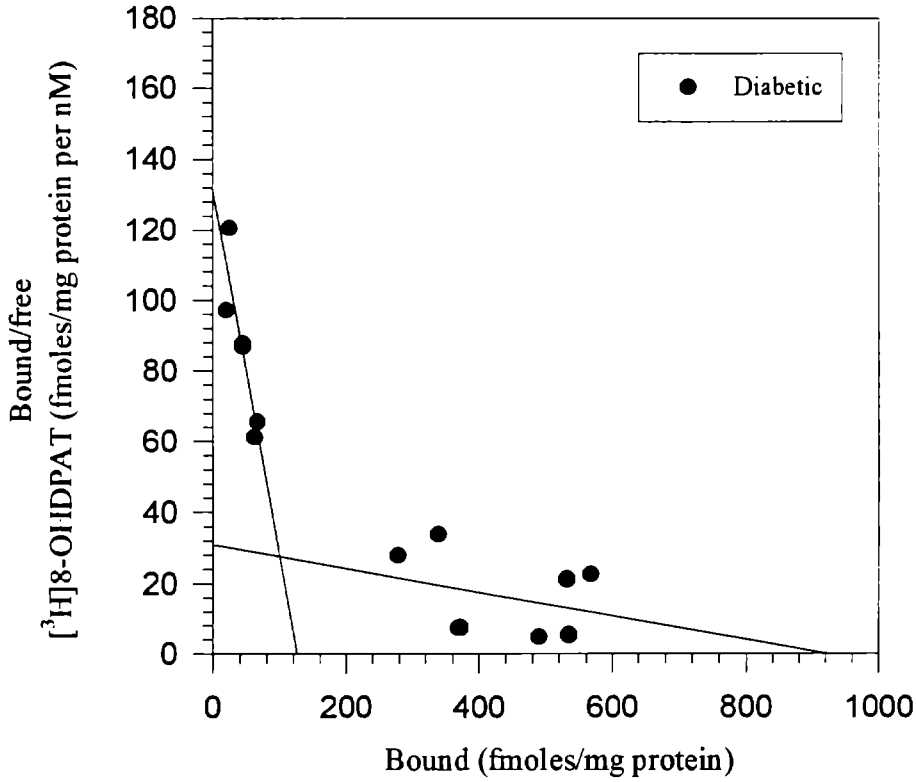


Figure-14c

Scatchard analysis of 5-HT_{1A} receptors with [^{3}H]8-OH-DPAT against 5-HT in cerebral cortex of diabetic + insulin treated rats

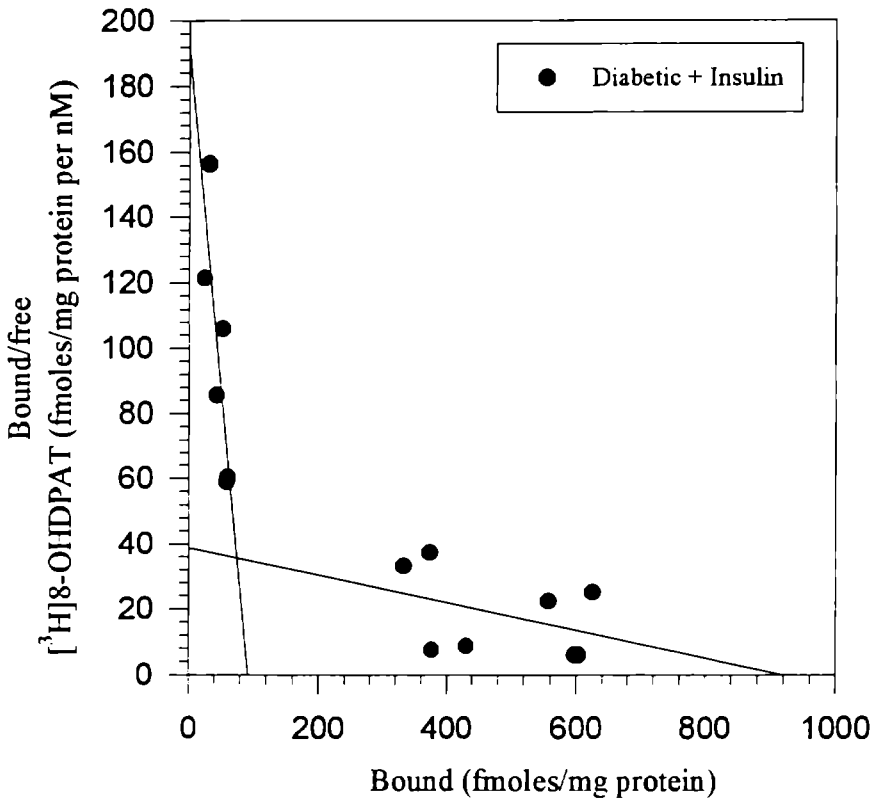


Figure-14d

Scatchard analysis of 5-HT_{1A} receptors with [^{3}H]8-OH-DPAT against 5-HT in cerebral cortex of diabetic + insulin + tryptophan treated rats

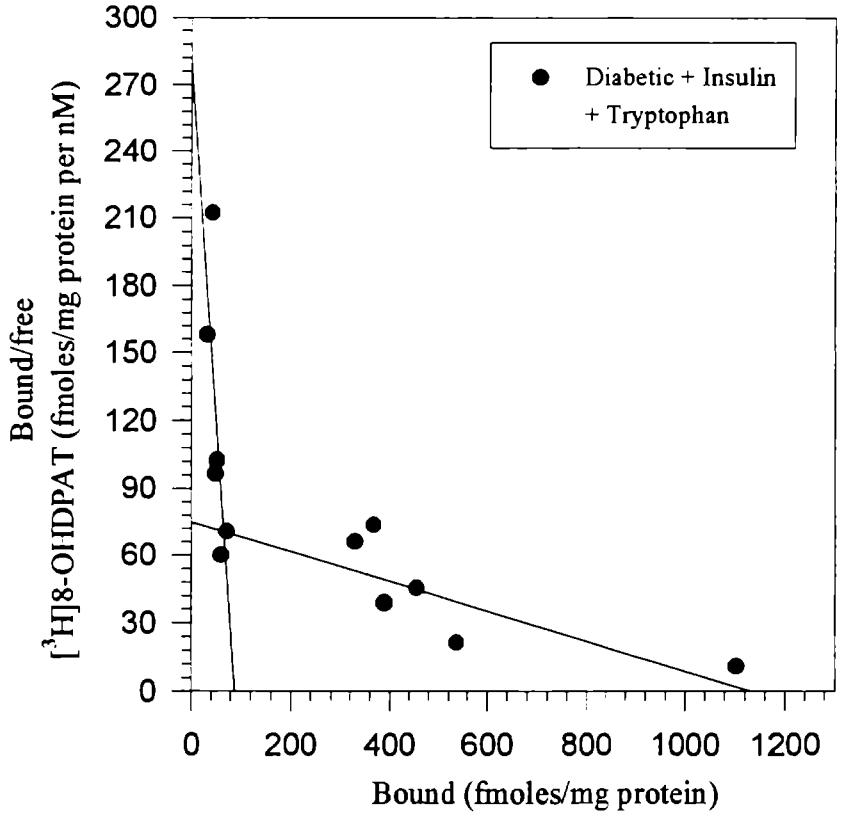
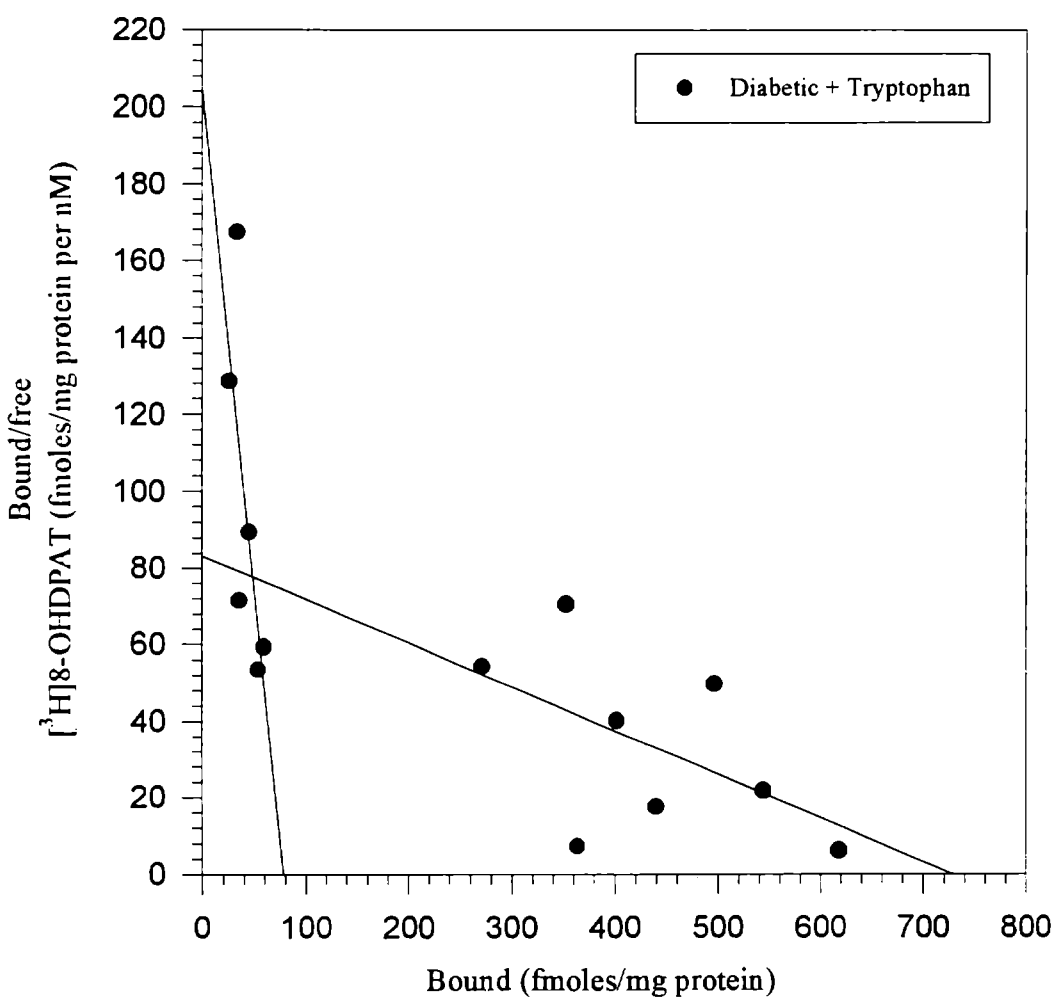


Figure-14e

Scatchard analysis of 5-HT_{1A} receptors with [^{3}H]8-OH-DPAT against 5-HT in cerebral cortex of diabetic + tryptophan treated rats



Displacement analysis using 5-HT against [^{3}H]8-OH-DPAT in cerebral cortex of experimental rats

Figure-15a

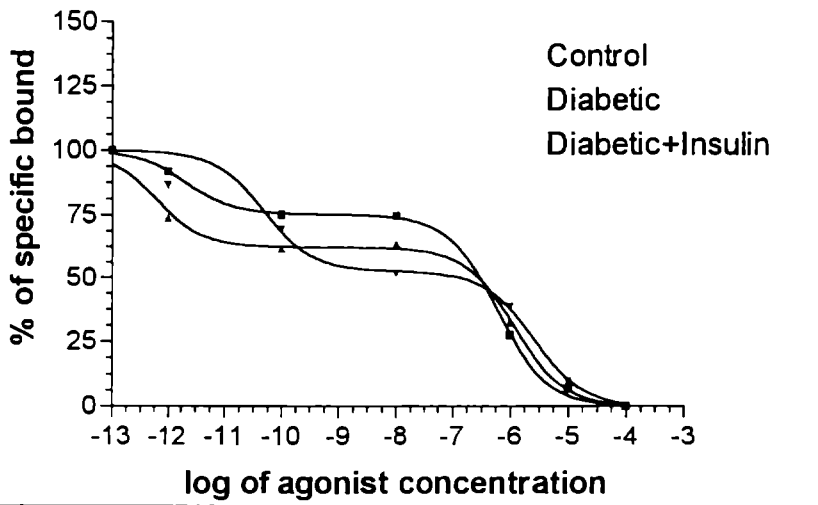


Figure-15b

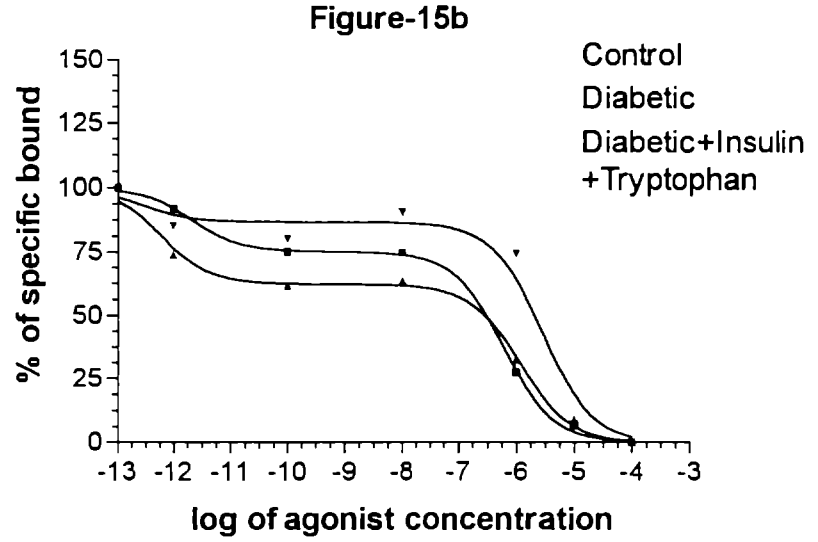
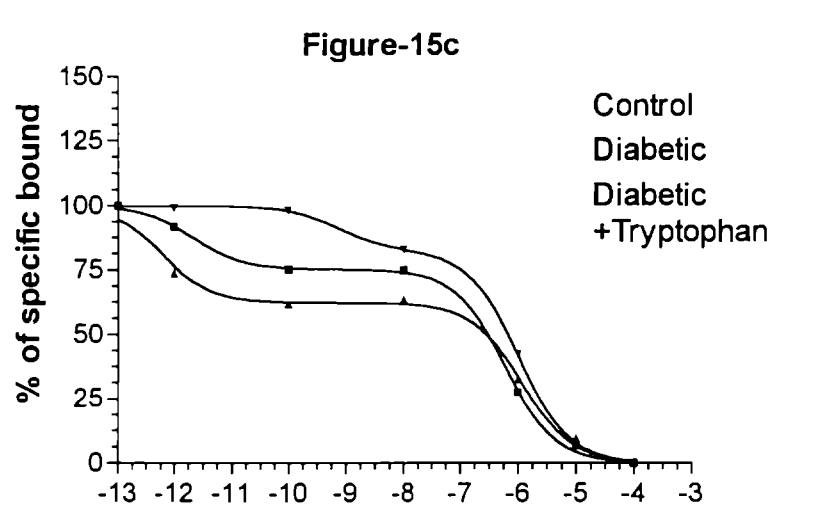


Figure-15c



Displacement analysis using 5-HT against [^{3}H]8-OH-DPAT in cerebral cortex of experimental rats with and without Gpp[NH]p

Figure-16a

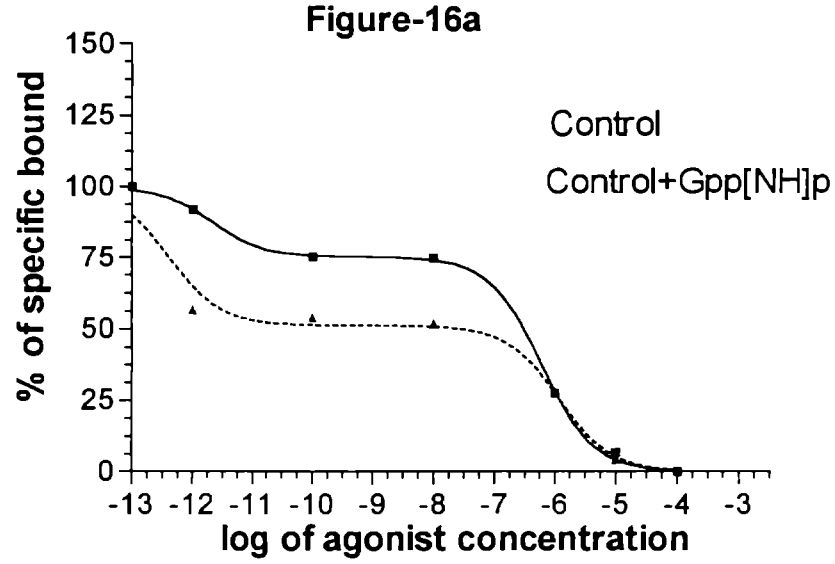


Figure-16b

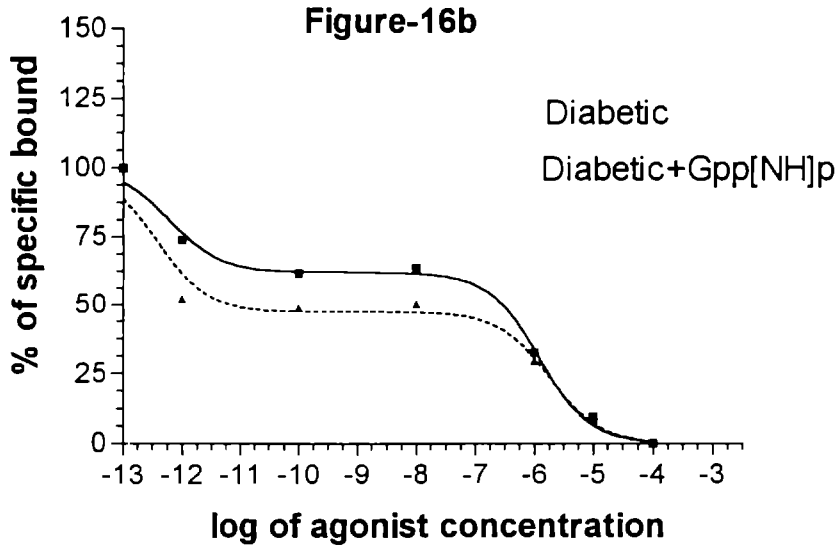


Figure-16c

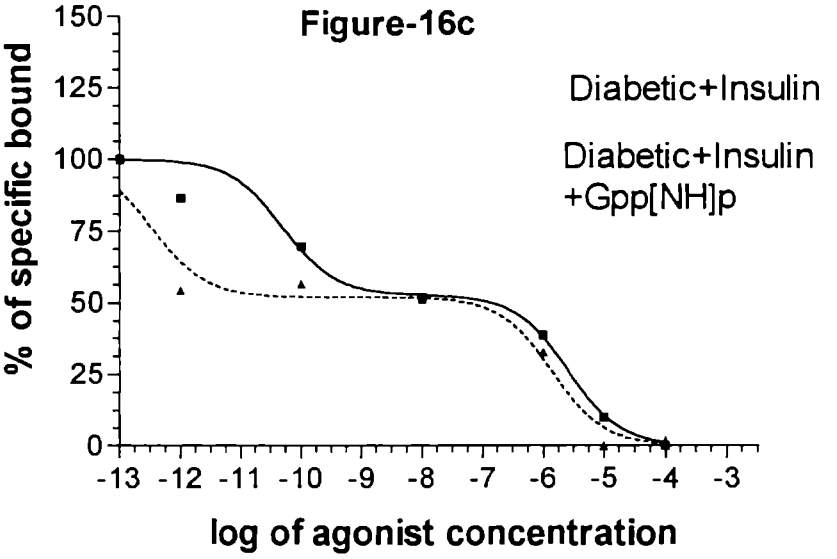


Figure-17a

Scatchard analysis of 5-HT_{1A} receptors with [^{3}H]8-OH-DPAT in brain stem of control rats

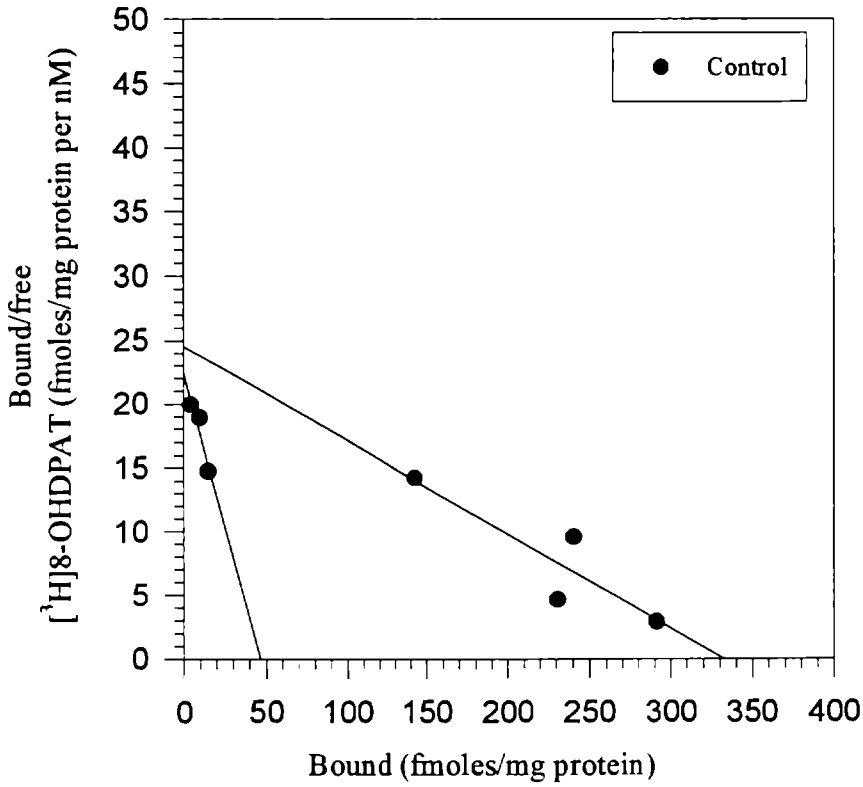


Figure-17b

Scatchard analysis of 5-HT_{1A} receptors with [^{3}H]8-OH-DPAT in brain stem of diabetic rats

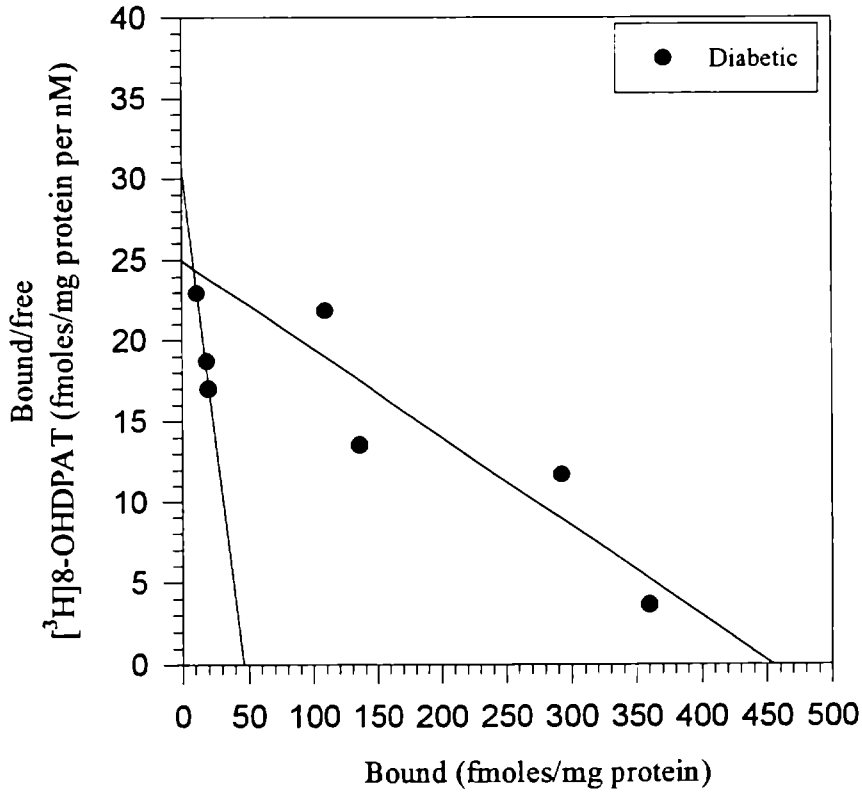


Figure-17c

Scatchard analysis of 5-HT_{1A} receptors with [^{3}H]8-OH-DPAT in brain stem of diabetic+Insulin treated rats

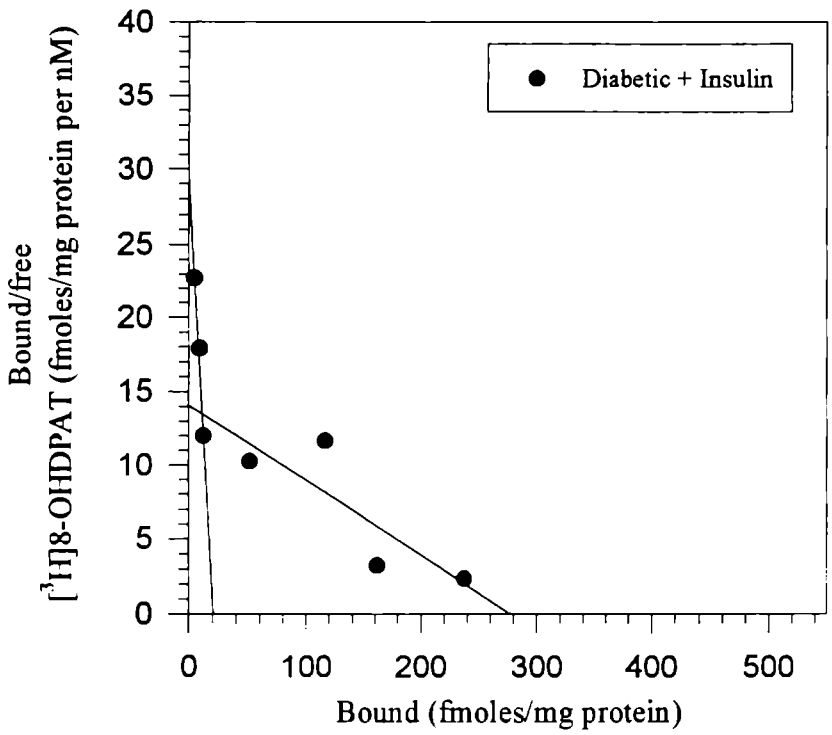


Figure-17d

Scatchard analysis of 5-HT_{1A} receptors with [^{3}H]8-OH-DPAT in brain stem of Diabetic + Insulin + Tryptophan treated rats

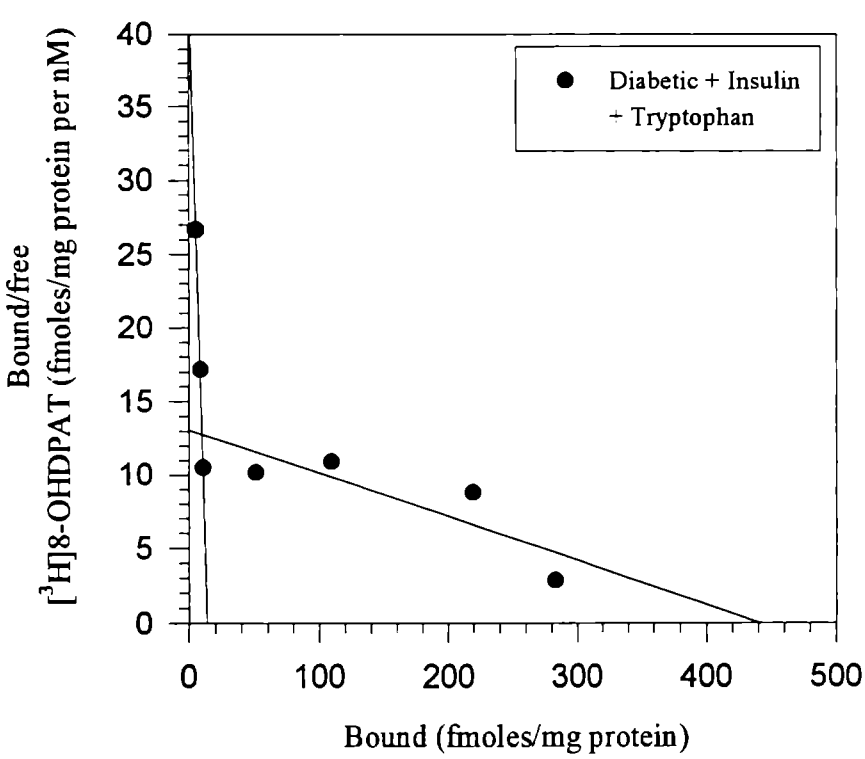
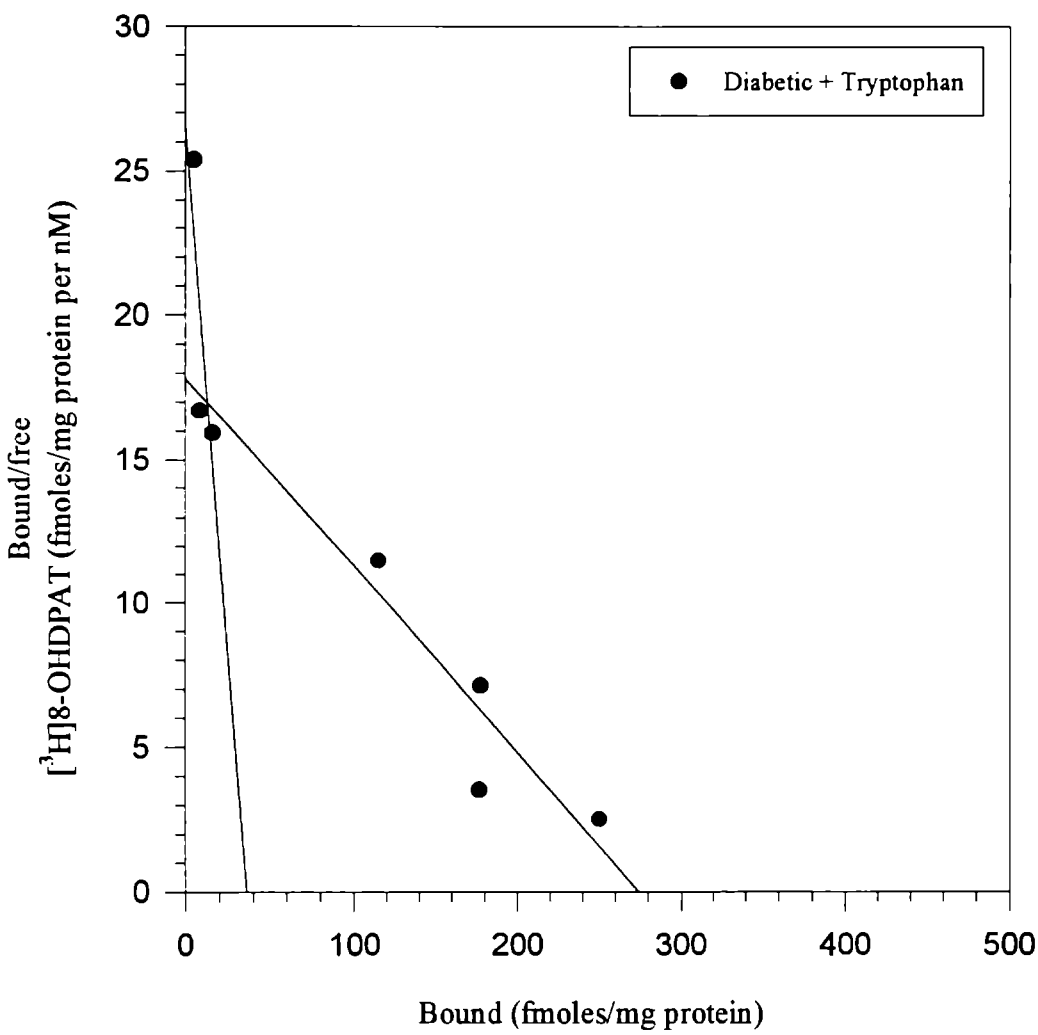


Figure-17e

Scatchard analysis of 5-HT_{1A} receptors with [^{3}H]8-OH-DPAT in brain stem of Diabetic + Tryptophan treated rats



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

Figure-18a

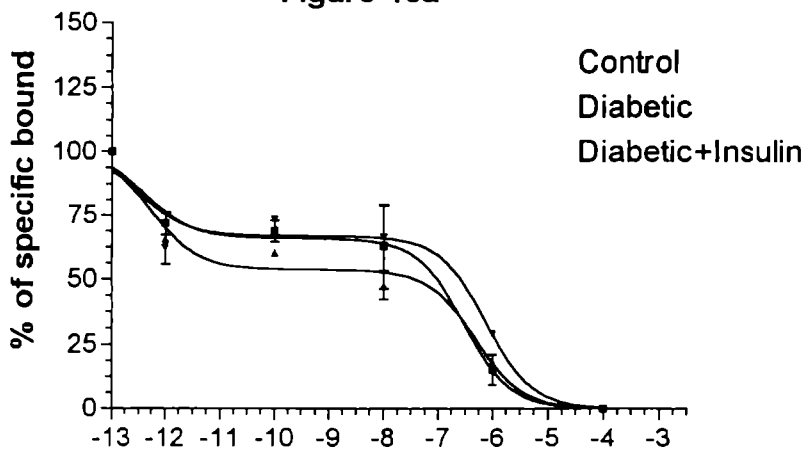


Figure-18b

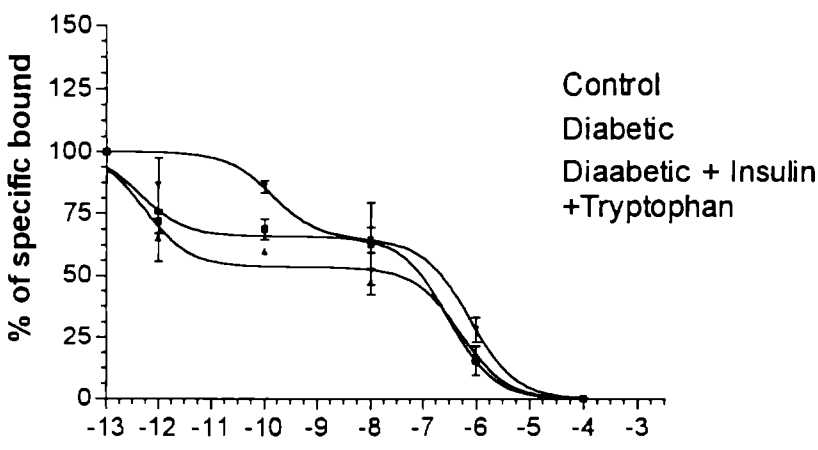
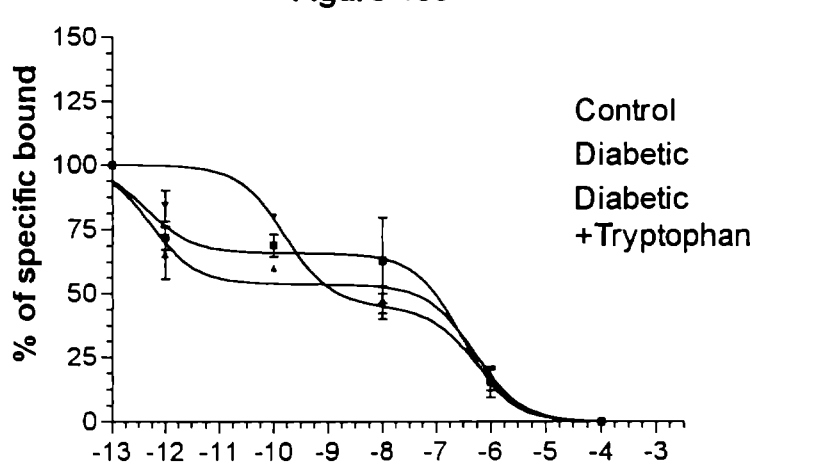


Figure-18c



Displacement analysis using 5-HT against [^{3}H]8-OH-DPAT in brain stem of experimental rats with and without Gpp[NH]p

Figure-19a

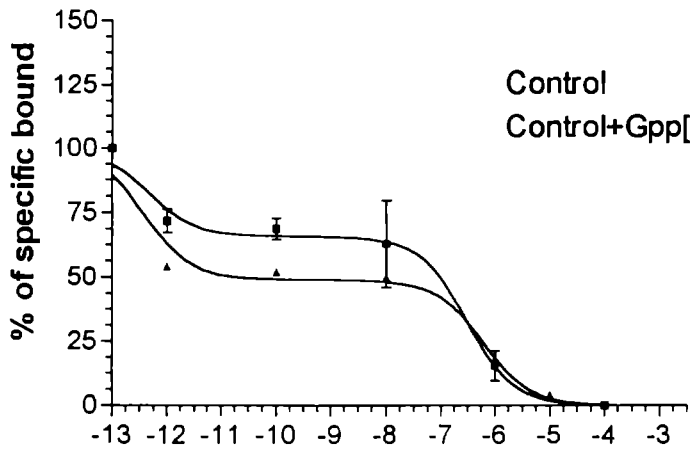


Figure-19b

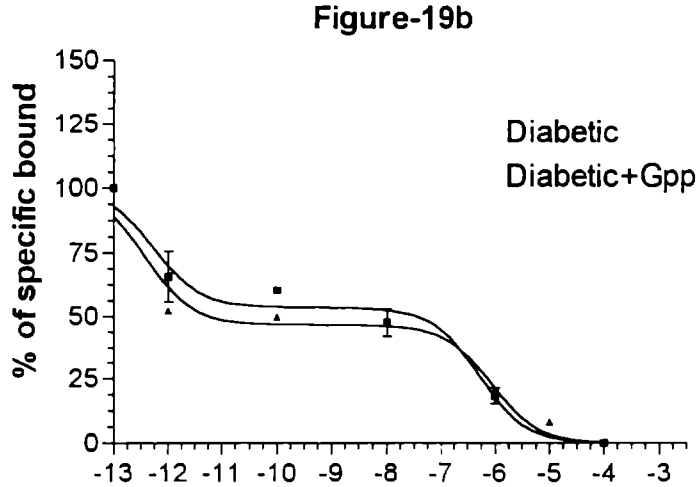
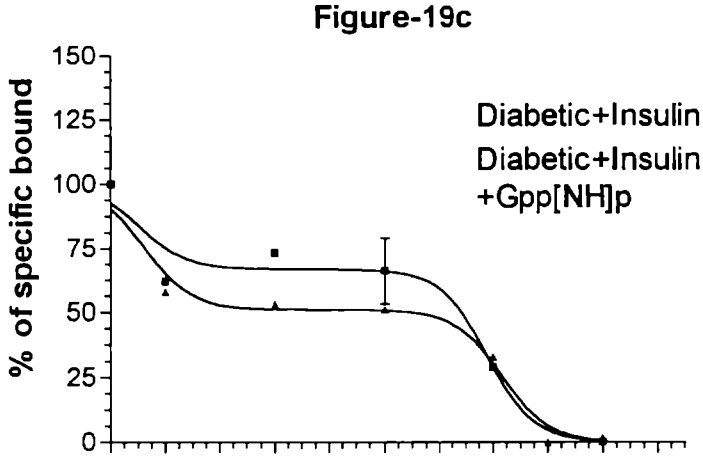
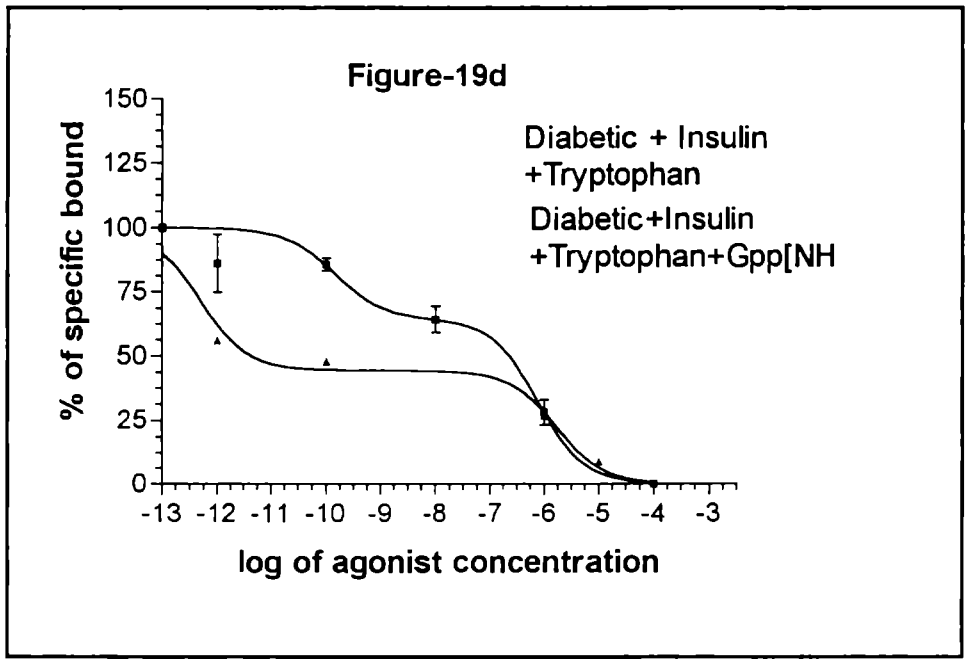


Figure-19c



Displacement analysis using 5-HT against [^{3}H]8-OH-DPAT in brain stem of experimental rats with and without Gpp[NH]p



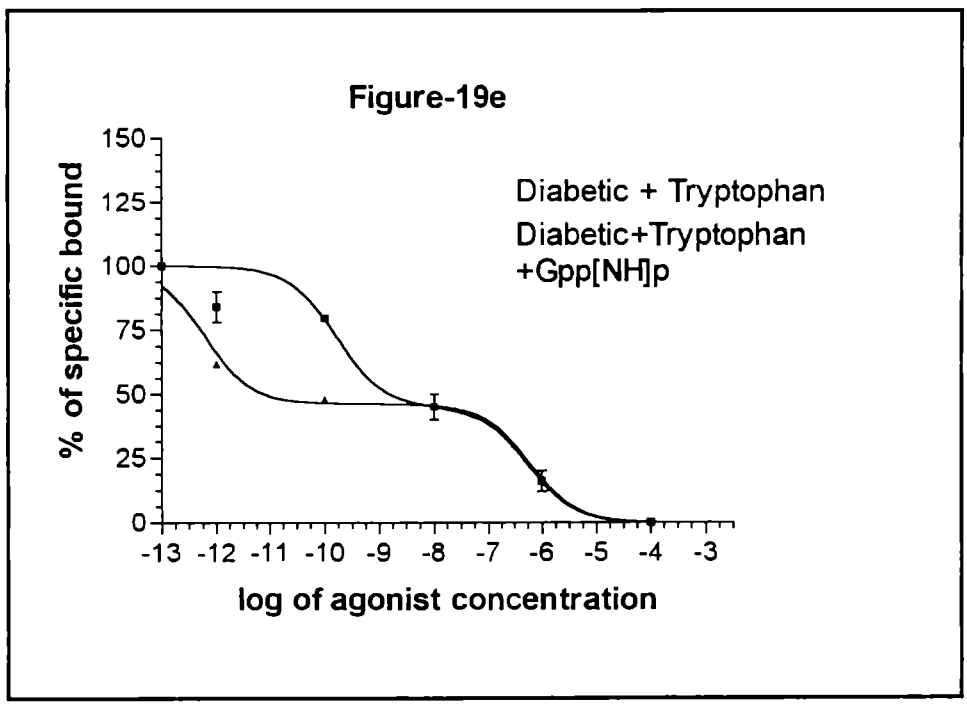


Figure-20a

Scatchard analysis of 5-HT_{1A} receptors with [^{3}H]8-OH-DPAT against 5-HT in hypothalamus of control rats

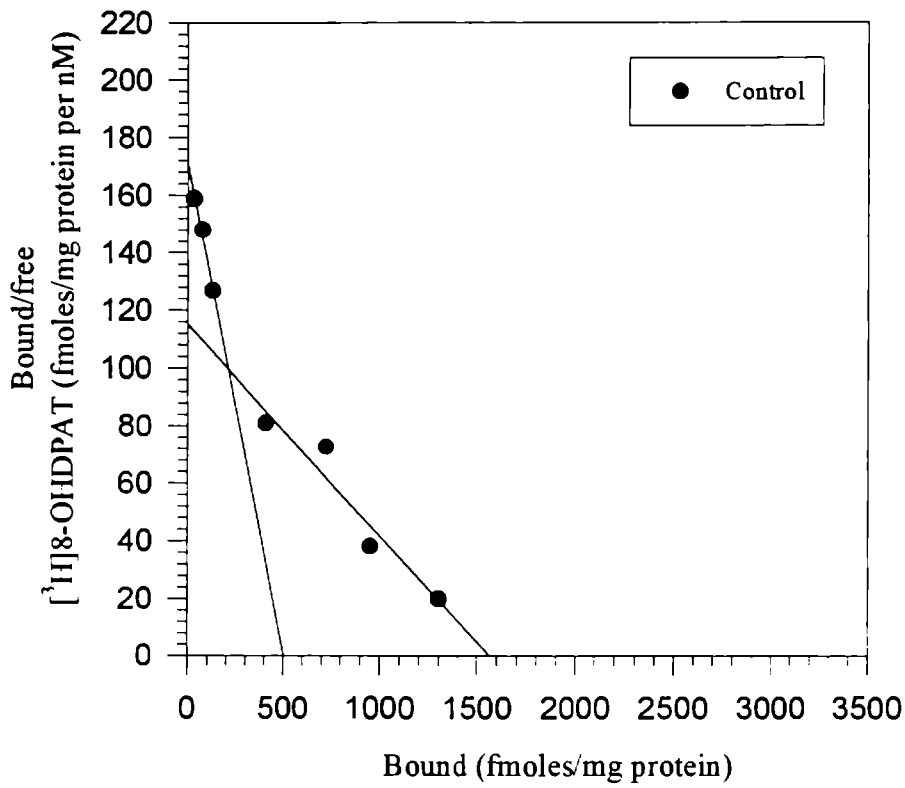


Figure-20b

Scatchard analysis of 5-HT_{1A} receptors with [^{3}H]8-OH-DPAT against 5-HT in hypothalamus of diabetic rats

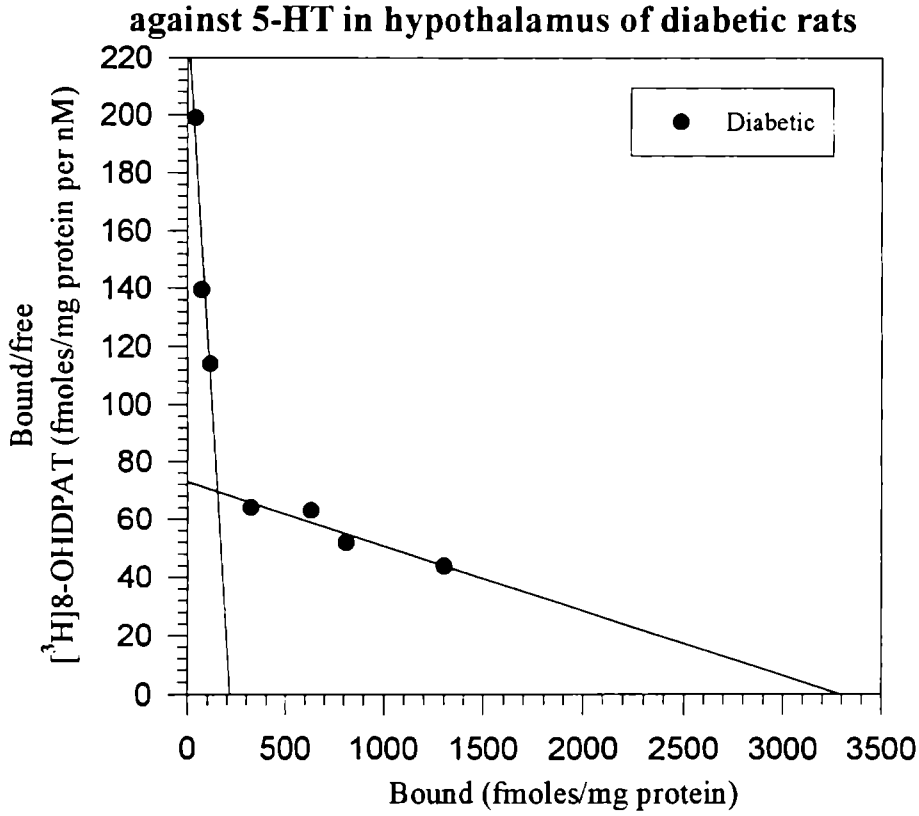


Figure-20c

Scatchard analysis of 5-HT_{1A} receptors with [^{3}H]8-OH-DPAT against 5-HT in hypothalamus of dia + Insulin treated rats

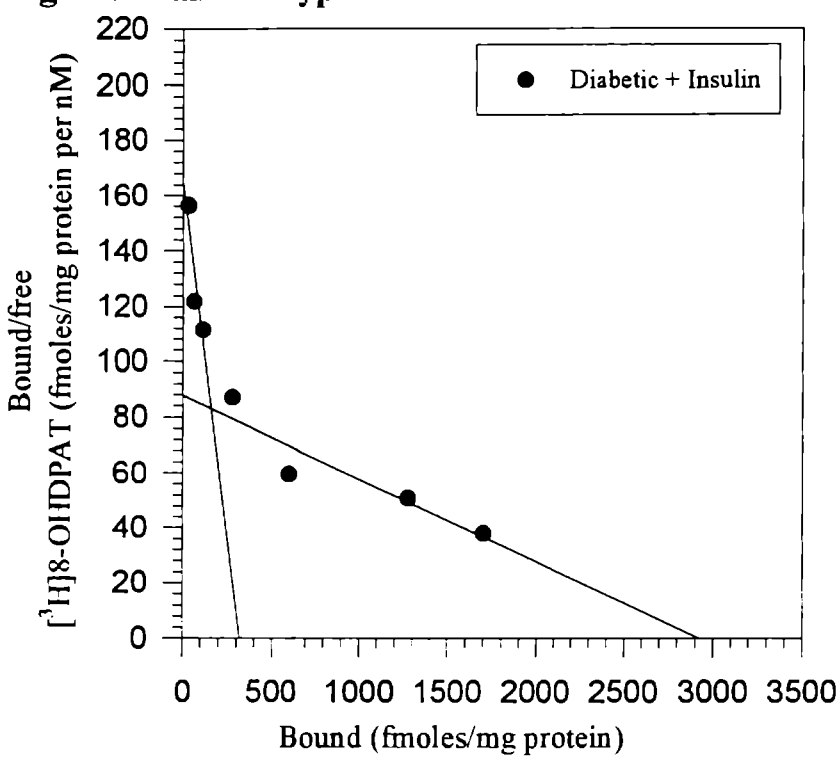


Figure-20d

Scatchard analysis of 5-HT_{1A} receptors with [^{3}H]8-OH-DPATagainst 5-HT in hypothalamus of dia + Insulin + tryptophan treated rats

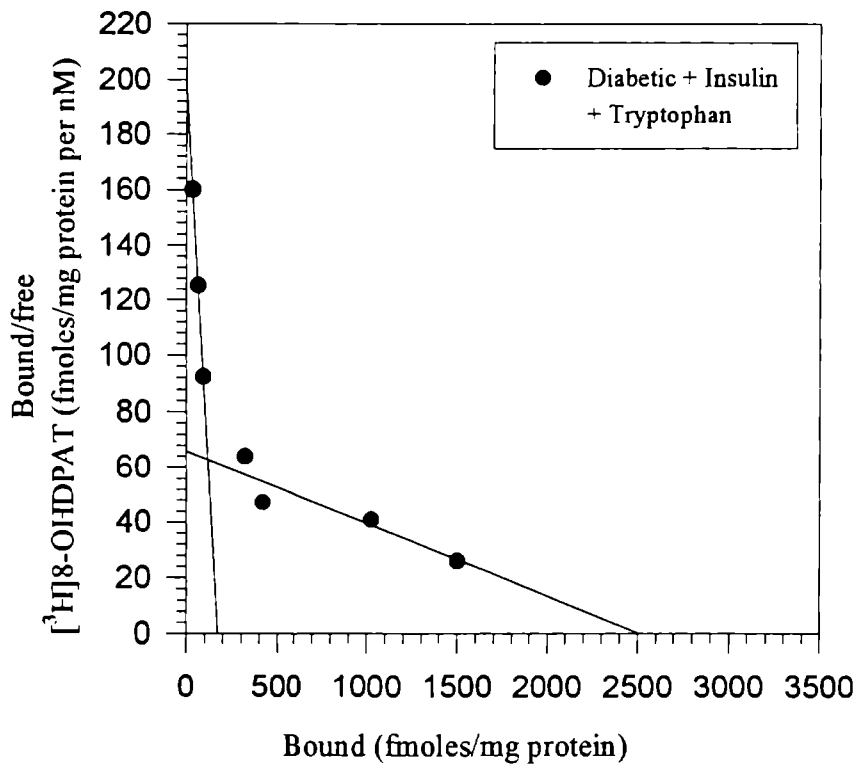
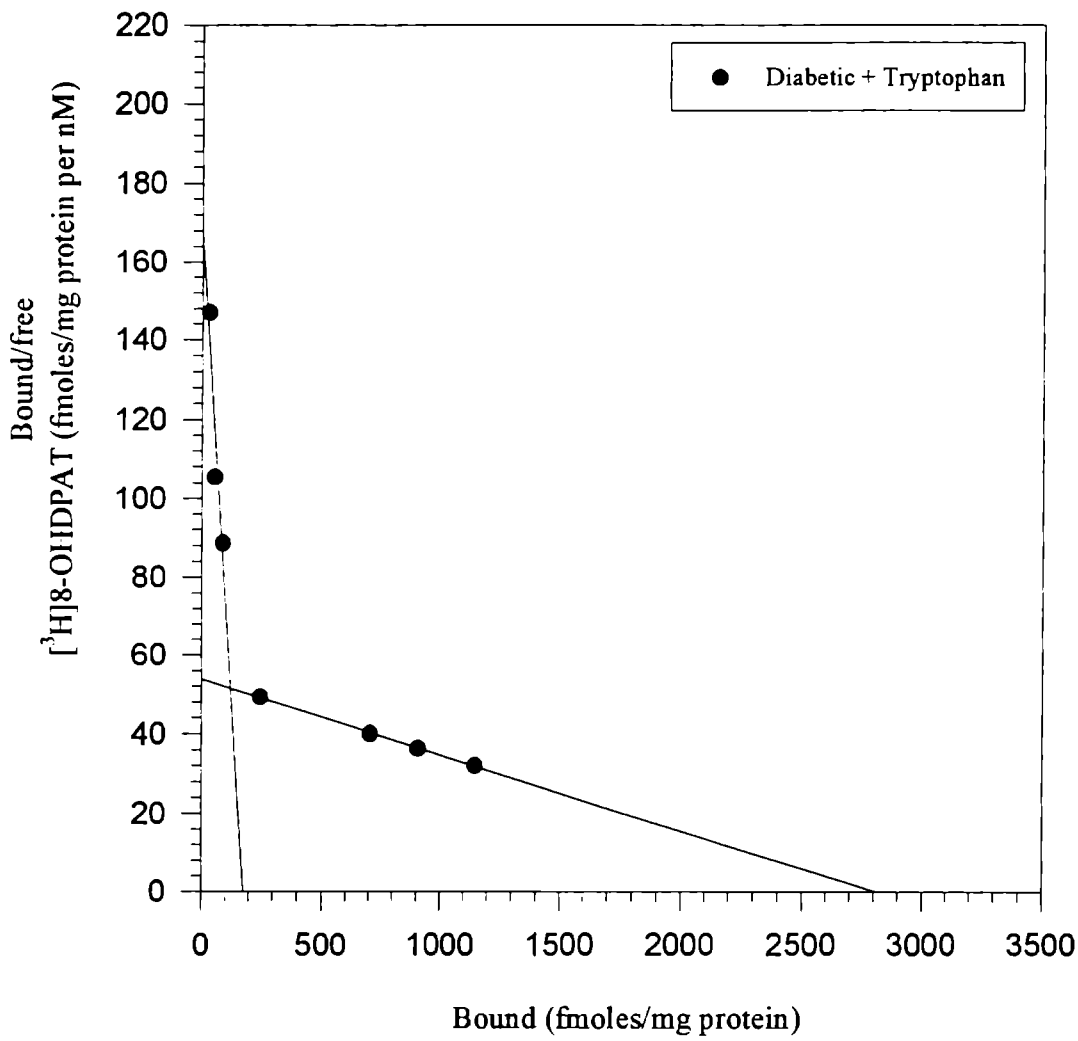


Figure-20e

Scatchard analysis of 5-HT_{1A} receptors with [^{3}H]8-OH-DPAT against 5-HT in hypothalamus of dia + tryptophan treated rats



Displacement analysis using 5-HT against [^{3}H]8-OH-DPAT in hypothalamus of experimental animals

Figure-21a

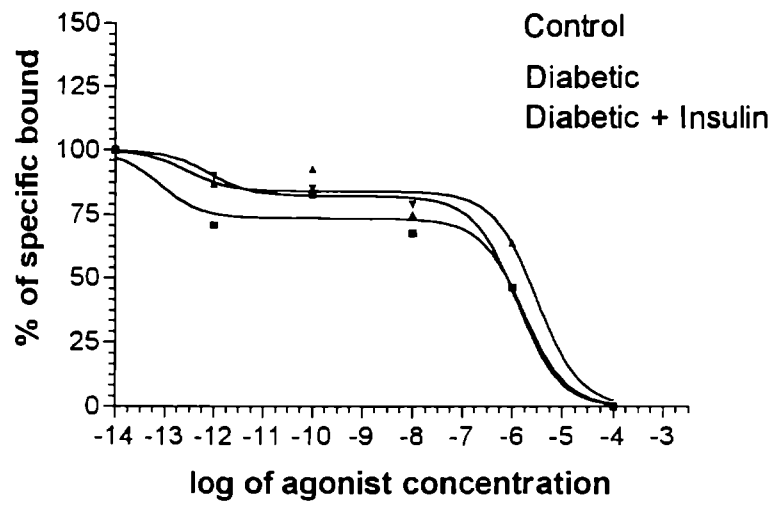


Figure-21b

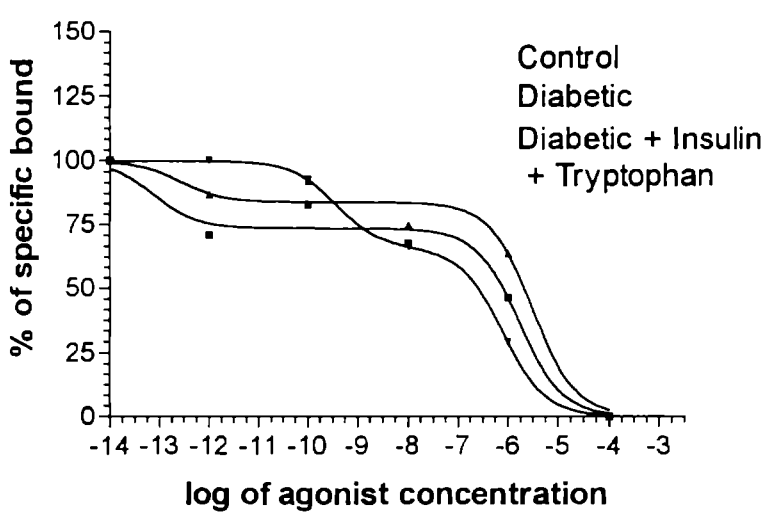


Figure-21c

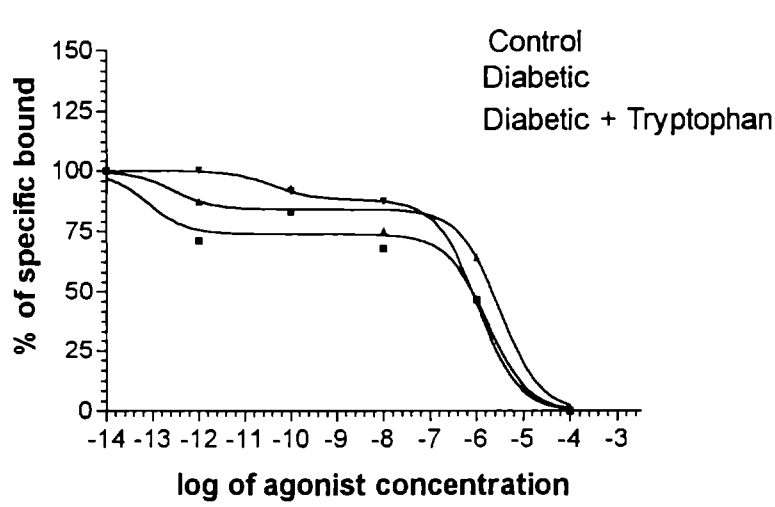
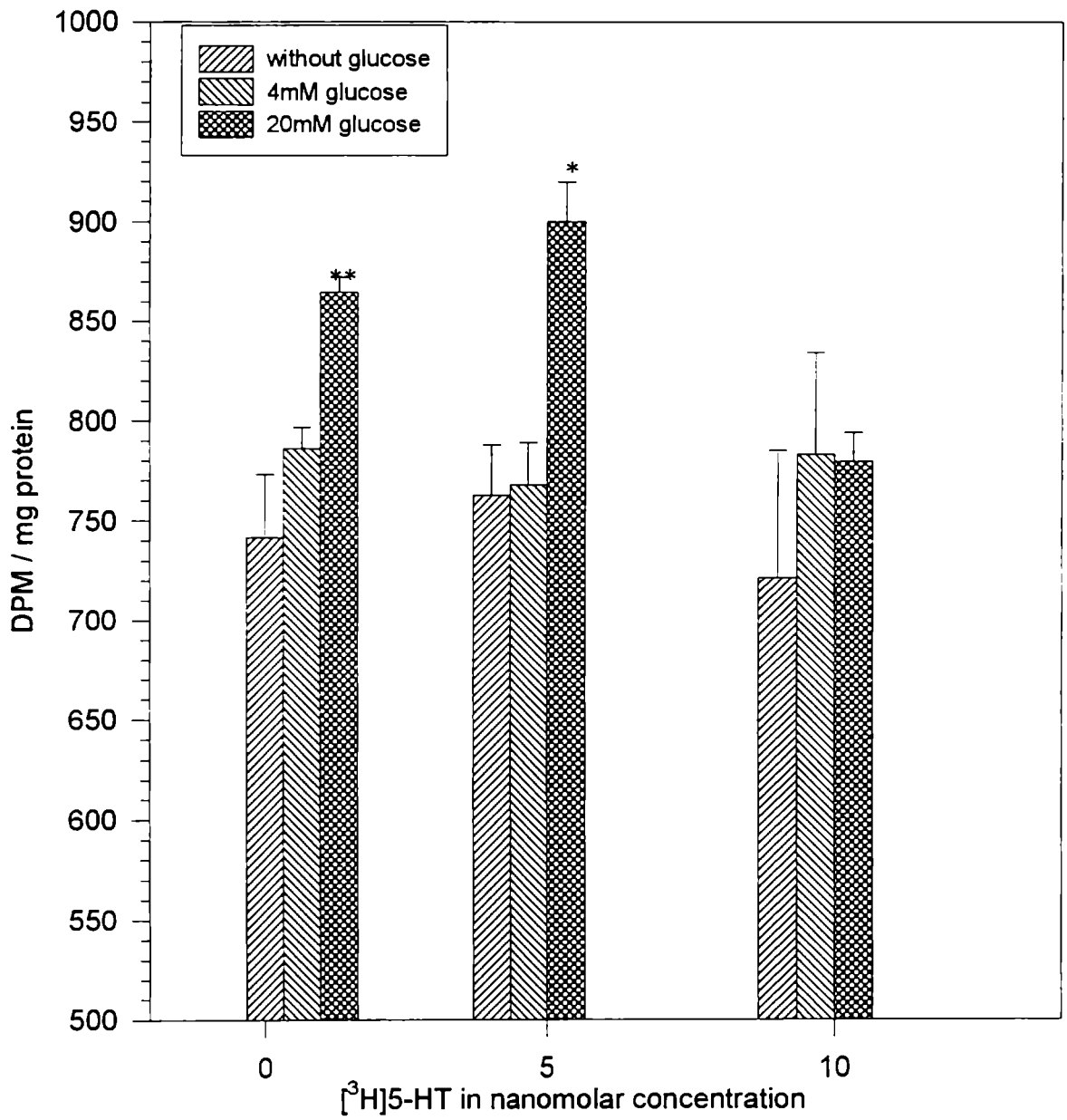


Figure-22

***In vitro* [^{3}H]5-HT uptake by pancreatic islets in the presence of different concentrations of glucose and ketanserin**

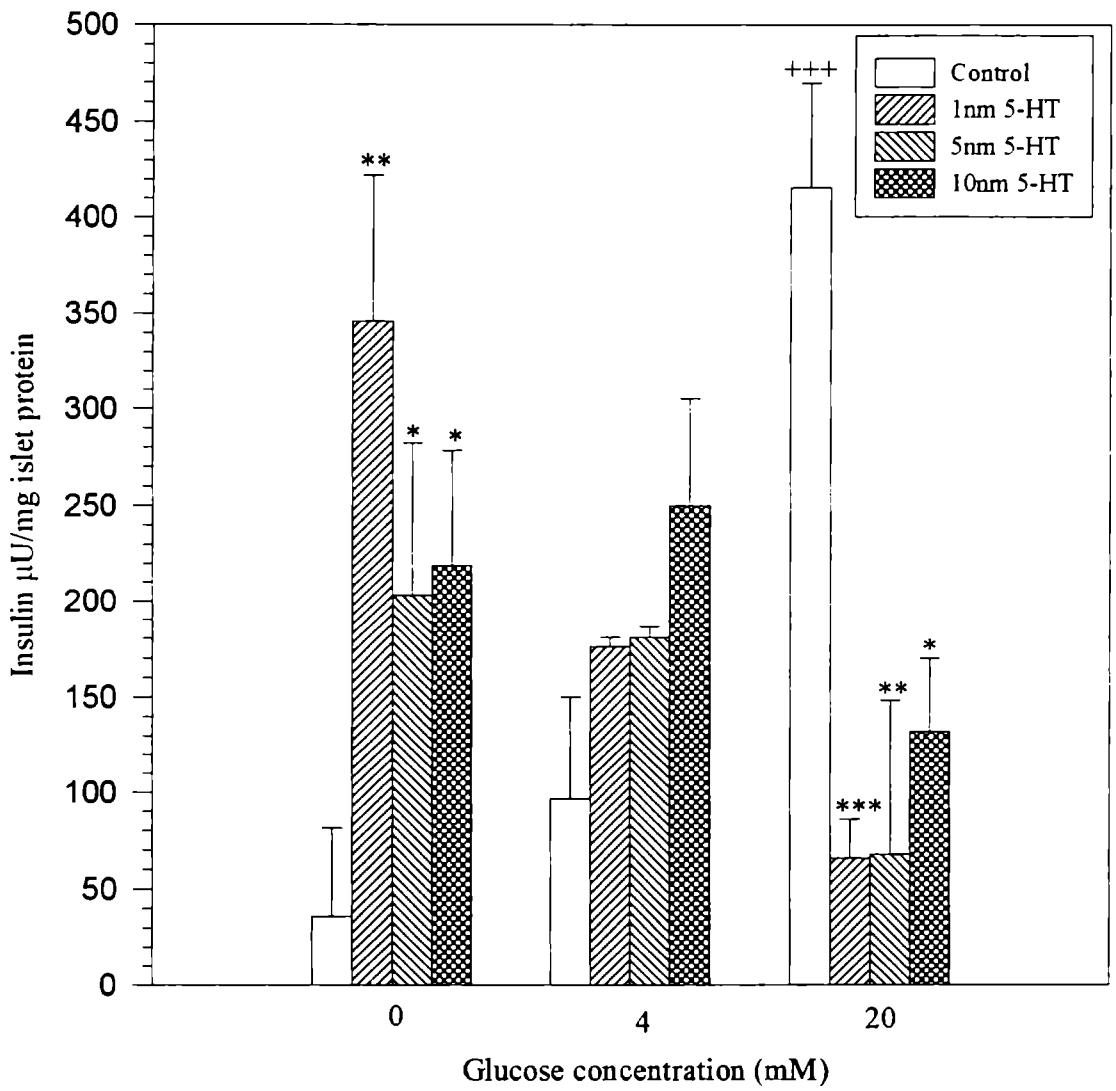


*p<0.05 compared to without glucose

**P<0.01 compared to without glucose

Figure-23

Effect of serotonin on glucose induced insulin secretion from pancreatic islets *in vitro*



*p<0.05 compared to control

**p<0.01 compared to control

***p<0.001 compared to control

+++p<0.001 compared to 0 mM and 4 mM glucose

Figure-24

[^{3}H]5-HT uptake and inhibition of insulin secretion at 20mM glucose concentration

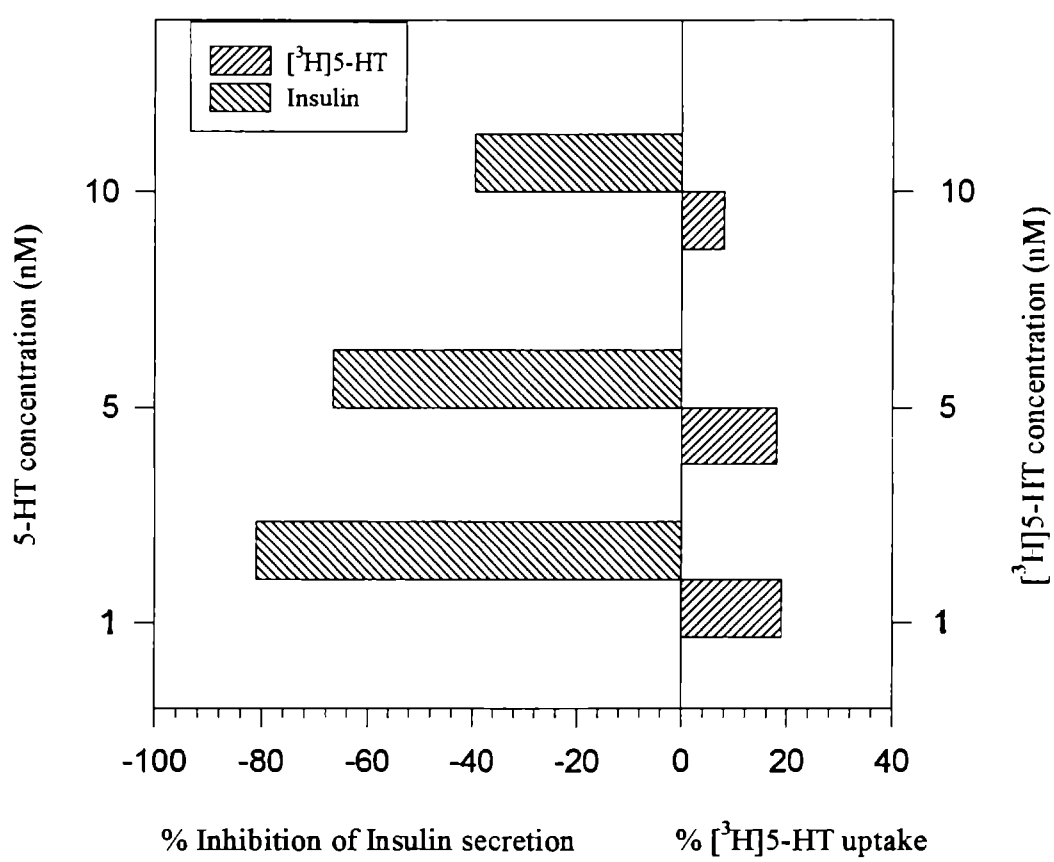


Figure-25a

Scatchard analysis of specific nuclear binding protein with [^{3}H]5-HT in pancreatic islets of control and experimental rats

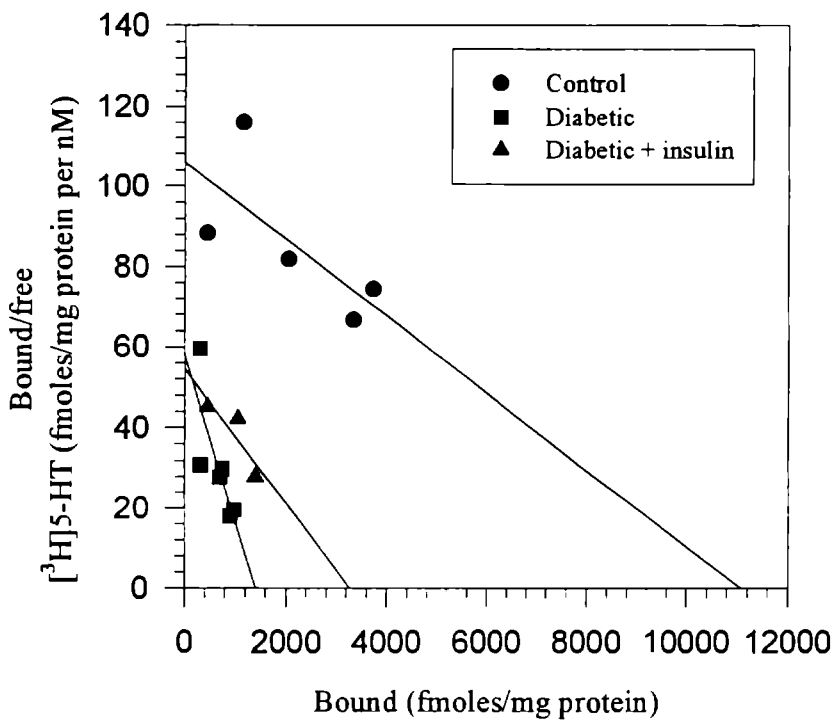


Figure-25b

Scatchard analysis of specific nuclear binding protein with [^{3}H]5-HT in pancreatic islets of control and experimental rats

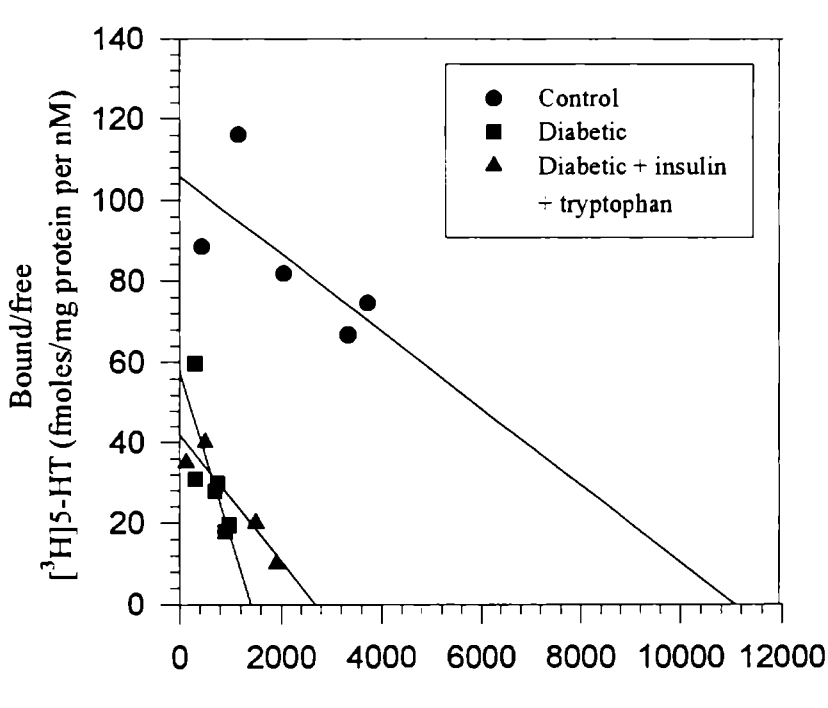
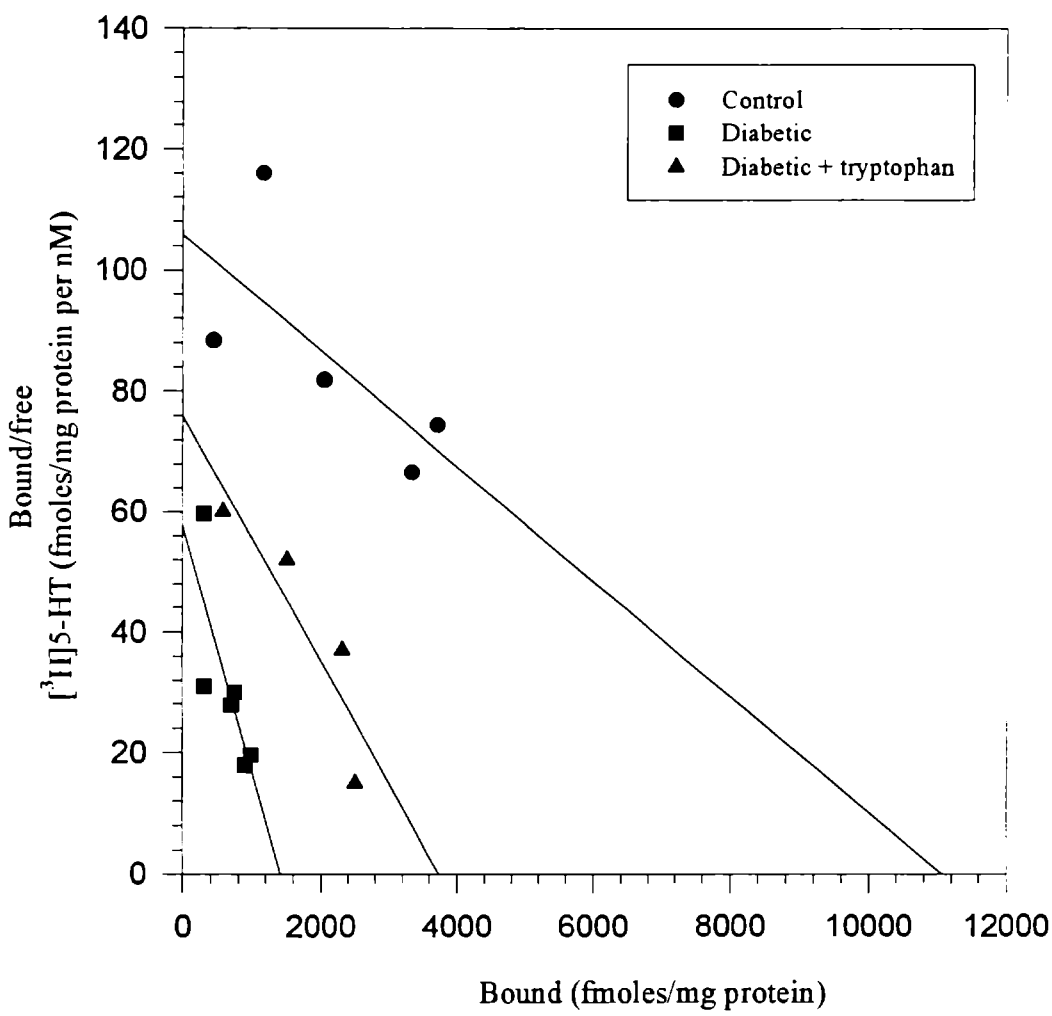


Figure-25c

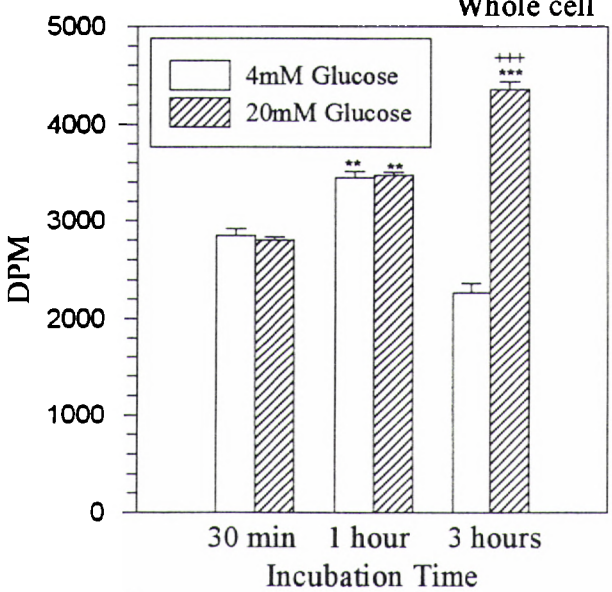
Scatchard analysis of specific nuclear binding protein with [^{3}H]5-HT in pancreatic islets of control and experimental rats



Uptake of [^{3}H]5-HT by pancreatic islets at different time intervals

Figure-26a

Whole cell



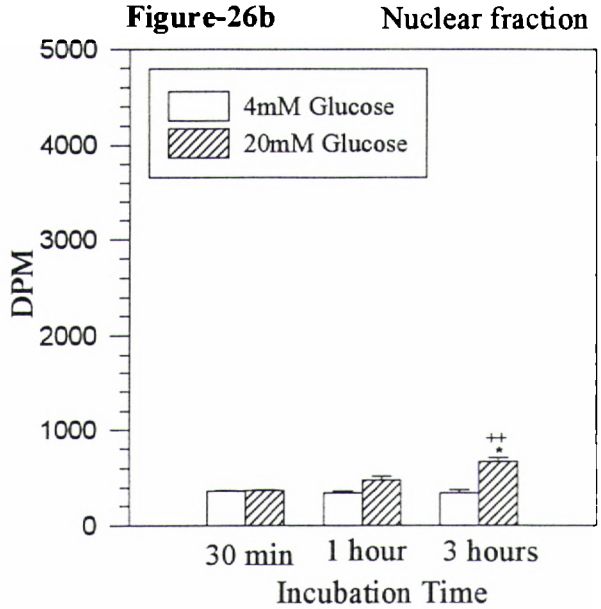
**P<0.01 compared to 30min

***P<0.001 compared to 30min

+++P<0.001 compared to 3 hours and 4mM glucose

Figure-26b

Nuclear fraction



*P<0.05 compared to 30min

++P<0.01 compared to 3 hours and 4mM glucose

Figure-26c

Mitochondrial + Membrane fraction

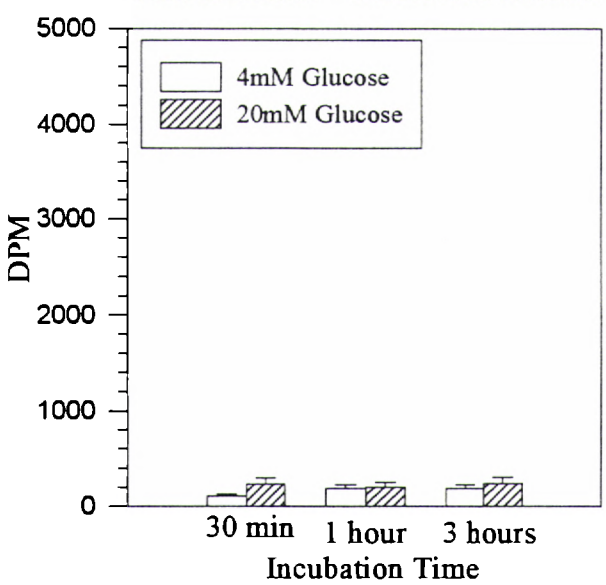
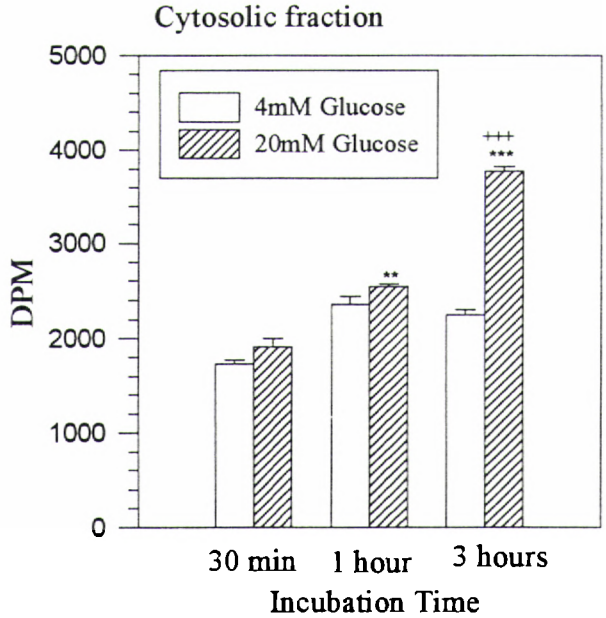


Figure-26d

Cytosolic fraction

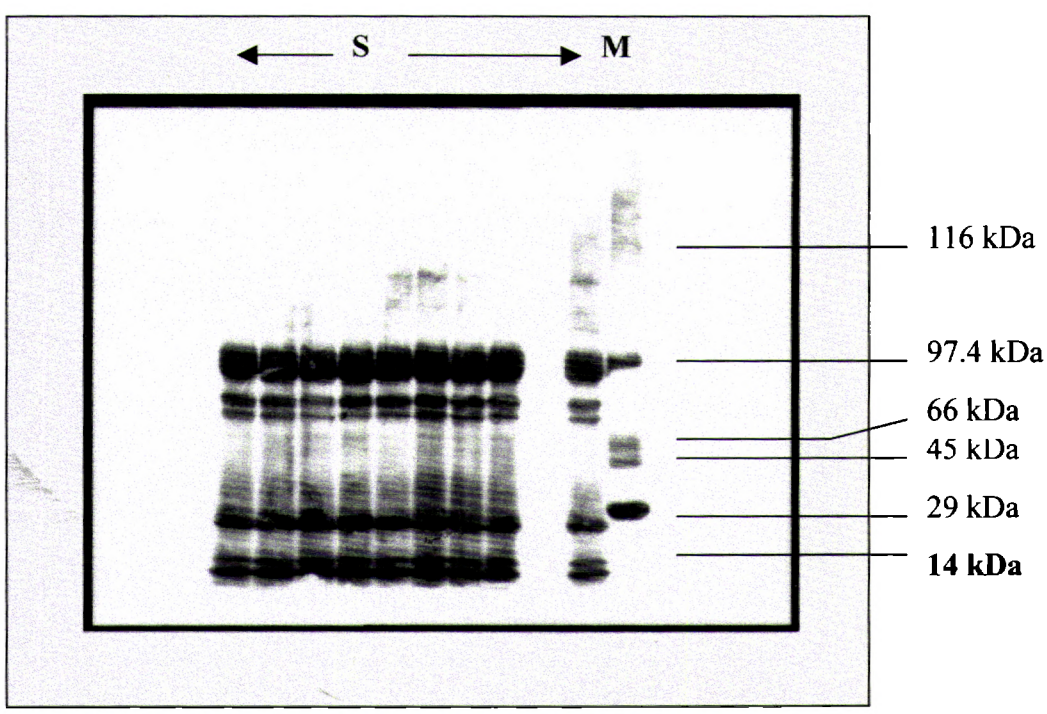


**P<0.01 compared to 30min

***P<0.001 compared to 30min

+++P<0.001 compared to 3 hours and 4mM glucose

PLATE-7



SDS-PAGE of specific nuclear 5-HT binding protein (14-kDa) in the pancreatic islet cells.
Lane- M- Molecular weight markers
Lanes- S- Samples (solubilised nuclear proteins from pancreatic islets)
Molecular weight markers used: β-galactosidase- 116-kDa, Phosphorylase- 97.4-kDa, Bovine albumin- 66-kDa, Egg albumin- 45-kDa, Carbonic anhydrase- 29-kDa.

Figure-27

Identification of [^{3}H]5-HT binding nuclear protein in pancreatic islets by ligand blotting method

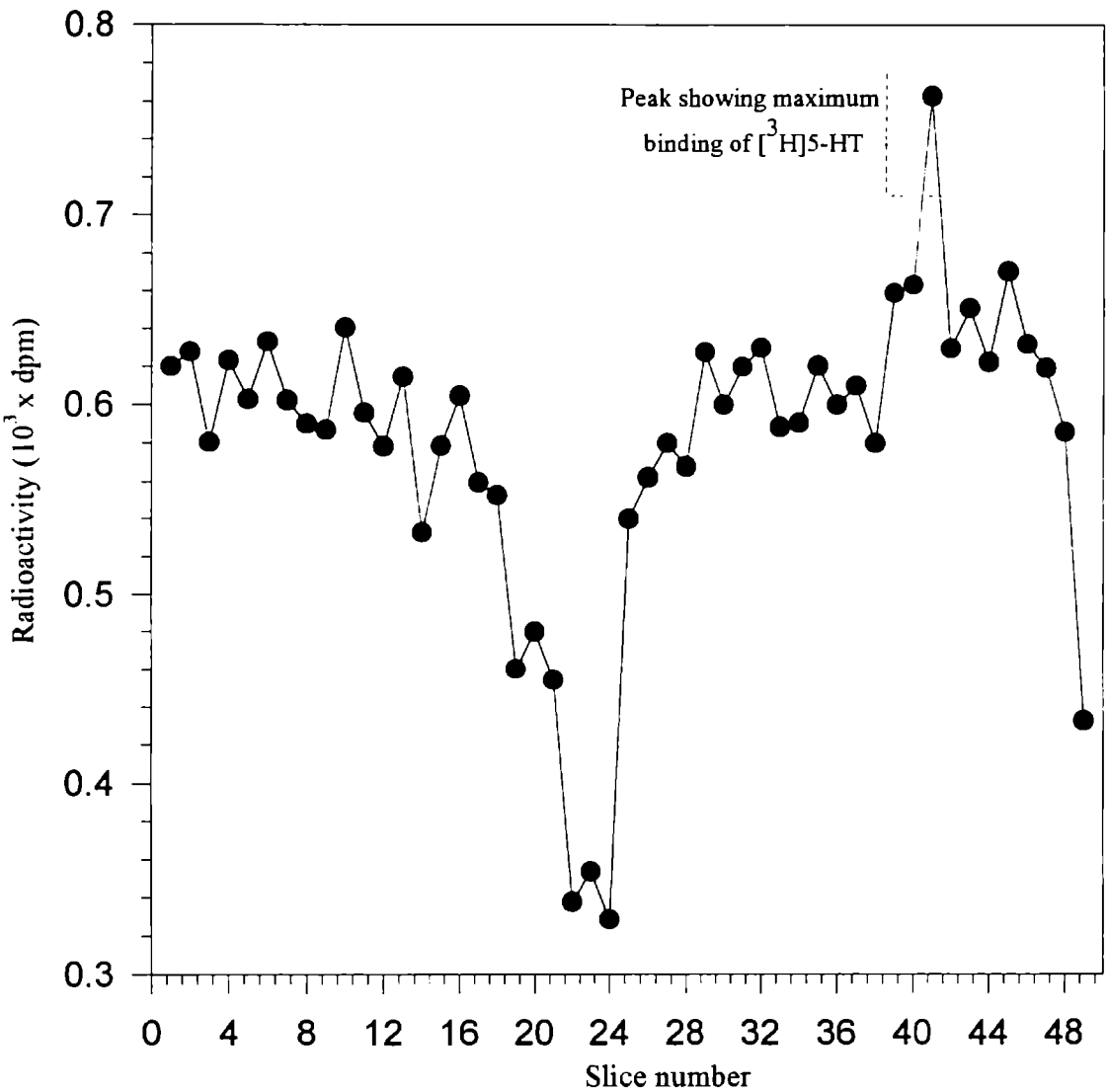
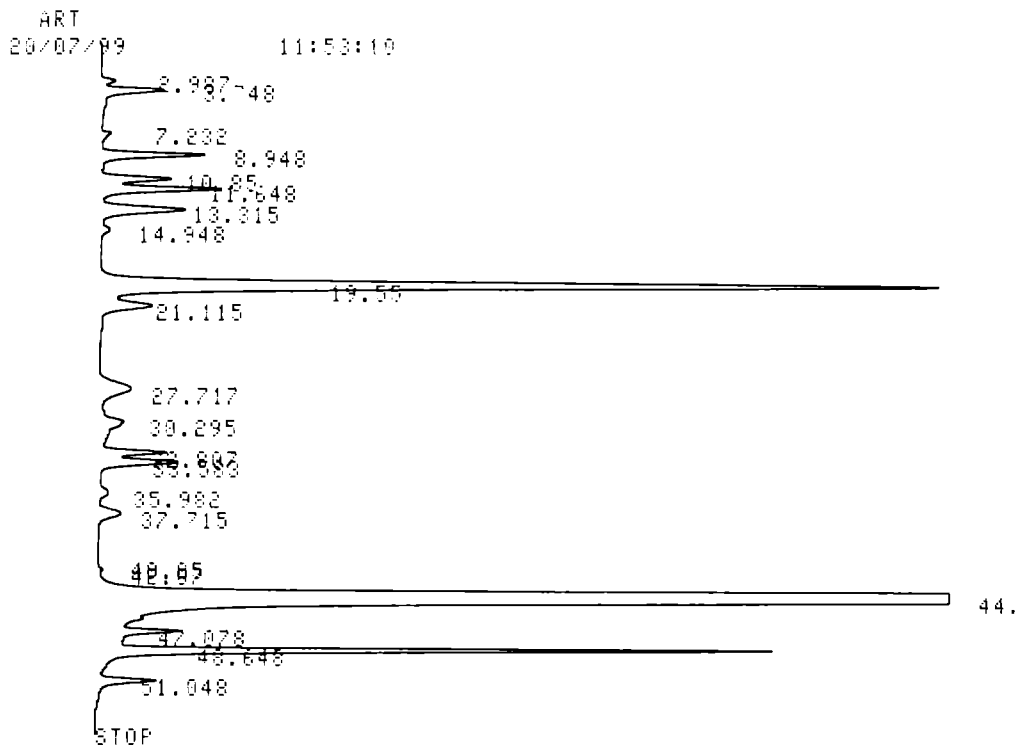


Figure-28

Chromatogram of aminoacid composition of 14-kDa nuclear specific binding protein from pancreatic islets



CHROMATOGRAM MEMORIZED

CHROMATOPAC C-R6A _E 0
SAMPLE NO 0 METHOD 44
REPORT NO 1276 SAMPLE WT 100

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	2.987					
2	3.748	1915				
	7.232	315				
4	948	198		1	0.0018	ASP
5	10.85	2378			0.0011	THR
	11.648	4107			0.0019	SER
	13.315	3852		4	0.0023	GLU
	14.948	434		5	0.0013	PRO
	19.55	36116			0.0229	GLY
10	21.115	2678			0.002	ALA
11	27.717	2832			0.0014	VAL
12	30.295	2445		9	0.0014	MET
13	32.807	2 16		11	0.001	ILE
14	33.583	3275		12	0.0016	LEU
15	35.982	715		1	0.001	TYR
16	37.715	1439		14	0.0007	PHE
17	40.05		V			
	42.07	21				
19	44.35	288670		15	0.0773	HIS
20	47.078	1492		16	0.0002	LYS
21	48.648	19106		17	0.0029	AMM
22	51.048	1819	T	1	0.0002	ARG
	TOTAL				1208	

DISCUSSION

5. DISCUSSION

The blood glucose level of diabetic rats was significantly elevated by administration of streptozotocin which specifically destroys the pancreatic β-cells (Junod *et al.*, 1969). Destruction of β-cells lead to a decrease in insulin secretory efficiency of these cells. Treatment of diabetic rats with insulin, tryptophan and insulin + tryptophan significantly reduced the blood glucose level. There was no significant change in the body weight of the experimental rats.

In our experiments we have used L-tryptophan instead of 5-HTP, since 5-HTP would lead to 5-HT formation at sites that do not form 5-HT physiologically The enzyme aromatic L-amino acid decarboxylase that converts 5-HTP to 5-HT is present in many other cells other than serotonergic neurons (Fuller, 1981). Administration of L-tryptophan will specifically increase the 5-HT content in serotonergic neurons. The disadvantage in use of L-tryptophan is that, only a small percentage of the administered L-tryptophan will be converted to 5-HT To overcome this a high dose treatment is required.

5.1. CENTRAL NERVOUS SYSTEM CONTROL OF INSULIN REGULATION

5.1.1. Brain 5-HT content is reduced during diabetes

In our experiments we have observed a significant reduction of 5-HT content in cerebral cortex (CC), brain stem (BS) and hypothalamus (Hypo) of diabetic rats. These finding agree with the previous reports of decreased 5-HT in brain regions during diabetes (Jackson & Paulose, 1999; Sandrini *et al.*, 1997; Sumiyoshi *et al.*, 1997; Thorre *et al* 1997; Shimizu, 1991, Chu *et al.*, 1986; Kulikov *et al* 1986). In cerebral cortex the decrease in 5-HT content is due to a reduction in the conversion of 5-HTP to 5-HT This is because of a significant decrease in 5-HTP content. Another contributing factor for the decreased 5-HT is the significant increase in the breakdown of 5-HT to 5-HIAA that is catalysed by monoamine oxidase which is known to regulate insulin secretion (Pizzinat *et al.*, 1999).

In case of brain stem the decrease in 5-HT content is brought about by a significant

increase in the rate of synthesis of 5-HT and its breakdown to 5-HIAA. There is also no significant increase of 5-HTP during diabetes. This leads to a decreased accumulation of 5-HT in the serotonergic neurons. There was no significant change in the hypothalamic 5-HT content of diabetic rats. But there was a significant reduction in the availability of the precursor 5-HTP in diabetic rats. A significant reduction in the conversion of 5-HTP to 5-HT is also observed during diabetes without any change in the breakdown of 5-HT Thus it appears that in hypothalamus the breakdown of 5-HT to 5-HIAA is reduced to compensate for the decreased availability of 5-HTP The observed reduction in 5-HT synthesis will be associated with reduced transmitter release (Carndall *et al.*, 1981).

Insulin treatment was able to significantly increase the 5-HT content in CC, BS and Hypo. This increase in the brain 5-HT content is due to the increase in tryptophan uptake through the BBB with other neutral amino acids. The carrier system for transport of tryptophan across the BBB is shared by several large neutral amino acids including tryptophan (Carndall *et al.*, 1981). Insulin tends to release the tryptophan bound to albumin hence increasing the concentration of free tryptophan in plasma (Trulson & Mackenzie, 1978; Curzon & Mursden, 1975). Treatment of diabetic rats with tryptophan and insulin + tryptophan also significantly increased the brain 5-HT content. Jamnicky *et al.*, (1993) have reported that administration of tryptophan in combination with insulin to diabetic rats have reversed the levels of brain tryptophan, 5-HT, 5-HIAA and serum concentrations of valine, leucine and isoleucine towards control. Oral administration of 5-HTP to diabetic patients have also increased brain 5-HT content (Rossi-Fanelli, 1998).

The NE content in BS and Hypo of diabetic rats were significantly increased. There was no significant change in the NE content in CC of diabetic rats. This result is concordant with the previously published reports (Tasaka *et al.*, 1992; Chen & Yang, 1991, Lackovic *et al.*, 1990). Insulin, tryptophan and insulin + tryptophan treatment reversed the increased NE content in BS and Hypo to control.

It has been well documented that long term hyperglycaemia in diabetic animals can lead to chronic hypofunction of central 5-HT neurons leading to decreased brain tryptophan, 5-HT and 5-HIAA (Sandrini *et al.*, 1997; Kwok & Juorio, 1987). The

decrease in brain 5-HT is due to the decreased availability of tryptophan in brain. The amount of tryptophan correlates with the 5-HT content in the brain (Fernstrom & Fernstrom, 1995; Fernstrom, 1991, Curzon & Mursden, 1975; Friedman *et al.*, 1972; Ecclestron *et al.*, 1965).

An increase in the level of insulin can result in decreased plasma concentrations of large neutral amino acids which compete with tryptophan for uptake into the brain (Cruzon & Fernando, 1977). STZ selectively destroys pancreatic β-cells and causes hypoinsulinemia leading to hyperglycaemia (Hohenegger & Rudas, 1971, Arison *et al* 1967). This decrease in the circulating insulin can increase the competition of other amino acids with tryptophan for uptake into brain thereby decreasing the level of tryptophan and 5-HT in the brain of diabetic rats. Consumption of a tryptophan rich diet can also increase the brain tryptophan. This will lead to an increase in circulating tryptophan, which will reduce the competition of tryptophan with other amino acid for uptake into brain. Hutson *et al* (1985) have reported a similar increase in 5-HT and 5-HIAA in the cerebrospinal fluid (CSF) as seen in brain after i.p. administration of tryptophan.

5.1.2. Plasma 5-HT and EPI concentrations is increased during diabetes

During diabetes there was a significant increase in plasma 5-HT concentration. This agrees with our earlier report on the increase in platelet 5-HT content during diabetes (Jackson *et al.*, 1997). There is also a significant increase in the plasma 5-HTP concentration in diabetic rats. The increase in plasma 5-HTP can be related to an increase in plasma tryptophan. In the absence of insulin, plasma tryptophan remains bound to albumin which is not taken up readily through the BBB since only free tryptophan is taken up through the BBB. This leads to an accumulation of tryptophan in the plasma of diabetic rats. Plasma EPI concentration was also significantly increased in diabetic rats. The increased NE and EPI concentrations in plasma is due to increased sympathetic stimulation during diabetes centrally and peripherally (Jackson *et al.* 1997; Chaouloff *et al.*, 1990a; McCall & Hornis, 1988; Hoyer, 1988c). Insulin, tryptophan and insulin + tryptophan treatment effectively reversed the 5-HT and EPI levels to control.

5.1.3. Brain 5-HT$_{2A}$ receptor activity and expression is increased during diabetes

One of the major findings of this study is that there is an increase in affinity of 5-HT$_{2A}$ receptors in cerebral cortex and hypothalamus without any change in its number and there is an appearance of a low affinity site during STZ-induced diabetes. In the case of brain stem 5-HT$_{2A}$ receptors there is an up-regulation of 5-HT$_{2A}$ receptors accompanied by a decrease in its affinity These alterations of 5-HT$_{2A}$ receptors in the brain regions is a compensatory mechanism for the decreased 5-HT content reported during diabetes in the brain regions (Jackson & Paulose, 1999; Sandrini *et al.*, 1997).

In our experiments, treatment of diabetic rats with insulin effectively reversed the altered 5-HT$_{2A}$ receptors to control. The increase in circulating insulin favours the increased uptake of tryptophan into the brain that in turn increases the brain 5-HT content thereby bringing a decrease in the 5-HT$_{2A}$ receptors. It is reported that the up-regulation of 5-HT$_{2A}$ receptors during diabetes is a secondary effect of hypoinsulinemia (Sumiyoshi *et al.*, 1997). This up-regulation of the receptor can have a possible role in the regulation of insulin secretion. The increased affinity and increase in number of 5-HT$_{2A}$ receptors in CC, Hypo and BS respectively can increase the sympathetic nerve discharge thereby increasing the circulating NE and EPI levels. This increased NE and EPI might then bind to α_2 adrenergic receptors and inhibit insulin secretion from pancreatic islets with simultaneous increase in glucagon level. It is already reported that the 5-HT$_2$ agonist 1-(2,5-di-methoxy-4-iodophenyl)-2-aminopropane (DOI) was able to produce a tremendous increase in sympathetic nerve discharge, thus increasing EPI concentration. 5-HT$_2$ antagonists, ketanserin and LY53857 were able to reverse the increase in sympathetic nerve discharge produced by DOI (Jackson *et al.*, 1997; Chaouloff *et al* 1990a; McCall & Hornis, 1988; Hoyer, 1988c). McDonald, (1996) have reported increased expression of 5-HT$_2$ receptor mRNA in islets maintained for 1day at 20mM glucose than in islets maintained at 1mM glucose. Trulson & Mackenzie, (1978) have reported that after 4 weeks of administration of streptozotocin, the brain tryptophan content was decreased by 27%. Insulin administration was able to bring back the brain tryptophan and 5-HIAA levels to normal. Tryptophan uptake across the BBB is increased in the presence insulin. Insulin enhances the uptake of branched chain amino acids into the muscles thereby decreasing their plasma concentration. Since these amino acids

compete with tryptophan for transport into brain, there is a resultant increase in brain tryptophan (Curzon & Mursden, 1975).

During diabetes there is a significant reduction of brain tryptophan, 5-HT and 5-HIAA content (Sandrini *et al* 1997; Kwok & Juorio, 1987). The decreased brain 5-HT content leads to an up-regulation of 5-HT_{2A} receptors in BS and an increased affinity of these receptors in cerebral cortex (Jackson & Paulose, 1999; Sandrini *et al.*, 1997; Sumiyoshi *et al.*, 1997). This leads to increased sympathetic stimulation and thereby decreases insulin secretion from pancreatic islets mediated by EPI release from adrenal glands (Chaouloff *et al.*, 1990d). An up-regulation of 5-HT_{2A} receptors also increases the risk of diabetes induced depression (Mann *et al.*, 1986; Stanley, 1983). The increase in brain 5-HT reverses the altered 5-HT_{2A} receptor binding parameters in cerebral cortex and brain stem and reduces sympathetic nerve stimulation thus reducing the inhibitory effect of EPI on insulin secretion.

It is reported that diet can also influence the brain 5-HT content. Consumption of tryptophan deficient diet can also lead to reduced circulating tryptophan and brain 5-HT content (Fernstrom, 1994). DeMarte & Enesco, (1985) maintained a group of mice for 78 weeks on tryptophan restricted, protein restricted and control diet. They found that brain 5-HT levels were significantly reduced only in mice on the tryptophan-restricted diet, but not for mice on the protein restricted diet. It is not only tryptophan that is influenced by the diet but other amino acids such as tyrosine that is the precursor for dopamine and norepinephrine. The same process is applicable for the uptake of choline which is the precursor of acetylcholine (Fernstrom, 1994). From this it appears that diet can also play an important role in the induction of diabetes through the serotonergic system by reducing the brain 5-HT content. In addition to the central 5-HT_{2A} receptors the peripheral 5-HT_{2A} receptors may also play a major role in regulation of insulin, since the pancreatic islets contain a large amount of endogenous serotonin (DeMarte & Enesco, 1985; Bird *et al.*, 1980).

Thus, from our study we conclude that STZ induced diabetes causes an increase in affinity of 5-HT_{2A} receptors in cerebral cortex and hypothalamus without any change in

the number of receptors. The brain stem 5-HT_{2A} receptors are up-regulated accompanied by the appearance of a low affinity site which was reversed to control by insulin treatment. The enhanced 5-HT_{2A} receptor binding observed in brain regions can mediate an increased sympathetic nerve discharge leading to inhibition of insulin release from pancreas and can also mediate diabetes induced depression. Alterations in the apparent number of 5-HT_{2A} receptors have also been reported in several central nervous system disorders such as schizophrenia, Parkinson's disease and Alzheimer's disease (Conn & Sanders-Bush, 1987).

We have observed an increase in the expression of 5-HT_{2A} receptors of diabetic BS. The CC and Hypo showed only an increase in affinity for 5-HT during diabetes without any change in its expression. This can be due to the fact that only BS has direct nerves originating from it and extending to the pancreas (Coldman & Dampney, 1998) The CC and Hypo does not have direct inervation to the end organ. The CC co-ordinates the overall function of the brain. Hypo acts through the hypathalamo-pituitary end organ axis. In CC and Hypo, a decrease in 5-HT content during diabetes brings about a compensatory increase in the affinity of 5-HT_{2A} receptors without increasing the mRNA levels. This compensation can be considered as a momentary change to overcome the decrease in 5-HT In the case of BS the decreased 5-HT is compensated by increasing the level of 5-HT_{2A} receptor mRNA which in turn will inhibit insulin secretion by direct sympathetic stimulation. This compensation is not a momentary change but it affects the transcription of the 5-HT_{2A} receptor gene. It has been reported that 5-HT can regulate the expression of 5-HT_{2A} receptors. The 5-HT dependent transcription activity depends upon the presence of functional 5-HT_{2A} receptors (Yun-Long *et al.*, 1995). Therefore, once there is a decrease in 5-HT in BS, the 5-HT_{2A} receptors increase its affinity to bind to the available 5-HT This in turn regulates the transcription of the 5-HT_{2A} receptors in BS. The significant in 5-HT_{2A} receptors observed during diabetes in pancreatic islets may be due to an increase in pancreatic 5-HT content.

5.1.4. Brain 5-HT_{1A} receptor activity is increased during diabetes

5-HT_{1A} has already been reported to have a similar role in the inhibition of insulin secretion. Scatchard analysis of high affinity 5-HT_{1A} receptors in CC and Hypo showed a significant decrease in the number of receptors without any significant change in its

affinity during diabetes. But the low affinity receptors in these two regions showed a significant increase in its number during diabetes. From these results it seems that the decreased 5-HT is able to up-regulate the low affinity 5-HT_{1A} receptor and down-regulate the high affinity 5-HT_{1A} receptors. As mentioned in the results the high affinity and low affinity 5-HT_{1A} receptors are actually two different proteins coded by two distinct mRNA and are not inter-convertable states of the same protein (Nenonene *et al* 1994). Therefore, such a differential regulation shows that in CC and Hypo, the alterations in the 5-HT_{1A} receptors during diabetes are mediated by the low affinity 5-HT_{1A} receptors. Treatment of diabetic rats with insulin, tryptophan and insulin + tryptophan did not reverse the altered high affinity and low affinity 5-HT_{1A} receptors. Nenonene *et al.*, (1994) have demonstrated the heterogeneity of [^{3}H]8-OH-DPAT binding in rat cerebral cortex and hippocampus. The first high affinity binding site, 5-HT_{1A}^{HIGH} represents the classic 5-HT_{1A} receptor based on its pharmacological profile and the effects of Gpp(NH)p The second binding site, 5-HT_{1A}^{LOW}, is also labelled by [^{3}H]8-OH-DPAT It has a micromolar affinity for 5-HT and it is not coupled to G proteins.

In case of brain stem, there is no significant change in number of low affinity 5-HT_{1A} receptor but there is an increase in its affinity during diabetic state. The high affinity 5-HT_{1A} receptors in BS show a significant increase in its number without any change in affinity This is reversed by insulin, tryptophan and insulin + tryptophan treatment. From these results it appears that in CC and Hypo the low affinity 5-HT_{1A} receptors are more involved during diabetes and in BS the high affinity 5-HT_{1A} receptors are involved in mediating the effect of decreased 5-HT An increase in the 5-HT_{1A} receptors in CC and BS has been previously reported (Sandrini *et al.*, 1997; Sumiyoshi *et al.*, 1997). But they have not reported any difference in the high and low affinity states of these receptors.

The increase in number of the 5-HT_{1A} receptors in response to the decreased 5-HT content in CC and BS will stimulate the sympathetic nerves and increase the vagal tone. An increased sympathetic activity will induce increased EPI output from the adrenal medulla that will inhibit insulin secretion (Bauhelal & Mir, 1993, Bauhelal & Mir, 1990a). This was proved by injecting the specific 5-HT_{1A} receptor agonist 8-OH-DPAT to normal

rats. The rats showed a very rapid increase in blood glucose level that reached its peak within 30min. A similar observation was reported from a number of laboratories (Laude *et al.*, 1990; Bauhelal *et al.*, 1990b; Chaouloff & Jeanrenard, 1988; Chaouloff & Jeanrenaud, 1987). Administration of 8-OH-DPAT also produced a sustained fall in blood pressure and heart rate. All these symptoms were abolished by idazoxan pre-treatment and by adrenalectomy (Bauhelal & Mir, 1993). 8-OH-DPAT treatment can also increase plasma levels of corticosterone that can also inhibit insulin secretion (Chaouloff *et al* 1990d; Aulakh *et al.*, 1988; Koenig *et al.*, 1987)

The 5-HT_{1A} is coupled to guanine nucleotide regulatory proteins or G proteins and its labelling by [^{3}H]8-OH-DPAT decreases in the presence of GTP (Hall *et al* 1985). GTP analogue caused a decrease in affinity of 5-HT_{1A} receptors to their natural ligand 5-HT in CC and BS of diabetic rats. This indicates a desensitisation of the 5-HT_{1A} receptors on G-protein association. In control rats the inhibition of 1nM [^{3}H]8-OH-DPAT binding by increasing concentrations of 5-HT shifted to the lower affinity state in the presence of Gpp[NH]p and the $Ki_{(H)}$ value increased in agreement with previous results using serotoninergic agonists (Hamon *et al.*, 1988). The reduction of 1nM [^{3}H]8-OH-DPAT high affinity confirms that this site corresponds to the known 5-HT_{1A} receptor The other component of 1nM [^{3}H]8-OH-DPAT binding i.e., the 5-HT_{1A}^{LOW} sites remained insensitive to 100μM Gpp[NH]p. A similar observation was reported by Emerit *et al* (1991).

5.1.5. Insulin synthesis is inhibited by stimulation of sympatho-adrenal activity through 5-HT_{1A} and 5-HT_{2A} receptors

In case of 5-HT_{1A} and 5-HT_{2A} receptors in brain regions of diabetic rats we have found a compensatory increase in either the number of the receptors or an increase in its affinity to bind to the decreased brain 5-HT content. Thus, from our study we conclude that the enhanced 5-HT_{2A} and 5-HT_{1A} receptor binding observed in brain regions of diabetic rats can mediate an increased sympathetic nerve discharge (Bauhelal & Mir, 1993; Bauhelal & Mir, 1990a). Simultaneously there is an increased EPI release from the adrenal medulla adding to the sympathetic stimulation. This lead to an inhibition of insulin release from pancreas.

5.1.6. Hypothalamo-pituitary-thyroid axis also plays an important role in inhibition of insulin secretion

The hypothalamo-pituitary-thyroid status is also affected during STZ induced diabetes. We have observed a significant decrease in hypothalamic 5-HT content and circulating T_3 and T_4 levels. This agrees with the previous reports of TSH, T_3 and T_4 reduction in STZ diabetes (VanHaasteren *et al.*, 1997; Wilber *et al.*, 1981). We have also observed an increase in affinity of 5-HT_{2A} and increase in number of low affinity 5-HT_{1A} receptors in hypothalamus of diabetic rats. A decrease in 5-HT content in hypothalamus will lead to decreased TSH secretion. Dakshinamurti *et al.* (1985) have reported a reduction in hypothalamic 5-HT content in pyridoxine-deficient hypothyroid rats. This decreased 5-HT reduces the synthesis and release of TSH from the pituitary through TRH secretion (Dakshinamurti *et al.*, 1986). Van Haasteren *et al* (1997) have reported that the reduced hypothalamic TRH release during diabetes is probably not caused by decrease in TRH synthesis or transport to the median eminence, but seems to be due to impaired TRH release from the median eminence which can be related to the lack of insulin. Therefore, the decreased T_3 and T_4 in circulation may be due to a decreased release of TRH from the hypothalamus that is mediated by an altered 5-HT_{2A} and 5-HT_{1A} receptors. The postulate that 5-HT neurons stimulate TSH secretion in rats is supported by the observation that injection of 5-HT into the third ventricle caused rapid increase in serum TSH (Smythe *et al.*, 1982). Balsa *et al.*, (1998) have reported that 5-HT can stimulate the secretion of GH, ACTH and LH acting directly at pituitary level on the posterior pituitary Treatment with insulin, tryptophan and insulin + tryptophan reversed the hypothalamic 5-HT content and the altered 5-HT_{1A} and 5-HT_{2A} receptors. But the T_3 and T_4 content in circulation was not reversed to control level by these treatments. This may be due to the delayed thyroid response to the treatments.

5.2. PERIPHERAL CONTROL OF INSULIN SECRETION

5.2.1. Pancreatic islet 5-HT content is increased during diabetes

Pancreatic islet is considered as a tissue rich in 5-HT (Bird *et al.*, 1980). Within the islets 5-HT is stored along with insulin granules (Bird *et al.*, 1980; Jain-Etcheverry &

Zieher, 1968; Falck & Hellmann, 1963). Our results show a significant increase in pancreatic 5-HT content in diabetic rats. There is also a significant increase in the level of 5-HTP which acts as precursor of 5-HT The presence of increased pancreatic content of 5-HTP in diabetic rats will lead to increased synthesis of 5-HT which is evident from our results. In presence of high glucose 5-HTP is rapidly taken up into the islets which stimulates insulin secretion. But the enzyme 5-Hydroxy tryptophan decarboxylase readily converts it into 5-HT that inhibits insulin secretion (Sundler *et al.*, 1990; Lindstrom & Sehlin, 1983). Tryptophan which is a precursor of 5-HTP, is reported to have a stimulatory effect on insulin release from hamster pancreas (Bird *et al.*, 1980). The presence of monoamine oxidase enzyme that catabolises 5-HT within the β-cells and specifically nuclear membrane show an effective metabolism of 5-HT within the pancreas (Pizzinat *et al.*, 1999; Gujrati *et al.*, 1996; Feldman & Chapman, 1975). Treatment with insulin, tryptophan and insulin + tryptophan effectively decreased the pancreatic 5-HTP content which in turn decreased the 5-HT content. The decreased 5-HT will in turn reduce the inhibition of insulin secretion. 5-HT can also act as a marker for insulin secretion. 5-HT is taken up into insulin granules and co-released with insulin on stimulation of pancreatic β-cells by glucose (Zhou & Misler, 1996). All these evidences show the presence of 5-HT within the pancreatic islets and has a role in the regulation of insulin secretion from the β-cells. The EPI content in pancreas of diabetic rat showed a significant increase. At high concentrations, EPI binds and stimulates α_2-adrenergic receptors which in turn inhibits insulin secretion.

5.2.2. Diabetes induces 5-HT uptake into pancreatic islets and inhibits insulin secretion

A number of laboratories have reported the presence of 5-HT and other monoamines within the pancreatic islets (Bird *et al.*, 1980), but there was no evidence for the actual mechanism by which 5-HT control insulin secretion from the β-cells. We have observed a significant uptake of [^{3}H]5-HT into the pancreatic islets in the presence of 20mM glucose, which can be considered equivalent to diabetic state *in vivo*. Incubation of islets with 1nM and 5nM [^{3}H]5-HT showed a marked increase in 5-HT uptake in presence of 20mM glucose concentration compared to islets incubated with 4mM glucose. But cells incubated with 10nM [^{3}H]5-HT did not show any significant increase in 5-HT uptake. These results show that 5-HT is taken up by the islets only in the presence of

glucose. Thus, it can be considered that glucose is the determining factor for the uptake of 5-HT into the islets. There are also reports which state that 5-HT is taken up into the insulin granules and secrete 5-HT/insulin in a pulsatile fashion on stimulation of pancreatic islet β-cells under physiologic conditions (Zhou & Misler, 1996). Our results confirm the earlier findings where in a normal islet, an increase in glucose level will lead to increased uptake of 5-HT into the islets. 5-HT is co-released with insulin on glucose stimulation maintaining a steady equilibrium. But in the case of diabetic islets the increase in glucose leads to increased uptake of 5-HT into the islets. Since the glucose induced stimulus for insulin secretion is less, there is a decreased insulin output. This leads to an increased accumulation of 5-HT within the islets. Extensive studies on transmembrane transport of 5-HT have been carried out with neurons and thrombocytes. These cells generally have similar uptake mechanisms (Sneddon, 1973; Abrams & Solomon, 1969). The following are the two hypotheses for the mechanism of 5-HT uptake into the cells:

(i) 5-HT transport is mediated by islet high affinity low capacity active transport mechanism as well as by passive diffusion (Stahl & Meltzer, 1978).

(ii) 5-HT has two active transport systems working in parallel - one with saturable high affinity and low capacity and another with non-saturable low affinity and high capacity (Shaskan & Snyder, 1970).

The high affinity mechanism is sodium dependant and inhibited by metabolic blockers, whereas the low affinity component is not sodium dependant (Stahl & Meltzer, 1978). It is also reported that metabolic inhibition and sodium deficiency reduced the initial uptake of 5-HT in the presence of low extracellular 5-HT concentration but had no or less pronounced effects at higher 5-HT concentrations. This explains the decreased 5-HT uptake into the islets at 10nM [^{3}H] 5-HT concentration seen in our results whereas at low [^{3}H] 5-HT concentration (1nM, 5nM) there was a significant increase in glucose dependant [^{3}H] 5-HT uptake.

To study the possible action of 5-HT on insulin regulation, we fractionated the islets into membrane + mitochondrial, cytosolic and nuclear fractions. Only the nuclear fraction showed positive binding to [^{3}H]5-HT in all the experimental groups compared to

the membrane and cytosolic fractions which did not show any direct binding. Further confirmation on the uptake and binding of 5-HT to the nuclear fraction was made by incubating the fractionated islets with 5nM [^{3}H]5-HT for different time intervals and at different glucose concentrations (4mM and 20mM). The whole cells showed a glucose dependant uptake of [^{3}H]5-HT as discussed above. The mitochondrial and membrane fractions did not show any significant change in binding in the presence of glucose whereas the cytosolic fractions showed a significant increase of [^{3}H]5-HT uptake/transport in the presence of 20mM glucose at 1hour and 3hours incubations. The nuclear fraction also showed a significant increase in glucose dependent [^{3}H]5-HT uptake at 3hour incubations. We conclude from these results that 5-HT is taken up into the islet in a glucose dependent manner and binds to the nuclear protein. The presence of increased amounts of [^{3}H]5-HT in the cytoplasm signifies the 5-HT taken up which then binds to the nuclear protein.

The 5-HT binding protein on the nuclear membrane was identified by ligand blotting as a 14-kDa protein. Amino acid composition of this protein showed the presence of high concentrations of histidine (79 08%) followed by glycine (6 18%) and glutamine (1 12%). This 14-kDa 5-HT binding protein showed a marked similarity and homology with respect to amino acid composition and molecular weight to a helix-loop-helix protein2 (bHLH2) which belongs to the basic helix-loop-helix family of transcription factors.

The catabolic breakdown of 5-HT and other monoamines are also carried out in the pancreatic islets. Catacholamines are inactivated mainly by two mechanisms, through the enzyme catechol-o-methyltransferase (CDMT) and monoamine oxidase (MAO). A number of reports have appeared with regard to the role of MAO in islets, since the process of deamination of biogenic and exogenic amines lead to concomitant production of hydrogen peroxide (H_2O_2). The generation of H_2O_2 may affect the redox state of the β-cell glutathione system, the balance of which is known to influence nutrient-induced insulin release (Miller, 1981).

Monoamine oxidase exists in two isoforms, MAO-A and MAO-B, which are separate gene products, exhibiting different substrate specificity and whose activity may be differentially inhibited by specific inhibitors (Pizzinat *et al.*, 1999; Fowler *et al.*, 1978; Johnston, 1968). 5-HT is the preferred substrate for MAO-A whereas phenylethylamine (PEA) is preferentially deaminated by MAO-B. Tyramine, EPI, NE and dopamine are common substrate for both isoforms. In addition to cytoplasm of the cell, monoamine oxidase has been report to be present in the nuclei of placental cells (Gujrati *et al.* 1996).

The uptake of 5-HT into the islets can directly inhibit insulin synthesis within the pancreatic islets. The islets incubated with 1nM and 5nM 5-HT in the presence of 20mM glucose showed a significant inhibition of insulin secretion. But the islets incubated with 10nM 5-HT showed a lesser degree of inhibition compared to 1nM and 5nM 5-HT This is due to the decreased uptake of 5-HT due to high concentration of 5-HT in the medium. In contrast to this, in the absence of glucose, 5-HT was able to stimulate insulin secretion. This stimulation was further reduced in the presence of 4mM glucose. These results show that the 5-HT acts as a stimulant for insulin secretion in the absence of glucose but inhibits insulin in the presence of glucose.

5.2.3. 14-kDa nuclear 5-HT binding protein is regulated by 5-HT

Scatchard analysis of the 5-HT binding protein (bHLH) in islet nuclear fraction shows a significant down-regulation during diabetes that is evident from the reduced B_{max}. The K_d of the diabetic group is also significantly reduced showing an increased affinity for 5-HT This can be a natural mechanism of down-regulation similar to the 5-HT_{2A} receptor by the increased content of 5-HT (Leysen & Pauwels, 1990; Eison *et al.*, 1989). This is consistent with the traditional adaptive pattern of regulation of brain monoamine receptors (Yun-Long *et al.*, 1995). The increased affinity of the nuclear 5-HT binding protein leads to increased binding of 5-HT present within the islets to this protein. This leads to a feedback down-regulation of the 5-HT binding protein and inhibits insulin gene transcription during diabetes since this protein acts as an enhancer for insulin gene transcription.

SEROTONIN (5-HT) AND INSULIN REGULATION IN NORMAL PANCREATIC β-CELL

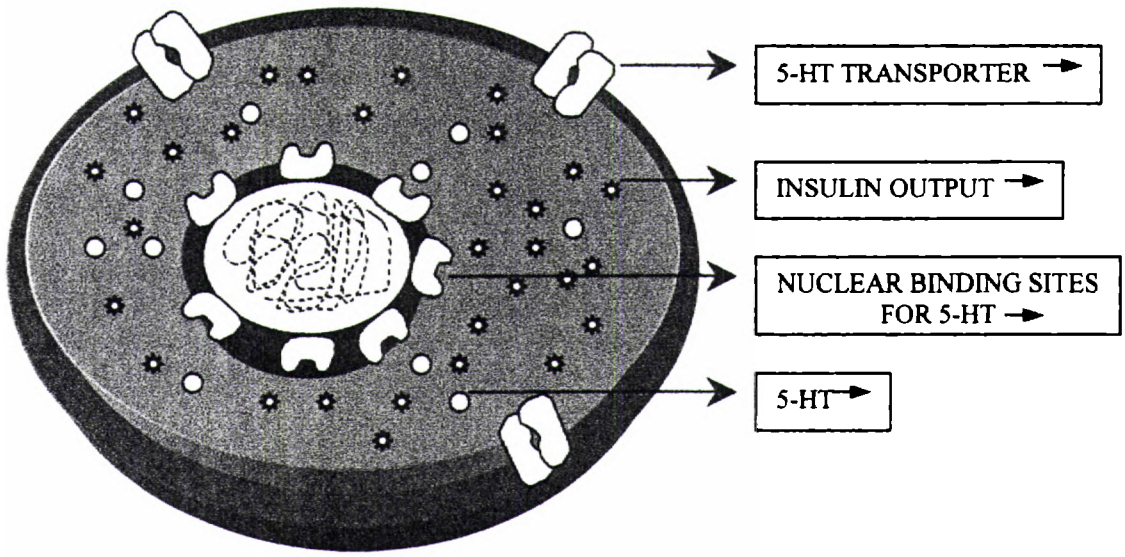


Figure shows normal pancreatic islet cell with normal amount of 5-HT, nuclear 5-HT binding protein and insulin output

SEROTONIN (5-HT) AND INSULIN REGULATION IN DIABETIC PANCREATIC β-CELL

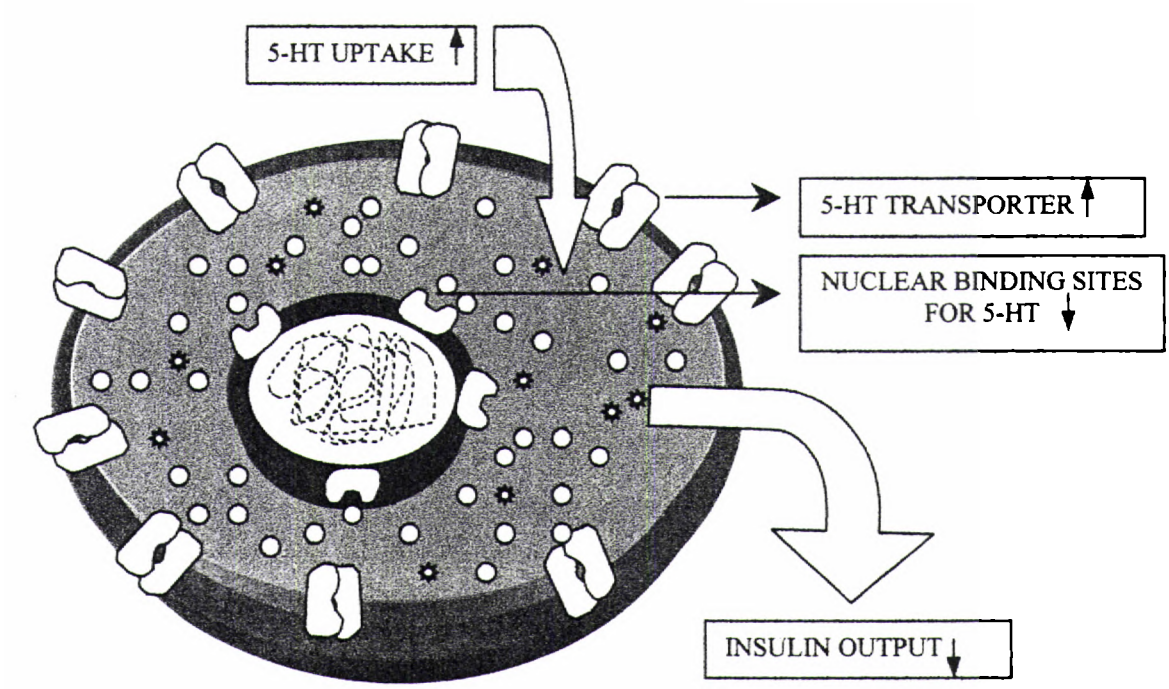


Figure shows inhibition of insulin secretion from a diabetic pancreatic β-cell. Glucose-induced 5-HT uptake down-regulates the nuclear 5-HT binding protein. Since this protein can act as an enhancer for insulin transcription, down-regulation of this protein reduces the insulin output.

Insulin replacement and a combination of treatment with insulin, tryptophan and insulin + tryptophan to diabetic rats failed to reverse the feedback control of the 5-HT binding protein. This is evident from the significant decrease in B_{max} compared to the controls. Thus, from our results we can infer that the feedback inhibition of the binding protein is triggered in the islets by hyperglycaemia induced increase in islet 5-HT. The down-regulation of the 5-HT binding protein cannot be completely corrected by insulin, tryptophan and insulin + tryptophan treatments. This is opposite to the results seen in the brain regions where we have observed a complete reversal of the $5\text{-}HT_{2A}$ binding parameters to control, by insulin, tryptophan and insulin + tryptophan treatments.

5.2.4. Possible transcriptional regulation of insulin gene by basic Helix-Loop-Helix protein2 (bHLH 2)

Based on the amino acid homology the 14-kDa nuclear 5-HT binding protein was identified as a basic helix-loop-helix protein. The bHLH2 protein acts as a transcriptional activator of insulin promoter They bind to E-box of the insulin promoter and enhance the transcription of the insulin gene (Vienna & Melson, 1995, Peers *et al* 1994; Robinson *et al.*, 1994). An array of A and E elements constitute symmetrical enhansers that co-operatively account for >90% of the transcriptional activity of the insulin gene promoter (Ohlsson *et al.*, 1988). The E elements contain a core sequence CANNTG, which are recognition motifs for transcription factors in the basic helix-loop-helix family E12 and E47 which activate the insulin promoter in close synergism with A element binding homeobox transcription factors, such as IDX-1 The A elements consist of a core sequence TAAT which constitutes a binding site for homeodomain transcription factors. In addition to transcriptional activation there are various repressors of insulin gene promoter. Chronic hyperglycaemia may contribute to the pancreatic β-cell dysfunction observed in patients with type II diabetes which is mainly due to the phenomenon of glucose toxicity (Robertson *et al.*, 1994). Studies, *in vivo* in animal models and *in vitro* studies with immortalised β-cell line have shown a reduction in insulin gene transcription mediated by glucose toxicity Such a condition is associated with loss of transactivator protein such as IDX-1/IPF-1/STF-1 and PIPE3b1-binding protein (Zangen *et al.*, 1997; Poitout *et al.*, 1996; Sharma *et al.*, 1995, Olson *et al.*, 1995; Olson *et al.*, 1993, Robertson

et al., 1992). Since insulin gene transcription is both positively and negatively controlled, the repressors also play an important role in insulin regulation. Glucose induced repression of insulin gene is mainly mediated through CCAAT/enhancer binding protein (C/EBP). C/EBPs are a family of transcription factors that regulate genes of the acute phase response, cell growth, differentiation and the expression of cell type specific genes (Mandrup & Lane, 1997; Pope *et al.*, 1994; Vasseur-Cognet & Lane, 1993; Descombes *et al* 1990; Poli *et al.* 1990).

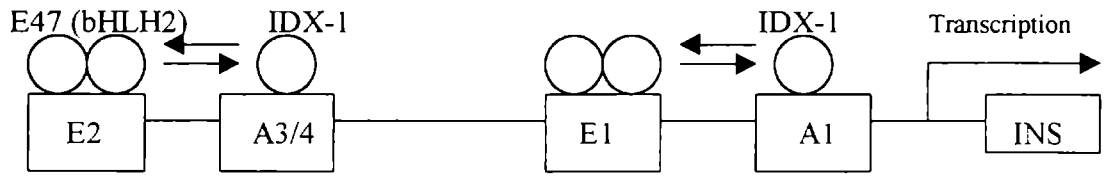
The C/EBPs bind to DNA exclusively as dimers and contain a conserved C-terminal basic region, Leucine-Zipper domain, that is characterised by a DNA-contacting basic region linked to a leucine-zipper dimerisation motif (Trautwein *et al.*, 1996). In pancreatic β-cell C/EBP specifically interacts by direct protein-protein interactions with a heptad leucine repeat sequence within activation domain 2 (AD2) of the basic helix-loop-heix transcription factor E47, thereby inhibiting the DNA binding activity and the transactivation potential of E47 This interaction leads to the inhibition of both dimerisation and DNA binding of bHLH2 protein to the E element of the insulin promoter that reduces insulin gene transcription.

In our results we have observed a decrease in 5-HT binding protein (considered as the bHLH2 protein based on amino acid homology) in islets incubated in hyperglycaemic medium. This decrease in bHLH2 protein can reduce its binding to the E element of the insulin promoter and decrease insulin gene transcription in diabetic state. The inhibition of insulin gene transcription is further aggravated by the induction of the repressor C/EBP in pancreatic β-cells by chronically elevated glucose levels (Ming *et al* 1997). This increased C/EBP can bind to the AD2 domain of bHLH2 protein and prevent it from binding to the E element of the insulin promoter thus inhibiting insulin gene transcription during diabetes.

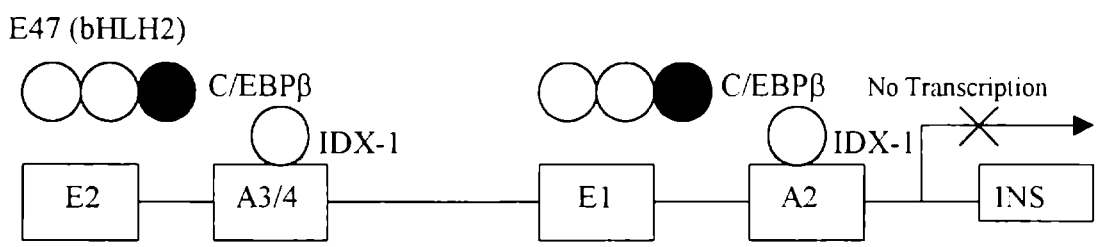
There is another report that agrees on the inhibition of insulin transcription by decreased bHLH2 protein (Dumonteil *et al.*, 1998). The bHLH2 protein E47 is able to dimmerise with another protein BETA2 (β-cell E-box transactivator2). This BETA2/E47 complex is able to regulate both insulin promoter and glucagon promoter by binding to the

E-box. Over expression of E47 (bHLH2 protein) is able to inhibit E-box mediated glucagon gene expression and stimulate E-box mediated insulin gene transcription.

NORMAL TRANSCRIPTION OF INSULIN GENE IN PRESENCE OF bHLH2 PROTEIN

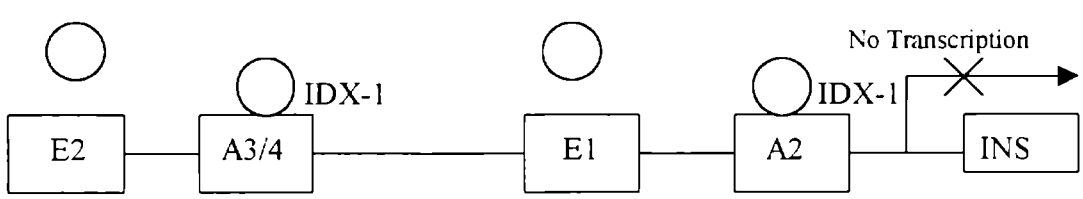


INHIBITION OF TRANSCRIPTION BY DIMERISATION OF bHLH2 WITH C/EBPβ INDUCED BY HYPERGLYCAEMIA



INHIBITION OF TRANSCRIPTION BY DOWN-REGULATION OF bHLH2 BY 5-HT

Decreased E47 (bHLH2)



In our experiment we have found a decrease in bHLH2 protein induced by hyperglycaemia. This prevents the inhibition of glucagon gene transcription leading to increased glucagon and glycogenesis thereby increasing blood glucose level. Therefore, the heterodimer BETA2/E47 can be considered as an islet specific factor whose ratio can control both insulin and glucagon gene transcription.

In addition to the above mentioned mechanisms these transcription factors can

influence insulin gene promoter by regulating glucose and hormones, which elevate β-cell [$Ca2^{-}$] and cAMP levels and possibly protein kinase C activity (Goodison *et al.* 1992).

5.3. CONCLUSION

We conclude from our studies that the serotonergic system can regulate insulin secretion from the pancreatic islets. The regulation is suggested to be mediated through the central nervous system directly and/or indirectly affecting the sympathetic stimulation and the peripheral control at the pancreatic level. We have observed a decrease of 5-HT content in CC, BS and Hypo. This decrease in 5-HT content led to an increase in the expression of 5-HT_{2A} receptors in BS along with an increase in its affinity for 5-HT in CC and Hypo. The high affinity 5-HT_{1A} receptors in BS was up-regulated and the low affinity 5-HT_{1A} receptors of CC and Hypo showed an increase in affinity for 5-HT This increased activity of the 5-HT_{1A} and 5-HT_{2A} receptors will inhibit insulin secretion by increasing the sympathetic activity during diabetes. An increased sympathetic activity will increase the circulating EPI content that directly controls insulin secretion. Peripherally 5-HT is rapidly taken up into pancreatic islets which inhibits insulin secretion. Our *in vitro* studies show that there is a significant decrease in the amount of glucose induced insulin secretion in the presence of 5-HT Within the islets 5-HT is able to bind to a novel 14-kDa nuclear protein and down-regulate it during diabetes. Based on the amino acid composition homology, this 14-kDa nuclear 5-HT binding protein was identified as a basic helix-loop-helix (bHLH2) transcription factor that acts as an enhancer of insulin gene promoter Since 5-HT can bind and down-regulate this 14-kDa bHLH2 protein, it will lead to inhibition of insulin secretion from the pancreatic β-cells. Treatment of diabetic rats with insulin, tryptophan and insulin + tryptophan reversed the altered brain 5-HT_{1A} and 5-HT_{2A} receptors by increasing the brain 5-HT content. But these treatments were not able to up-regulate the nuclear 5-HT binding protein.

Thus we conclude that serotonin can regulate insulin secretion through increased 5-HT_{2A} receptor gene expression, up-regulation of 5-HT_{1A} receptors in brain regions and down-regulation of nuclear 5-HT binding bHLH2 protein in pancreatic islets. Manipulations of these receptor gene expressions at the molecular level can have a clinical significance in the control of diabetes mellitus.

5.4. SUMMARY

1. Streptozotocin induced diabetic rats were used as model to study the role of 5-HT and its receptors in insulin regulation.

2. The 5-HT content is decreased in cerebral cortex, brain stem and hypothalamus of diabetic rats. This is due to decrease in uptake of L-tryptophan through the blood-brain-barrier during diabetes.

3. RT-PCR studies confirmed an increase in expression of brain stem 5-HT_{2A} receptor and an increase in its affinity in cerebral cortex and hypothalamus. The high affinity 5-HT_{1A} receptors in brain stem was up-regulated and the low affinity 5-HT_{1A} receptors of cerebral cortex and hypothalamus showed an increase in affinity for 5-HT

4. The increased function of the brain 5-HT_{1A} and 5-HT_{2A} receptors will lead to inhibition of insulin secretion by increasing the sympathetic activity An increase in sympathetic activity will decrease insulin secretion from the pancreatic islets.

5. Treatment of diabetic rats with insulin, tryptophan and insulin + tryptophan reversed the altered 5-HT_{1A} and 5-HT_{2A} receptor binding parameters in cerebral cortex, brain stem. The 5-HT_{2A} receptor binding parameters were reversed by these treatments in hypothalamus but 5-HT_{1A} receptors did not reverse to the control state.

6. *In vitro* incubation of pancreatic islets with [^{3}H]5-HT and increased concentration of glucose showed a glucose mediated 5-HT uptake into the islets.

7 Glucose mediated insulin secretion was inhibited in islets incubated with high glucose and 5-HT concentrations.

8. During hyperglycaemic state the increased 5-HT in the islets can bind and down-regulate a novel 14-kDa nuclear 5-HT specific binding protein. Based on amino acid

composition homology, this 14-kDa protein was identified as a basic helix-loop-helix (bHLH2) transcription factor that acts as an enhancer of insulin gene promoter

9. Down-regulation of the bHLH2 protein during diabetes by 5-HT will inhibit insulin gene transcription, since this protein acts as an enhancer of insulin gene promoter

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