MATERIALS AND METHODS

BIOCHEMICALS AND THEIR SOURCES

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

Chemicals used for this study

Biochemicals:

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

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

Radiochemicals

[3H] Dopamine (Sp. activity- 45.1 Ci/mmol), [3H] Spiperone (Sp. activity 16.5 Ci/mmol) and [3H]YM-09151-2 (cis-N-(1-benzyl-2-methylpyrrolidine-3-yl)-5-chloro-2-methoxy-4-methylaminobenzamide Sp. activity - 85.0 Ci/mmol) were purchased from NEN Life Sciences Products, Inc. Boston, USA.

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

Molecular biology chemicals

Reverse transcriptase enzyme MuMLV was purchased from Amersham Biosciences, USA, Taq polymerase, random hexamers, Human RNAse inhibitor, DNA
molecular weight markers, dNTPs were purchased from Bangalore Genei Pvt. Ltd. India. Tri-reagent kit was purchased from Sigma Chemical Co., USA. Dopamine D\textsubscript{2} receptor primers for PCR and β-actin primers for PCR were synthesised by Sigma Chemical Co., USA.

**ANIMALS**

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*.

**Induction of diabetes**

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). Animals were divided into the following groups as i) Control [C] ii) Diabetic [D] iii) Insulin treated diabetic rats [D+I]. Each group consisted of 4-6 animals. The insulin treated diabetic group received a daily dose (1 Unit/kg body weight) of Lente and Plain insulin (Boots India). The dose was increased daily according to the blood glucose level (Sasaki & Bunag, 1983).

Streptozotocin (65mg/kg body wt.) was injected to adult male Wistar rats and corpus striatal dopamine and HVA content were measured at different time periods (3 hrs, 12 hrs, 24 hrs and 48 hrs). In the dose dependent study different doses of streptozotocin (5, 10, 20, 40 & 65 mg/kg body wt.) were injected to adult male Wistar rats and the striatal dopamine and HVA changes were quantified after 48 hrs. Control rats were injected with citrate buffer.

**Tissue preparation**

Rats were sacrificed by decapitation on the 14\textsuperscript{th} day of the experiment. The cerebral cortex, corpus striatum, brain stem and hypothalamus were dissected out quickly over ice according to the procedure of Glowinski & Iversen, (1966). The tissues were stored at -70\textdegree C until assay.
The rats were sacrificed by decapitation in time dependent studies at intervals - 3 hrs, 12 hrs, 24 hrs and 48 hrs and in dose dependent studies after 48 hrs of STZ injection. Brain was dissected out quickly over ice according to the procedure of Glowinski & Iversen, (1966) and the corpus striatum was used for both the experiments.

**Estimation of Blood glucose**

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

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

\[
\text{Glucose} + O_2 + H_2O \xrightarrow{\text{(GOD)}} \text{Glucose acid} + H_2O_2.
\]

The hydrogen peroxide formed in this reaction reacts with 4-aminoantipyrine and 1-hydroxybenzoic acid in the presence of peroxidase (POD) to form N-(4-antipyryl)-p-benzoquinoneimine. 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.

**HISTOLOGY**

Brain region - corpus striatum, cerebral cortex and hypothalamus from control, diabetic and insulin treated diabetic rats were used to make paraffin sections of 0.5μm for histological studies. These were stained using Nissl’s periodic acid stain (PAS) and studied under light microscope for large accumulation of glycogen scattered in the brain tissues.

The pancreas from control, diabetic and insulin treated diabetic rats were used to make paraffin sections of 0.5μm. They were stained using hematoxylin-eosin stain and studied under light microscope for showing the degeneration in the pancreas.

**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) 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 column 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.

In the case of pancreas and adrenals the tissues were homogenised in 0.1N perchloric acid. The homogenate was centrifuged at 5000xg for 10 minutes at 4°C (Kubota refrigerated centrifuge) and the clear supernatant was filtered through 0.45 μm HPLC grade filters and used for HPLC analysis. Data from pancreas and adrenals of the experimental and control rats were statistically analysed and tabulated.

Determination of Plasma Monoamincs and their metabolites

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

**PROTEIN DETERMINATION**

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

**DOPAMINE RECEPTOR BINDING STUDIES USING [³H] RADIOLIGANDS IN THE BRAIN REGIONS OF CONTROL, DIABETIC AND INSULIN TREATED DIABETIC RATS**

Dopamine DA receptor binding studies using [³H] Dopamine

Dopamine DA receptor assay was done using [³H] DA as per Madras, et al., (1988) and Hamblin & Creese, (1982). Brain tissues were homogenised in a polytron homogeniser with 10 volumes of cold 50mM Tris-HCl buffer, along with EDTA 1mM, ascorbic acid 0.01%, MgCl₂ 4mM, CaCl₂ 1.5 mM pH 7.4 and centrifuged at 38,000xg for 30min. at 4°C. The pellet was washed twice by rehomogenization and centrifuged twice at 38,000 g for 30min. at 4°C. This was resuspended in appropriate volume of the buffer containing the above mentioned composition.

Binding assays were done using different concentrations i.e., 0.25nM-1.5nM of [³H] DA in 50mM Tris-HCl buffer, along with EDTA 1mM ascorbic acid 0.01%, MgCl₂ 1mM, CaCl₂ 2 mM, NaCl 120mM, KCl 5mM pH 7.4 in a total incubation volume of 300μl containing 200-300 μg of proteins. Specific binding was determined using 100μM unlabelled butaclamol. Competition studies were carried out with 0.25nM [³H] DA in each tube with unlabelled ligand concentrations varying from 10⁻⁹ - 10⁻⁴M of DA.

Tubes were incubated at 25°C for 60 min. and filtered rapidly through GF/B filters (Whatman). The filters were washed quickly by three successive washing with 5.0 ml of ice cold 50mM Tris buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.
Corpus striatal dopamine D$_2$ receptor binding studies using [$^3$H] spiperone of control, diabetic and insulin treated diabetic rats.

Dopamine D$_2$ receptor binding assay in the striatum was done according to the modified procedure of Trulson and Himmel, (1983), Grigoridias and Seeman, (1985) and Paulose et al., (1981). Three samples of striatum were pooled from each group. The tissues were weighed and homogenized in 10 volumes of ice cold 50mM Tris-HCl buffer, along with 1mM EDTA, 4mM MgCl$_2$, 1.5mM CaCl$_2$, 5mM KCl pH 7.4. The homogenate was centrifuged at 480g for 10 min and the supernatant was saved and the pellet was resuspended in 10 volumes of the buffer and centrifuged at 480g for 10 min. The final pellet was discarded and the supernatant were again centrifuged at 48,000xg for 30 minutes. The pellet was resuspended in 50 volumes of 50mM Tris HCl, pH 7.4 and recentrifuged at 48,000xg for 30 minutes. This was resuspended in appropriate volume of the buffer containing the above mentioned composition.

Binding assays were done using different concentrations i.e., 0.01nM-1.5nM of [$^3$H] spiperone in 50mM Tris-HCl buffer, along with 1mM EDTA, 4mM MgCl$_2$, 1.5 mM CaCl$_2$ 5mM KCl with 12μM pargyline and 0.1% ascorbic acid in a total incubation volume of 300μl containing 150-200 μg proteins. For the study of dopamine D$_2$ receptors to preclude serotonergic component of the striatum 40nM ketanserine was used. Specific binding was determined using 1.0μM unlabelled butaclamol. Competition studies were carried out with 0.25nM [$^3$H]spiperone in each tube with unlabelled ligand concentrations varying from $10^{-12}$ - $10^{-4}$M of spiperone.

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

Dopamine D$_2$ receptor binding studies using [$^3$H] YM-09151-2 in brain regions of control, diabetic and insulin treated diabetic rats.

Dopamine D$_2$ receptor binding assay was done according to the modified procedure of Unis, et al., (1998) and Madras, et al., (1988). The dissected brain tissues corpus striatum, hypothalamus, cerebral cortex tissues and brain stem were weighed and
homogenized in 10 volumes of ice cold 50mM Tris-HCl buffer, along with 1mM EDTA, 5mM MgCl₂, 1.5 mM CaCl₂, 120mM NaCl, 5mM KCl pH 7.4. The homogenate was centrifuged at 48,000xg for 30 min. The pellet was washed and recentrifuged with 50 volumes of the buffer at 48,000xg for 30 min. This was suspended in appropriate volume of the buffer containing the above mentioned composition.

Binding assays were done using different concentrations i.e., 0.25nM-2.0nM of [³H] YM-09151-2 in 50mM Tris-HCl buffer, along with 1mM EDTA, 5mM MgCl₂, 1.5 mM CaCl₂, 120mM NaCl, 5mM KCl with 10μM pargyline and 0.1% ascorbic acid in a total incubation volume of 300μl containing 200-300μg of protein. Specific binding was determined using 5.0 μM unlabelled sulpiride. Competition studies were carried out with 0.25nM [³H] YM-09151-2 in each tube with unlabelled ligand concentrations varying from 10⁻¹² - 10⁻⁴M of YM-09151-2.

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

DOPAMINE RECEPTOR BINDING STUDIES USING [³H] RADIOLIGANDS IN THE PANCREATIC ISLETS OF CONTROL, DIABETIC AND INSULIN TREATED DIABETIC RATS

Dopamine DA receptor binding studies using [³H] Dopamine in the islets of control, diabetic and insulin treated rats

Pancreatic islets of control, diabetic and insulin treated diabetic rats were isolated by standard collagenase digestion procedure using aseptic techniques [Howell, 1968]. The islets were isolated in HEPES-buffered sodium free Hanks Balanced Salt Solution (HBSS) [Pipeleers, 1985] with the following composition: 137mM Choline chloride, 5.4mM KCl, 1.8mM CaCl₂, 0.8mM MgSO₄, 1mM KH₂PO₄, 14.3mM KHCO₃, 10mM HEPES with 0.2% (w/v) BSA (Fraction V), equilibrated with 5% CO₂ and pH 7.3 at room temperature. Autoclaved triple distilled water was used in the preparation of the buffer.
Pancreatic islets isolated as per the above mentioned procedure was homogenised in a polytron homogeniser with 10 volumes of cold 50mM Tris-HCl buffer, along with EDTA 1mM ascorbic acid 0.01%, MgCl₂ 4mM, CaCl₂ 1.5 mM pH 7.4 and centrifuged at 38,000xg for 30min. at 4°C. The pellet was washed twice by rehomogenization and centrifuged twice at 38,000xg for 30min. at 4°C. This was resuspended in appropriate volume of the buffer containing the above mentioned composition.

Binding assays were done using different concentrations i.e., 0.1nM-2.0 nM of [³H] DA in 50mM Tris-HCl buffer, along with EDTA 1mM ascorbic acid 0.01%, MgCl₂ 1mM, CaCl₂ 2 mM, NaCl 120mM, 5mM KCl 10μM pargyline pH 7.4 in a total incubation volume of 300μl containing 50-75μg of proteins. Specific binding was determined using 100μM unlabelled butaclamol. Competition studies were carried out with 0.5nM [³H] DA in each tube with unlabelled ligand concentrations varying from 10⁻⁹ - 10⁻⁴M of DA.

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

Dopamine D₂ receptor binding studies using [³H] YM-09151-2 in the islets of control, diabetic and insulin treated diabetic rats.

The assay was done in a similar way as in brain regions. After isolation the islets were homogenized in 10 volumes of ice cold 50mM Tris-HCl buffer, along with 1mM EDTA, 5mM MgCl₂, 1.5 mM CaCl₂, 120mM NaCl, 5mM KCl pH 7.4. The homogenate was centrifuged at 48,000xg for 30 min. The pellet was washed and recentrifuged with 50 volumes of the buffer at 48,000xg for 30 min. This was suspended in appropriate volume of the buffer containing the above mentioned composition.

Binding assays were done using different concentrations i.e., 0.1nM-2.00nM of [³H] YM-09151-2 in 50mM Tris-HCl buffer, along with 1mM EDTA, 5mM MgCl₂, 1.5 mM CaCl₂, 120mM NaCl, 5mM KCl with 10μM pargyline and 0.1% ascorbic acid in a total incubation volume of 300μl containing 50-75 μg of protein. Specific binding was determined using 5.0 μM unlabelled sulpiride. Competition studies were carried out with
0.5nM $[^3]$H YM-09151-2 in each tube with unlabelled ligand concentrations varying from $10^{-12}$ - $10^{-4}$M YM-09151-2.

ANALYSIS OF THE RECEPTOR BINDING DATA

Linear regression analysis for Scatchard plots

The data was analysed according to Scatchard (1949). The specific binding was determined by subtracting non-specific binding from the total. The binding parameters, maximal binding ($B_{\text{max}}$) and equilibrium dissociation constant ($K_d$), were derived by linear regression analysis by plotting the specific binding of the radioligand on X-axis and bound/free on Y-axis. The maximal binding is a measure of the total number of receptors present in the tissue and the equilibrium dissociation constant is the measure of the affinity of the receptors for the radioligand. The $K_d$ is inversely related to receptor affinity.

Nonlinear regression analysis for displacement curve

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

$[^3]$H Dopamine uptake studies by pancreatic islets in vitro

Pancreatic islets of male Wistar rats were aseptically dissected out into a sterile petridish containing ice cold Hanks Balanced Salt Solution (HBSS) and isolated by standard collagenase digestion procedure (Howell, 1968). The islets were isolated in HEPES-buffered sodium free (Pipeleers, 1985) with the following composition: 137mM Choline chloride, 5.4mM KCl, 1.8mM CaCl$_2$, 0.8mM MgSO$_4$, 1mM KH$_2$PO$_4$, 14.3mM KHCO$_3$, 10mM HEPES with 0.2% (w/v) BSA (Fraction V), equilibrated with 5% CO$_2$ and pH 7.3 at room temperature. Autoclaved triple distilled water was used in the preparation of the buffer. 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. This filtrate was transferred to a sterile petri dish 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 were used for all other experiments.

The islets isolated by the above mentioned method were resuspended in HEPES buffered HBSS with 4mM glucose and pre-incubated for 1 hour at 37°C (Howell & Taylor, 1968). The islet suspension was centrifuged at 4°C at 500xg to remove inherent insulin. The pre-incubated islets were then washed thrice with cold 10mM Tris HCl buffer, pH 7.4 and finally resuspended in HBSS without glucose. 200μl of islet suspension was transferred to tubes containing 10⁻⁸ M, 10⁻⁷ M, 10⁻⁶ M, 10⁻⁵ M, 10⁻⁴ M concentrations of [³H] DA. [³H] DA concentrations were used along with 4mM and 20mM glucose. The final incubation volume was made up to 0.5ml. The tubes were incubated for 2 hours at 37°C in a shaking water bath.

The tubes were centrifuged after incubated for one hour at 1,500xg for 10min at 4°C. The supernatant was aspirated out and pellet washed superficially with 0.2ml of HBSS twice to remove free [³H] DA. The pellet was digested with 100μl of 1M KOH overnight and counted in a liquid scintillation counter with Cocktail-T to measure the [³H] DA uptake.

Effect of Norepinephrine on dopamine uptake by pancreatic islets in vitro

The islets prepared as per the above mentioned procedure were transferred to tubes containing 10⁻⁸ M, 10⁻⁴ M concentrations of [³H] DA and 10⁻⁸ M, 10⁻⁴ M concentrations of NE.

[³H] DA concentrations were used along with 4mM and 20mM glucose. NE was used to study the [³H] DA uptake along with glucose in this experiment. The
final incubation volume was made up to 0.5ml. The tubes were incubated for 2 hours at 37°C in a shaking water bath.

The tubes were centrifuged after incubated for one hour at 1,500xg for 10 min at 4°C. The supernatant was aspirated out and pellet washed superficially with 0.2 ml of HBSS twice to remove free[^3]H] DA or NE. The pellet was digested with 100 μl of 1M KOH overnight and counted in a liquid scintillation counter with Cocktail-T to measure the[^3]H] DA uptake.

In vitro insulin secretion in the presence of different concentrations of dopamine, its antagonist and agonist

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

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

Studies were also done in islets incubated for 1 hour at 37°C with combinations of DA and NE at different concentrations to know the effect of NE on the role of DA in insulin secretion.

Radioimmuno Assay of Insulin

Principle of the assay

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

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

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

\[
\text{Corrected average count of standard or sample} \times 100 \\
\text{Corrected average count of zero standard}
\]

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

EXPRESSION STUDIES OF DOPAMINE D2 RECEPTOR IN DIFFERENT BRAIN REGIONS OF CONTROL, DIABETIC AND INSULIN TREATED DIABETIC RATS.

REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

Preparation of RNA

RNA was isolated from the different brain regions of control, diabetic and insulin treated diabetic experimental rats using the Tri reagent from Sigma Aldrich.

Isolation of RNA

Tissue (25-50 mg) homogenates were made in 0.5 ml Tri Reagent and was centrifuged at 12,000xg for 10 minutes at 4°C. The clear supernatant was transferred to a fresh tube and it was allowed to stand at room temperature for 5 minutes. 100μl of chloroform was added to it, mixed vigorously for 15 seconds and allowed to stand at
room temperature for 15 minutes. The tubes were then centrifuged at 12,000xg for 15 minutes at 4°C. Three distinct phases appear after centrifugation. The bottom red organic phase contained protein, interphase contained DNA and a colourless upper aqueous phase contained RNA. The upper aqueous phase was transferred to a fresh tube and 250μl of isopropanol was added and the tubes were allowed to stand at room temperature for 10 minutes. The tubes were centrifuged at 12,000xg for 10 min at 4°C. RNA precipitate forms a pellet on the sides and bottom of the tube. The supernatants were removed and the RNA pellet was washed with 500μl of 75% ethanol, vortexed and centrifuged at 12,000xg for 5 min at 4°C. The pellets were semi dried and dissolved in minimum volume of DEPC-treated water. 2 μl of RNA was made up to 1 ml and absorbance were measured at 260nm and 280nm. For pure RNA preparation the ratio of absorbance at 260/280 was ≥ 1.7. The concentration of RNA was calculated as one absorbance 260 = 42μg.

RT-PCR Primers

The following primers were used for dopamine D2 receptors and β-actin mRNA expression studies.

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-GCC AAA CCA GAG AAG AAT GG-3' 5'-GAT GTG CGT ATG AAG GAA GG-3'</td>
<td>500bp</td>
</tr>
<tr>
<td>5'-CAA CTT TAC TCT GGC CAC TAC C-3' 5'-TAC GAC TGC AAA CAC TCT ACA CC-3'</td>
<td>150bp</td>
</tr>
</tbody>
</table>

RT-PCR of dopamine D2 receptors and β-actin

RT-PCR was carried out in a total reaction volume of 20μl reaction mixture in 0.2ml tubes. RT-PCR was performed on an Eppendorf Personal thermocycler. cDNA synthesis of 2mg RNA was performed in a reaction mixture containing MuMLV reverse transcriptase (40U/reaction), 2mM dithiothreitol, 4 units of human placental RNAse
inhibitor, 0.5μg of random hexamer and 0.25mM (dNTPS dATP, dCTP, dGTP and dTTP). The tubes were then incubated at 42°C for one hour. Then reverse transcriptase, MuMLV, was inactivated by heating at a temperature of 95°C.

**Polymerase Chain Reaction**

Polymerase Chain Reaction (PCR) was carried out in a 20μl volume reaction mixture containing 4μl of cDNA, 0.25mM dNTPS - dATP, dCTP, dGTP and dTTP -, 0.5 units of Taq DNA polymerase and 10 picomoles of specific primers. The three primers used have the same annealing temperature.

Following is the thermocycling profile used for PCR:

- 94°C -- 5 min --- Initial Denaturation
- 94°C -- 30 sec --- Denaturation
- 56°C -- 30 sec --- Annealing  36 cycles
- 72°C -- 30 sec --- Extention
- 72°C -- 7 min --- Final Extension

**Analysis of RT-PCR product**

The Polymerase Chain Reaction product was loaded on a 2.0% agarose gel with ethidium bromide. Bromophenol blue was used as the indicator dye. 60V current was used for all the run. The image was captured using an Imagemaster gel documentation system (Pharmacia Biotec) and the bands were densitometrically analysed using Total Lab software. Dopamine D2 receptor mRNA expression in the brain regions-CS, CC, HYPO and BS of control, diabetic and insulin treated diabetic rats were analysed.

**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).