MATERIALS AND METHODS

BIOCHEMICALS AND THEIR SOURCES

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

Important Chemicals Used for the Present Study

i) *Biochemicals*

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

ii) *Radiochemicals*

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

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

iii) *Molecular biology chemicals*

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

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

ANIMALS

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

PARTIAL PANCREATECTOMY

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

Sacrifice and Tissue Preparation

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

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

Estimation of Blood Glucose

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

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

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

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

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

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

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

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

ESTIMATION OF CIRCULATING INSULIN BY RADIOIMMUNOASSAY

Principle of the assay

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

Assay Protocol

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

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

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

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

ISOLATION OF PANCREATIC ISLETS

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

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

5-HT QUANTIFICATION BY HPLC

Brain 5-HT HPLC determinations were done by electrochemical detection (Paulose *et al.*, 1988). The tissues from brain regions were homogenised in 0.4N perchloric acid. The homogenate was centrifuged at 5000 x g for 10 minutes at 4°C (Kubota Refrigerated Centrifuge, Japan) and the clear supernatant was filtered through 0.22 μm HPLC grade filters and used for HPLC analysis in Shimadzu HPLC

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

Adrenal monoamines

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

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

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

Analysis of circulating Norepinephrine

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

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

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

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

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

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

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

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

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

BINDING STUDIES IN THE PANCREATIC ISLETS

5-HT_{1A} Receptor Binding Assays

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

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

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

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

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

Protein Estimation

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

Receptor Binding Parameters Analysis

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

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

Displacement Curve analysis

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

REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

Isolation of RNA

RNA was isolated from the pancreas and brain regions of sham and pancreatectomised rats using Tri reagent (Sigma Chemical Co., USA). Brain tissues and islets isolated by collagenase digestion procedure (25-50 mg) were homogenised in 0.5 ml Tri Reagent. The homogenate was centrifuged at 12,000 *g* for 10 minutes at 4°C. The clear supernatant was transferred to a fresh tube and it was allowed to stand at room temperature for 5 minutes. 100µl of chloroform was added to it, shaken vigorously for 15 seconds and allowed to stand at room temperature for 15 minutes. The tubes were centrifuged at 12,000 *g* for 15 minutes at 4°C. Three distinct phases appear after centrifugation. The bottom red organic phase contained protein, interphase contained DNA and a colourless upper aqueous phase contained RNA. The upper aqueous phase was transferred to a fresh tube and 250µl of isopropanol was added and the tubes allowed to stand at room temperature for 10 minutes. The tubes were centrifuged at 12,000 *g* for 10 minutes at 4°C. RNA precipitate forms a pellet on the sides and bottom of the tube. The supernatant was removed and the RNA pellet was washed with 500µl of 75% ethanol, vortexed and centrifuged at 12,000 *g* for 5 minutes at 4°C. The pellet was semi dried and dissolved in minimum volume of DEPC-treated water. 2µl of RNA was made up to 1ml and optical density was measured at 260nm and 280nm. For pure RNA preparation the ratio of optical density at 260/280 was ≥ 1.7. The concentration of RNA was calculated as one optical density$_{260}$ = 42µg.

RT-PCR Primers

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

5'- TGG CTT TCT CAT CTC CAT CC -3' 5'- CTC ACT GCC CCA TTA GTG C –3' PRODUCT SIZE: 357bp	5-HT_{1A}
5'- CCA ACG AAC ACC TTC TTT CC -3' 5'- GCA TTG TGC AGT TTC TTC TCC -3' PRODUCT SIZE: 252bp	5-HT_{2C}
5'- CAA CTT TAC CTT GGC CAC TAC C -3' 5'- TAC GAC TGC AAA CAC TCT ACA CC -3' PRODUCT SIZE: 150bp	β-ACTIN

RT-PCR of 5-HT_{1A}, 5-HT_{2C} Receptor and β-Actin

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

Polymerase Chain Reaction (PCR)

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

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

Following is the thermocycling profile used for PCR

94°C – 5 minutes --- Initial Denaturation

94°C – 30 seconds --- Denaturation

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

72°C – 30 seconds --- Extension

72°C – 5 minutes --- Final Extension

Analysis of RT-PCR product

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

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

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

Insulin secretion study - 1 hour

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

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

Insulin secretion study - 24 hrs

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

PANCREATIC DNA SYNTHESIS STUDIES *IN VITRO*

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

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

STATISTICS

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