Materials and Methods

CHEMICALS USED IN THE STUDY AND THEIR SOURCES

Biochemicals
Sulpiride, SCH 23390, dextrose, bovine serum albumin, ethylene diamine tetra acetic acid (EDTA), sucrose, magnesium chloride, calcium chloride, pargyline, ascorbic acid, Tris HCl, foetal calf serum (heat inactivated), D-glucose and paraformaldehyde (PFA) were purchased from Sigma Chemical Co., St. Louis, USA. All other reagents were of analytical grade purchased locally from SRL, India. Tissue freezing medium, Jung was purchased from Leica Microsystems Nussloch GmbH, Germany.

Radiochemicals
\(^{3}H\)SCH 23390 (Sp. activity 83Ci/mmol) and \(^{3}H\)YM-09151-2 (cis-N-(1-benzyl-2-methylpyrrolidine-3-yl)-5-chloro-2-methoxy-4-methylaminobenzamide Sp. activity - 85.0Ci/mmol) were purchased from NEN Life Sciences Products, Inc. Boston, USA. The \(^{3}H\)IP3 and \(^{3}H\)cAMP were purchased from American Radiolabelled Chemicals, USA.

Molecular Biology Chemicals
Tri-reagent kit was purchased from Sigma Chemical Co., St. Louis, USA. ABI PRISM High Capacity cDNA Archive kit and Taqman probes for Real-Time PCR were purchased from Applied Biosystems, Foster City, CA, USA. Dopamine D1 (Rn_02043440), Dopamine D2 (Rn_00561126), CREB (Rn_00578826), Bax (Rn_01480160), SOD (Rn01477289), GPx (Rn_00577994), Akt 1 (Rn00583646), NF-kB (Rn01399583), Caspase-8 (Rn00574069), BDNF (Rn01484924), GDNF (Rn00569510), GLUT 1
Materials and Methods

(Rn_00567331), phospholipase C (Rn_01647142) and TNF α (Rn00562500_m1) primers were used for the gene expression studies using Real-Time PCR.

Confocal Dyes

Rat specific primary antibody for Dopamine D1 (No: NRG 01691597 Millipore), Dopamine D2. (No: LV 1583420 Millipore) and secondary antibody of FITC (No: AB7130F, Chemicon) were used for the immunohistochemistry studies using confocal microscope.

Animals

Wistar neonatal (postnatal day, P7) rats weighing 10.0–12.0 g were used for all experiments. They were purchased from Kerala Agriculture University, Mannuthy, India and Amrita Institute of Medical Sciences, Kochi, India. All groups of neonatal rats were maintained with their mothers under optimal conditions—12-h light and 12-h dark periods—and were fed standard food and water ad libitum. Adequate measures were also taken to minimize pain and discomfort of the animals. All animal care procedures were in accordance with Institutional, Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA – Reg. No: 383/01/a/CPSCEA) and the National Institute of Health guidelines.

Plant Material

Specimens of *Bacopa monnieri* were collected from Cochin University area. The plants were taxonomically identified and authenticated by Mr. K.P. Joseph, Head of the Dept. of Botany (Retd), St. Peter’s College, Kolencherry and voucher specimens are deposited at the herbarium of the Centre for Neuroscience, Dept. of Biotechnology, Cochin University of Science and Technology, Cochin, Kerala.
Materials and Methods

**Preparation of Bacopa monnieri Plant Extract**

Crude whole plant extract was used to study the anti-hypoglycemic effect in insulin induced neonatal hypoglycemia. *Bacopa monnieri* plant extract was prepared by the procedure of Paulose et al., (2008). Fresh, whole *Bacopa monnieri* plant (6–8 months old) was collected (in the month of March) and washed. Leaves, roots and stems of *Bacopa monnieri* plant were cut into small pieces and dried in shade. About 100 g fresh plant dried in shade yielded 15 g powder. Homogenate was extracted at required concentration (300 mg fresh plant/Kg body weight) by dissolving 450 mg of dried powder in 80 ml distilled water and used to study the anti-hypoglycemic effect in insulin induced neonatal hypoglycemia.

**Preparation of Bacoside A**

Bacoside A was a generous gift from the Natural Remedies Pvt Ltd. Veerasandra Industrial Area, Bangalore, India and the extraction procedure was follows. Bacoside A was extracted according to the protocol of Pal & Sarin, 1992.

**EXPERIMENTAL DESIGN**

**Determination of Anti-hypoglycemic Potential of Bacopa monnieri and Bacoside A**

Experimental Animals were divided into following Groups:

i. Control (C)

ii. Neonatal Hypoglycemia (H)

iii. Neonatal Hypoglycemia treated with Glucose (H+G)

iv. Neonatal Hypoglycemia treated with Bacoside A (H+D)

v. Neonatal Hypoglycemia treated with *Bacopa monnieri* (H+B)

vi. Neonatal Hypoglycemia treated with Glucose and Bacoside A (H+G+D)

vii. Neonatal Hypoglycemia treated with Glucose and *Bacopa monnieri* (H+G+B)

Each group consisted of 4-6 animals.
Materials and Methods

Induction of neonatal hypoglycemia

The control neonatal rats were intra-peritoneally injected with saline (Oliver et al. 1999) and hypoglycemia was induced in the experimental groups (P7) using human regular insulin (Actrapid) in a dose of 10 IU/kg intra peritoneally followed by fasting for 240 min. The target blood glucose was <40 mg/dL, a value conventionally used to define hypoglycemia in newborn infants (Burns et al. 2008). Recurrent hypoglycemia followed by treatment was induced for 10 days (P7 – P16).

Treatment

Glucose (500 mg/kg body wt) was intra-peritoneally (i.p.), administered to the hypoglycemic neonatal rats, a dose that corrects brain glucose concentration in hypoglycemic newborn rats (Vannucci & Vannucci, 1997), Bacoside A (50 mg/kg body wt) and *Bacopa monnieri* (100 mg/kg body wt) was orally administered (Amee et al., 2009) to the hypoglycemic neonatal rats.

Determination of Body Weight

Body weight of all experimental group of rats were determined gravimetrically with animal weighing balance (Essae Teraoka, India) on 0th and 10th day of the experiment.

Determination of Blood Glucose

The hypoglycemic state of animals was assessed by measuring blood glucose concentrations at 3 hours after insulin treatment. The rats with a blood sugar level < 40 mg/dl were selected as hypoglycemic rats.

Sacrifice and Tissue Preparation

The control and experimental rats were sacrificed on the 11th day (P17) by decapitation. The brain regions –cerebral cortex, corpus striatum, cerebellum and brain stem were dissected out quickly over ice according to the procedure of
Glowinski and Iversen, (1966). The blood samples were collected and plasma was separated by centrifugation. The tissue samples and plasma were kept at -80° C until experiment.

**DOPAMINE D1 AND DOPAMINE D2 RECEPTOR BINDING STUDIES USING [3H] RADIOOLIGANDS**

*Dopamine D1 receptor binding studies using [3H] SCH 23390*

Dopamine D1 receptor binding assay using [3H] SCH 23390 in the brain regions were done according to the modified procedure of Mizoguchi et al., (2000). The tissues were weighed and homogenized in 10 volumes of ice cold 50mM Tris-HCl buffer, along with 1mM EDTA, 4mM MgCl2, 1.5mM CaCl2, 5mM KCl, pH 7.4. The homogenate was centrifuged at 40,000 x g for 30min. The pellet was washed and centrifuged with 50 volumes of the buffer at 40,000 x g for 30min. This was suspended in appropriate volume of the buffer containing the above mentioned composition.

Binding assays were done using different concentrations i.e., 0.5 - 5.0nM of [3H]SCH 23390 in 50mM Tris-HCl buffer, along with 1mM EDTA, 4mM MgCl2, 1.5 mM CaCl2, 5mM KCl with 12µM pargyline and 0.1% ascorbic acid in a total incubation volume of 250µl containing 100-200µg protein with 50µM unlabelled SCH 23390.

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 5.0ml of ice cold 50mM Tris buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Perkin Elmer Tri-Carb 2810 TR liquid scintillation analyser.

*Dopamine D2 receptor binding studies using [3H] YM-09151-2*

Dopamine D2 receptor binding assay was done according to the modified procedure of Unis et al., (1998). The dissected brain tissues were weighed and homogenized in 10 volumes of ice cold 50mM Tris-HCl buffer, along with 1mM EDTA, 5mM MgCl2, 1.5mM CaCl2, 120mM NaCl, 5mM KCl, pH 7.4. The
Materials and Methods

homogenate was centrifuged at 40,000 x g for 30 min. The pellet was washed and centrifuged with 50 volumes of the buffer at 40,000 x g 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., 0.1 - 2.0 nM of [³H]YM-09151-2 in 50 mM Tris-HCl buffer, along with 1 mM EDTA, 5 mM MgCl₂, 1.5 mM CaCl₂, 120 mM NaCl, 5 mM KCl with 10 µM pargyline and 0.1% ascorbic acid in a total incubation volume of 250 µl containing 100-200 µg of protein. Specific binding was determined using 5.0 µM unlabelled sulpiride. 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 50 mM Tris buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Perkin Elmer Tri-Carb 2810 TR liquid scintillation analyser.

Protein determination

Protein was measured by the method of Lowry et al., (1951) using bovine serum albumin as standard. The intensity of the purple blue colour formed was proportional to the amount of protein which was read in a spectrophotometer (Shimadzu UV-1700) at 660 nm.

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_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
Materials and Methods

dissociation constant is the measure of the affinity of the receptors for the radioligand. The $K_d$ is inversely related to receptor affinity.

GENE EXPRESSION STUDIES IN DIFFERENT BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

Preparation of RNA

RNA was isolated from the different brain regions - cerebral cortex, corpus striatum, cerebellum and brain stem of control and experimental rats using Tri reagent from Sigma Chemical Co., St. Louis, USA.

Isolation of RNA

Tissue (25-50 mg) homogenates were made in 0.5ml Tri Reagent and was centrifuged at 12,000 x 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, mixed vigorously for 15 seconds and allowed to stand at room temperature for 15 minutes. The tubes were then centrifuged at 12,000 x 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 250 µl of isopropanol was added and the tubes were allowed to stand at room temperature for 10 minutes. The tubes were centrifuged at 12,000 x g for 10 min at 4°C. RNA precipitated as 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 12,000 x g for 5 min at 4°C. The pellets were semi dried and dissolved in minimum volume of DEPC-treated water. 2µl of RNA was made up to 1ml and absorbance was measured at 260 nm and 280 nm in spectrophotometer (Shimadzu
Materials and Methods

UV-1700). 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.

cDNA Synthesis

Total cDNA synthesis was performed using ABI PRISM cDNA Archive kit in 0.2ml microfuge tubes. The reaction mixture of 20µl contained 0.2µg total RNA, 10X RT buffer, 25X dNTP mixture, 10X Random primers, MultiScribe RT (50U/µl) and RNase free water. The cDNA synthesis reactions were carried out at 25°C for 10 minutes and 37°C for 2 hours using an Eppendorf Personal Cycler. The primers and probes were purchased from Applied Biosystems, Foster City, CA, USA designed using Primer Express Software Version (3.0).

Real-Time PCR Assay

Real Time PCR assays were performed in 96-well plates in ABI 7300 Real Time PCR instrument (Applied Biosystems). To detect gene expression, a TaqMan quantitative 2-step reverse transcriptase polymerase chain reaction (RT-PCR) was used to quantify mRNA levels. First-strand cDNA was synthesized with use of a TaqMan RT reagent kit, as recommended by the manufacturer. PCR analyses were conducted with gene-specific primers and fluorescently labeled TaqMan probe (designed by Applied Biosystems). Endogenous control, β-actin, was labelled with a reporter dye (VIC). All reagents were purchased from Applied Biosystems. The probes for specific gene of interest were labeled with FAM at the 5' end and a quencher (Minor Groove Binding Protein - MGB) at the 3' end. The real-time data were analyzed with Sequence Detection Systems software version 1.7. All reactions were performed in duplicate.

The TaqMan reaction mixture of 20µl contained 25ng of total RNA-derived cDNAs, 200nM each of the forward primer, reverse primer and TaqMan probes, endogenous control (β-actin) and 12.5µl of TaqMan 2X Universal PCR Master Mix (Applied Biosystems). The volume was made up with RNase free water. Each run contained both negative (no template) and positive controls.
Materials and Methods

The thermocycling profile conditions were as follows:

- 50°C -- 2 minutes --- Activation
- 95°C -- 10 minutes --- Initial Denaturation
- 95°C -- 15 seconds --- Denaturation (40 cycles)
- 50°C -- 30 seconds --- Annealing
- 60°C -- 1 minute --- Final Extension

Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence. The ΔΔCT method of relative quantification was used to determine the fold change in expression. This was done by first normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control β-actin in the same samples (ΔCT = CT_{Target} - CT_{β-actin}). It was further normalized with the control (ΔΔCT = ΔCT - CT_{Control}). The fold change in expression was then obtained (2^{ΔΔCT}).

**cAMP CONTENT IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS IN VIVO**

Brain tissues (cerebral cortex, corpus striatum, cerebellum and brain stem) were homogenised in a polytron homogeniser with cold 50mM Tris-HCl buffer, pH 7.4, containing 1mM EDTA to obtain a 15% homogenate. The homogenate was then centrifuged at 40,000 x g for 15min and the supernatant was transferred to fresh tubes for cAMP assay using[^3]H cAMP Biotrak Assay System kit.

**Principle of the assay**

cAMP assay kit was used. The assay is based on the competition between unlabelled cAMP and a fixed quantity of[^3]H cAMP for binding to a protein which has a high specificity and affinity for cAMP. The amount of labeled protein-cAMP complex formed is inversely related to the amount of unlabelled cAMP...
Materials and Methods

present in the assay sample. Measurement of the protein-bound radioactivity enables the amount of unlabelled cAMP in the sample to be calculated.

\[
\text{Free } [\text{H}] \text{cAMP} + \text{Binding cAMP protein} = \text{Bound } [\text{H}] \text{cAMP-binding protein}
\]

Separation of the protein bound cAMP from unbound nucleotide is achieved by adsorption of the free nucleotide on to a coated charcoal followed by centrifugation. An aliquot of the supernatant is then removed for liquid scintillation counting. The concentration of unlabelled cAMP in the sample is then determined from a linear standard curve.

Assay Protocol

The tubes were placed on a water bath at 0°C. The assay mixture consisted of different concentrations of standard, [\text{H}] cAMP and binding protein in case of standards; buffer, [\text{H}] cAMP and binding protein for zero blank and unknown samples. The mixture was incubated at 2°C for 2h. Cold charcoal reagent was added to the tubes and the tubes were immediately centrifuged at 12,000 x g for 2min at 2°C. Aliquots of the supernatant was immediately transferred to scintillation vials and mixed with cocktail-T and counted in a liquid scintillation counter (Perkin Elmer Tri-Carb, 2810).

\[ \frac{C_o}{C_x} \] is plotted on the Y-axis against picomoles of inactive cAMP on the X-axis of a linear graph paper, where \( C_o \) is the counts per minute bound in the absence of unlabelled cAMP and \( C_x \) is the counts per minute bound in the presence of standard or unknown unlabelled cAMP. From the \( \frac{C_o}{C_x} \) value for the sample, the number of picomoles of unknown cAMP was calculated.
Materials and Methods

**IP₃ CONTENT IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS IN VIVO**

Brain tissues (cerebral cortex, corpus striatum, cerebellum and brain stem) were homogenised in a polytron homogeniser in 50mM Tris-HCl buffer, pH 7.4, containing 1mM EDTA to obtain a 15% homogenate. The homogenate was then centrifuged at 40,000 x g for 15 minutes and the supernatant was transferred to fresh tubes for IP₃ assay using [³H] IP₃ Biotrak Assay System kit.

**Principle of the assay**

The assay was based on competition between [³H] IP₃ and unlabelled IP₃ in the standard or samples for binding to a binding protein prepared from bovine adrenal cortex. The bound IP₃ was then separated from the free IP₃ by centrifugation. The free IP₃ in the supernatant was then discarded by simple decantation, leaving the bound fraction adhering to the tube. Measurement of the radioactivity in the tube enables the amount of unlabelled IP₃ in the sample to be determined.

**Assay Protocol**

Standards, ranging from 0.19 to 25 pmoles/tube, [³H] IP₃ and binding protein were added together and the volume was made up to 100µl with assay buffer. Samples of appropriate concentration from experiments were used for the assay. The tubes were then vortexed and incubated on ice for 15min and they were centrifuged at 2000 x g for 10min at 4°C. The supernatant was aspirated out and the pellet was resuspended in water and incubated at room temperature for 10min. The tubes were then vortexed and decanted immediately into scintillation vials. The radioactivity in the suspension was determined using liquid scintillation counter (Perkin Elmer Tri-Carb, 2810).

A standard curve was plotted with %B/Bo on the Y-axis and IP₃ concentration (pmoles/tube) on the X-axis of a semi-log graph paper. %B/Bo was calculated as:
(Standard or sample cpm – NSB cpm)

\[ \frac{B_0 \text{ cpm} - \text{NSB cpm}}{\text{NSB- non specific binding and } B_0 \text{ - zero binding. IP}_3 \text{ concentration in the}} \]

samples was determined by interpolation from the plotted standard curve.

**DAD1 AND DAD2 EXPRESSION STUDIES IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS USING CONFOCAL MICROSCOPE**

Anaesthetized animals were transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (pH 7.4). After perfusion, the brain was dissected and immersion fixed in 4% paraformaldehyde for 1 hr and then equilibrated with 30% sucrose solution in 0.1M PBS. 30 µm sections were cut using Cryostat (Leica, CM1510 S). The sections were treated with PBST (PBS in 0.05% Triton X-100) for 20 min. Brain sections were blocked with 5% normal goat serum for 4 hours. Brain sections were then incubated overnight at 4 °C with either rat specific primary antibody for Dopamine D1 (No: NRG 01691597 Millipore, 1: 500 dilution in a 1X PBS solution containing 5% normal goat serum) or Dopamine D2. (No: LV 1583420 Millipore, 1: 500 dilution in a 1X PBS solution containing 5% normal goat serum). After overnight incubation brain sections washed with PBS and then incubated for 1 hour with secondary antibody conjugated with FITC (No: AB7130F, Chemicon, 1:1000 dilution in a 1X PBS solution containing 5% normal goat serum) in brain regions. After the incubations brain sections were washed with PBS. Remove the excess PBS off from the slides and mount cover glass with anti-fade mounting media. The sections were observed and photographed using confocal imaging system (Leica SP 5).
**Materials and Methods**

Expressions were analysed using pixel intensity method. Quantification was done using Leica application suit advanced fluorescence (LASAF) software by considering the mean pixel intensity of the image. The fluorescence obtained depends on the number of receptors specific to the added primary antibody. The mean pixel intensity was directly related to the fluorescence emitted from the sections and calculated with the LASAF software. All the imaging parameters in the confocal imaging system like PMT, pinhole and zoom factor were kept same for imaging the sections of all experimental groups.

**STATISTICS**

Statistical evaluations were done by ANOVA using InStat (Ver.2.04a) computer programme. Linear regression Scatchard plots were made using Sigma Plot software (version 2.0, Jandel GmbH, Erkrath, Germany). Relative Quantification Software was used for analyzing Real-Time PCR results.