**Materials and Methods**

**CHEMICALS USED AND THEIR SOURCES**

**Biochemicals**

5-hydroxytryptophan, 5-hydroxy indole acetic acid, ketanserin, bicuculline, baclofen, atropine, sodium octyl sulfonic acid, ethylene diamine tetra acetic acid-EDTA, D-glucose, calcium chloride, bovine serum albumin fraction V, Superoxide dismutase from bovine erythrocytes were purchased from Sigma Chemical Co., St. Louis, USA. All other reagents were of analytical grade purchased locally. HPLC solvents were of HPLC grade obtained from SRL and MERCK, India. Tissue freezing medium Jung was purchased from Leica Microsystems Nussloch GmbH, Germany.

**Radiochemicals**

4-amino-n-[2,3-\(^3\)H]butyric acid (Specific activity- 84.0 Ci/mmole) was purchased from Amersham Bioscience, USA. Baclofen, (-)-[butyl-4-\(^3\)H(N)] (Specific activity- 42.9 Ci/mmole), Bicuculline methyl chloride, (-)-[methyl-\(^3\)H] (Specific activity- 82.9 Ci/mmole), Quinuclidinylbenzilate, L-[Benzilic-4,4'-\(^3\)H]-[4-\(^3\)H] (Sp. Activity 42 Ci/mmole) were purchased from NEN Life Sciences Products, Inc., Boston USA. 5-Hydroxy [G-\(^3\)H] tryptamine creatine sulphate ([\(^3\)H]5-HT, 18.4Ci/mmole) and [Ethylene-\(^3\)H]-Ketanserin Hydrochloride (Sp. Activity 63.3Ci/mmole) were purchased from Perkin Elmer Nen Life and Analytical Sciences, Boston, MA, USA.

Radioimmunoassay kit for insulin and triiodothyronine was purchased from Baba Atomic Research Centre (BARC), Mumbai, India. The [\(^3\)H] IP3, [\(^3\)H]cGMP and [\(^3\)H]cAMP Biotrak Assay Systems were purchased from G.E Healthcare UK Limited, UK.
Molecular Biology Chemicals

Tri-reagent kit was purchased from Sigma Chemical Co., St. Louis, USA. ABI PRISM High Capacity cDNA Archive kit, Primers and TaqMan probes for Real-Time PCR were purchased from Applied Biosystems, Foster City, CA, USA. GABA\(_{\alpha 1}\) (Rn 00788315_m1), GABA\(_{\alpha 5}\) (Rn 00568803_m1), GABA \(_{A_\delta}\) (Rn 00568740_m1), GABA\(_{\gamma 3}\) (Rn 00577639_m1), GABA\(_B\) (Rn 00578911_m1), GAD1 (Rn 00690304_g1), Hif1A (Rn 00577560_m1), 5HT\(_{2A}\) (Rn01468302_m1), 5HTT (Rn00564737_m1), insulin receptor (Rn 00567070_m1), muscarinic M1 (Rn 00589936_s1), muscarinic M2 (Rn 02532311_s1), muscarinic M3 (Rn 00560986_s1), choline acetyl transferase (Rn 01453446_m1), acetyl choline esterase (Rn 00596883_m1), SOD (Rn 01477289), GPx (Rn00577994), BAX (Rn 01480160_g1), Phospholipase C (Rn 01647142), CREB (Rn 00578826_m1) primers were used for the study.

Confocal Dyes

Primary antibody for GABA\(_{\alpha 1}\) (No-31775), 5-HT\(_{2A}\) (No: RA24288 BD PharmenginTM), 5-HTT (No: AB9726 Chemicon) and rhodamine coated secondary antibody (No- AP307R) and FITC (No: AB7130F, Chemicon) were used for immunohistochemistry studies using confocal microscope.

ANIMALS

Pups with dams were purchased from Amrita Institute of Medical Sciences, Kochi. Neonatal rats of four days old were weighed and used for experiments. All groups of neonatal rats were maintained with their mothers under optimal conditions 12 hour light and 12 hour dark periods and were fed standard food and water ad libitum.
INDUCTION OF ACUTE HYPOXIA IN NEONATAL RATS

Wistar neonatal rats of 4-days old (body weight, 6.06 ± 0.45g) were used for the experiments and were grouped into seven as follows: (i) Control neonatal rats were given atmospheric air (20.9% oxygen) for 30 minutes (C); (ii) Hypoxia was induced by placing the neonatal rats in a hypoxic chamber provided with 2.6% oxygen for 30 minutes (Hx); (iii) Hypoxic neonatal rats were injected 10% dextrose (500mg/ Kg body wt) intra-peritoneally (i.p.) (Hx+G). (iv) Hypoxic neonatal rats were supplied with 100% oxygen for 30 minutes (Hx+O); (v) Hypoxic neonatal rats were injected 10% dextrose (500mg/ Kg body wt. i.p.) and treated with 100% oxygen for 30 minutes (Hx+G+O). (vi) Hypoxic neonatal rats were injected 10% dextrose (500mg/ Kg body wt), epinephrine (0.1µg/Kg body wt. i.p.) and treated with 100% oxygen for 30 minutes (Hx+G+E+O) (vii) Hypoxic neonatal rats were injected with epinephrine (0.10g/Kg body wt) i.p. (Hx + E). The experimental animals were maintained in the room temperature for one week.

Neonatal experimental rats were kept for one month for behavioural studies. Body weights were measured before the experiment.

TISSUE PREPARATION

Control and experimental neonatal rats were sacrificed by decapitation on postnatal day 14. The brain regions and body parts were dissected out quickly over ice according to the procedure of Glowinski and Iversen (1966) and the tissues were stored at -80°C for various experiments.

All animal care and procedures were in accordance with Institutional and National Institute of Health guidelines.

ESTIMATION OF BLOOD GLUCOSE

Blood glucose was estimated by GOD-POD glucose estimation kit from Biolab Diagnostics Pvt. Ltd. The glucose was estimated on post natal day 14. The
spectrophotometric method using glucose oxidase-peroxidase reactions was as follows:

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

\[
\text{Glucose} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{GOD}} \text{Gluconic acid} + \text{H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 + \text{Phenol} + 4\text{- aminoantipyrene} \xrightarrow{\text{PEROXIDASE}} \text{Coloured complex} + \text{H}_2\text{O}
\]

The hydrogen peroxide formed in this reaction reacted with 4-aminoantipyrine and 4-hydroxybenzoic acid in the presence of peroxidase (POD) to form N-\((4\text{-antipyryl})\)-p-benzo quinoneimine. The addition of mutarotase accelerated the reactions. The amount of dye formed was proportional to the glucose concentration. The absorbance was read at 500nm in (Shimadzu UV-1700 pharmaSPEC) spectrophotometer.

**KINETICS OF SUPEROXIDE DISMUTASE (SOD)**

The ability of the flavonoid to inhibit the reduction of nitro blue tetrazolium (NBT) by superoxide generated by the reduction of photoreduced riboflavin and oxygen was assayed. SOD assay was done in heart and cerebral cortex homogenate as described previously (Winterbourn et al., 1975).

The SOD concentration (U/mg) that established IC\(_{50}\) (50% inhibition of the reaction) was determined using a standard SOD (2000-10000 U/mg protein). Then, the dilution rate of heart and cerebral cortex homogenates that established IC\(_{50}\) was determined and the unit (U/mg) of the extract was calculated by the SOD concentration that established IC\(_{50}\) determined using standard SOD. One unit of SOD was defined as the amount of protein that inhibited the rate of NBT reduction by 50%. Kinetic parameters \(V_{\text{max}}\) and \(K_m\), were calculated from the data of SOD assay measured at substrate concentrations of 0.03 mM, 0.06 mM, 0.12 mM, 0.15 mM and 0.2 mM.
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**KINETICS OF CATALASE (CAT)**

Catalase assay was done in heart and cerebral cortex homogenate as described previously (Aebi, 1984). The reaction mixture contained 40 mM H$_2$O$_2$ in a 50 mM phosphate buffer pH 7.0, and 0.1 ml pure enzyme in a total volume of 3 ml. CAT activity was estimated by decrease in absorbance of H$_2$O$_2$ at 240 nm. Kinetic parameters $V_{max}$ and $K_m$, were calculated from the data of catalase assay measured at substrate concentrations of 200 µM, 400 µM, 600 µM and 800 µM.

**ESTIMATION OF TRIIODOTHYRONINE (T3) BY RADIOIMMUNOASSAY**

**Principle of the assay**

The assay was done according to the procedure of BARC radioimmunoassay kit. The radioimmunoassay method was based on the competition of unlabelled endogenous T3 with $[^{125}\text{I}]$ T3 for the limited binding sites on the antibody (Ab1) made specially for T3. The antibody was in the form of a complex with second antibody (Ab2). At the end of incubation, the T3 (Ag) bound to the antibody-second antibody complex (Ag-Ab1-Ab2) and free T3 was separated by the addition of PEG. The amount bound to the antibody complex in the assay tubes were compared with values of known T3 standards and the T3 concentration in the samples were calculated.

**Assay Protocol**

Standards, ranging from 0.15 to 2.5ng, T3 free serum, $[^{125}\text{I}]$ T3 and antiserum complex were added together and the volume was made up to 275µl with assay buffer. Samples of appropriate concentration from experiments were used for the assay. They were incubated at 37°C for 45 minutes. The PEG was added to all tubes 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.

T3 concentrations in the samples were determined from the standard curve plotted using MultiCalc™ software (Wallac, Finland).

A standard curve was plotted with %B/B₀ on the Y-axis and insulin concentration/ml on the X-axis of a log-logit graph. %B/B₀ was calculated as:

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

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

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 was 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 were 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 200µU/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 50µl [¹²⁵I] Insulin was added and incubated at room temperature for 3 hrs. 50µl second antibody was added 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/B₀ on the Y-axis and insulin concentration/ml on the X-axis of a log-log it graph. %B/B₀ was calculated as:

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

Corrected average count of zero standard

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

QUANTIFICATION OF GABA IN THE EXPERIMENTAL GROUPS OF NEONATAL RATS

GABA content in the brain regions of control and experimental rats were quantified by displacement method using the procedure of Kurioka et al. (1981) where the incubation mixture contained 30 nM [³H]GABA with and without GABA at a concentration range of 10⁻⁹ M to 10⁻³ M. The unknown concentrations were determined from the standard displacement curve using appropriate dilutions and calculated for µmoles/g wt. of the tissue.

QUANTIFICATION OF BRAIN SEROTONIN (5-HT) AND ITS METABOLITE IN THE EXPERIMENTAL GROUPS OF NEONATAL RATS

The monoamines were assayed according to the modified procedure of Dakshinamurti et al., (1988). The brain stem and cerebellum of experimental
groups of rats was homogenised in 0.4N perchloric acid. The homogenate was then centrifuged at 5000xg for 10 minutes at 4°C in a Sigma 3K30 refrigerated centrifuge and the clear supernatant was filtered through 0.22 µm HPLC grade filters and used for HPLC analysis.

5-Hydroxy indole Acetic Acid (5-HIAA) and 5-HT contents were determined in high performance liquid chromatography (HPLC) with electrochemical detector (ECD) (Waters, USA) fitted with CLC-ODS reverse phase column of 5 µm particle size. The mobile phase consisted of 50mM sodium phosphate dibasic, 0.03M citric acid, 0.1mM EDTA, 0.6mM sodium octyl sulfonate and 15% methanol. The pH was adjusted to 3.25 with orthophosphoric acid, filtered through 0.22 µm filter (Millipore) and degassed. A Waters model 515, Milford, USA, pump was used to deliver the solvent at a rate of 1 ml/minute. The neurotransmitters and their metabolites were identified by amperometric detection using an electrochemical detector (Waters, model 2465) with a reduction potential of +0.80 V. Twenty microlitre aliquots of the acidified supernatant were injected into the system for quantification. The peaks were identified by relative retention times compared with external standards and quantitatively estimated using an integrator (Empower software) interfaced with the detector. Data from different brain regions of the experimental and control rats were statistically analysed and tabulated.

GABA RECEPTOR BINDING STUDIES USING [³H] RADIOLIGANDS

Total GABA Receptor Binding studies

[³H]GABA binding to the GABA receptor was assayed in Triton X-100 treated synaptic membranes (Kurioka et al., 1981). Crude synaptic membranes were prepared using sodium-free 10mM tris sulphate buffer (pH 7.4). Each assay tube contained a protein concentration of 0.3-0.4 mg. In saturation binding
experiments, 5-40 nM of $[^3]$H GABA was incubated with and without excess of unlabelled GABA (100µM) and in competition binding experiments the incubation mixture contained 30 nM of [3H]GABA with and without GABA at a concentration range of $10^{-9}$M to $10^{-4}$M. The incubation was continued for 20 min at 0-4°C and terminated by centrifugation at 35,000 xg for 20 min. Bound radioactivity was counted with cocktail-T in a liquid scintillation counter. Specific binding was determined by subtracting non-specific binding from the total binding.

**GABA$_A$ Receptor Binding studies**

$[^3]$H bicuculline binding to the GABA receptor was assayed in Triton X-100 treated synaptic membranes (Kurioka et al., 1981). Crude synaptic membranes were prepared using sodium-free 10mM Tris sulphate buffer, pH 7.4. Each assay tube contained a protein concentration of 0.3-0.4 mg. In saturation binding experiments, 5nM to 75nM concentrations of $[^3]$H bicuculline was incubated with and without excess of 100µM unlabelled bicuculline. The incubation was continued for 20 min at 0-4°C and terminated by centrifugation at 35,000xg for 20 min. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. Specific binding was determined by subtracting non-specific binding from the total binding.

**GABA$_B$ Receptor Binding studies**

$[^3]$H baclofen binding to GABA receptor in the synaptic membrane preparations were assayed as previously described (Hills et al., 1987). Crude synaptic membrane preparation was suspended in 50mM Tris sulphate buffer, pH 7.4 containing 2mM CaCl$_2$ and 0.3-0.4 mg protein. In saturation binding experiments, 10-100nM of $[^3]$H baclofen was incubated with and without excess of 100µM unlabelled baclofen. The incubations were carried out at 20°C for 20
min. The binding reactions were terminated by centrifugation at 14000xg for 10
min. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid
scintillation counter. Specific binding was determined by subtracting non-specific
binding from the total binding.

SEROTONIN RECEPTOR BINDING STUDIES USING [3H]
RADIOLIGANDS

5-HT Receptor Binding studies

5-HT receptor assay was done using [3H]-5-hydroxytryptamine binding in

5-HT receptor assay was done using [3H]-5-hydroxytryptamine binding in
crude synaptic membrane preparations of cerebral cortex, brain stem, cerebellum
and corpus striatum by the modified method of Uzbekov et al., (1979). Crude
membrane preparation was suspended in 50 mM Tris-HCl buffer, pH 8.5,
containing 1.0 µM paragyline. The incubation mixture contained 0.3-0.4 mg
protein. In the saturation binding experiments, assays were done using different
concentrations i.e., 1.0nM-30nM of [3H] 5-HT incubated with and without excess
of unlabelled 10µM 5-HT. Tubes were incubated at 37°C for 15 minutes 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 8.5.
Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid
scintillation counter.

5-HT2A Receptor Binding Studies

5-HT2A receptor assay was done using [3H] Ketanserin binding in crude
synaptic membrane preparations of cerebellum by the modified method Leysen et
al., (1982). Crude membrane preparation was suspended in 50 mM Tris-HCl
buffer, pH 7.6. The incubation mixture contained 0.3-0.4 mg protein. In the
saturation binding experiments, assays were done using different concentrations
i.e., 0.1nM-2.5nM of [3H] Ketanserin which was incubated with and without
excess of unlabelled 10µM Ketanserin. Tubes were incubated at 37°C for 15 minutes and filtered rapidly through GF/B filters (Whatman). The filters were washed quickly by three successive washings with 5.0 ml of ice cold 50mM Tris buffer, pH 7.6. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

**TOTAL MUSCARINIC RECEPTOR BINDING STUDIES USING [³H] RADIOLIGANDS**

[³H] QNB binding assay in cerebral cortex, cerebellum, brain stem, and corpus striatum were done according to the modified procedure of Yamamura and Snyder (1981). Brain tissues were homogenised with 20 volumes of cold 50mM Tris-HCl buffer, pH 7.4, containing 1mM EDTA. The supernatant was then centrifuged at 30,000xg for 30 minutes and the pellets were resuspended in appropriate volume of the buffer. Total muscarinic binding parameter assays were done using 0.1-2.5nM of [³H] QNB in the incubation buffer, pH 7.4 in a total incubation volume of 250µl. The non-specific binding was determined using 100µM atropine for total muscarinic receptor. Total incubation volume of 250 µl contains 200-250µg protein concentration. Tubes were incubated at 22°C for 60 minutes and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washings with 5.0 ml of ice cold 50mM Tris-HCl buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non-specific binding determined showed 10% in all our experiments.

**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.
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

Competitive binding data was analyzed using non-linear regression curve-fitting procedure (GraphPad PRISM™, San Diego, USA). The data of the competitive binding assays were represented graphically with the log of concentration of the competing drug on x-axis and percentage of the radioligand bound on the y-axis. The steepness of the binding curve can be quantified with a slope factor, often called a Hill slope. A one-site competitive binding curve that follows the law of mass action has a slope of 1.0 and a two site competitive binding curve has a slope less than 1.0. The concentration of competitor that competes for half the specific binding was defined as $EC_{50}$, which is same as $IC_{50}$. The affinity of the receptor for the competing drug is designated as $K_i$ and is defined as the concentration of the competing ligand that binds to half the binding sites at equilibrium in the absence of radioligand or other competitors.
GENE EXPRESSION STUDIES IN DIFFERENT BRAIN REGIONS OF CONTROL AND EXPERIMENTAL NEONATAL RATS

Isolation of RNA

RNA was isolated from the different brain regions of control and experimental neonatal rats using the Tri reagent from Sigma Chemicals Co., St. Louis, MO, U.S.A. 25-50mg tissue 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 5min. 100µl of chloroform was added to it, mixed vigorously for 15sec and allowed to stand at room temperature for 15min. The tubes were then centrifuged at 12,000 x g for 15min 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 10min. The tubes were centrifuged at 12,000 x g for 10min 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 12,000 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 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.

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 10min 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 a 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 MasterMIX (Applied Biosystems). The volume was made up with RNAsfree water. Each run contained both negative (no template) and positive controls.

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
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60°C -- 1 minutes --- 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 ∆∆CT 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 (Δ∆CT= ΔCT − CT_{Control}). The fold change in expression was then obtained (2^{Δ∆CT}).

IMMUNOHISTOCHEMISTRY OF GABAA\textsubscript{α1} RECEPTOR, SEROTONIN RECEPTOR SUBTYPE 5-HT\textsubscript{2A}, SEROTONIN TRANSPORTER 5-HTT IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL NEONATAL RATS USING CONFOCAL MICROSCOPE

Control and experimental rats were deeply anesthetized with ether. The rat was transcardially perfused with PBS, pH- 7.4 followed by 4% paraformaldehyde in PBS. After perfusion the brains were dissected out and fixed in 4% paraformaldehyde for 1 hr and then equilibrated with 30% sucrose solution in PBS (0.1 M). 30 µm cerebellum sections were cut using cryostat (Leica, CM1510 S). The sections were treated with PBS. To block unspecific binding the sections were incubated for 1 hour at room temperature with 5% bovine serum albumin in normal goat serum. Brain slices were incubated overnight at 4 °C with rat primary antibody for GABAA\textsubscript{α1} (diluted in PBST at 1: 1000 dilution), 5-HT\textsubscript{2A} (No: RA24288 BD PharmenginTM, diluted in PBST at 1: 500 dilution) and 5HTT (No: AB9726 Chemicon Temecula, diluted in PBST at 1: 500 dilution). After overnight incubation, the brain slices were rinsed with PBS and then incubated with FITC (No: AB7130F, Chemicon, diluted in PBS at 1: 1000 dilution) coated secondary
antibody. The sections were observed and photographed using confocal imaging system (Leica SP 5).

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

Brain tissue from corpus striatum was 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 15min, 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 25pmoles/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.
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The radioactivity in the suspension was determined using liquid scintillation counter.

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:

\[
\frac{(\text{Standard or sample cpm} - \text{NSB cpm})}{(B_0 \text{ cpms} - \text{NSB cpm})} \times 100
\]

NSB- non specific binding and B_0 - zero binding. IP3 concentration in the samples was determined by interpolation from the plotted standard curve.

cGMP CONTENT IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS IN VIVO

Brain tissue from corpus striatum was 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 cGMP assay using \[^3\text{H}\]cGMP Biotrak Assay System kit.

Principle of the assay

The assay is based on the competition between unlabelled cGMP and a fixed quantity of the \[^3\text{H}\]cGMP for binding to an antiserum, which has a high specificity and affinity for cGMP. The amount of \[^3\text{H}\]cGMP bound to the antiserum is inversely related to the amount of cGMP present in the assay sample. Measurement of the antibody bound radioactivity enables the amount of unlabelled cGMP in the sample to be calculated. Separation of the antibody bound cGMP from the unbound nucleotide was done by ammonium sulphate precipitation, followed by centrifugation. The precipitate which contains the
antibody bound complex was dissolved in water and its activity was determined by liquid scintillation counting. The concentration of unlabelled cGMP in the sample was determined from a linear standard curve.

**Assay Protocol**

Standards, ranging from 0.5 to 4.0 pmoles/tube, and [³H]cGMP 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 antiserum was added to all the assay tubes and then vortexed. The tubes were incubated for 90min at 2 - 8°C. Ammonium sulphate was added to all tubes, mixed and allowed to stand for 5min in ice bath. The tubes were centrifuged at 12000 x g for 2min at room temperature. The supernatant was aspirated out and the pellet was dissolved in water and decanted immediately into scintillation vials. The radioactivity in the suspension was determined using liquid scintillation counter.

A standard curve was plotted with $C_o/C_x$ on the Y-axis and cGMP concentration (pmoles/tube) on the X-axis of a linear graph paper. $C_o$- the cpm bound in the absence of unlabelled cGMP; $C_x$- the cpm bound in the presence of standard/unknown cGMP. cGMP concentration in the samples was determined by interpolation from the plotted standard curve.

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

Brain tissues- BS and CB 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 [³H]cAMP Biotrak Assay System kit.
Principle of the assay

A cAMP assay kit was used. The assay is based on the competition between unlabelled cAMP and a fixed quantity of tritium labeled compound 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 present in the assay sample. Measurement of the protein-bound radioactivity enables the amount of unlabelled cAMP in the sample to be calculated.

\[
\text{Free } [^3\text{H}]\text{cAMP} + \text{Binding protein} = \text{Bound } [^3\text{H}]\text{cAMP-binding protein} + \text{cAMP}
\]

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, [^3H]cAMP and binding protein in case of standards; buffer, [^3H]cAMP and binding protein for zero blank and unknown samples, [^3H]cAMP and binding protein for determination of 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 (Wallac, 1409).

\[ C_0/C_x \] was plotted on the Y-axis against picomoles of inactive cAMP on the X-axis of a linear graph paper, where \( C_0 \) is the counts per minute bound in the absence of unlabelled cAMP and \( C_x \) was the counts per minute bound in the presence of standard or unknown unlabelled cAMP. From the \( C_0/C_x \) value for the sample, the number of picomoles of unknown cAMP was calculated.

**BEHAVIOURAL STUDIES**

**Wire maneuver test**

For wire maneuver test, rat was hung by both forelimbs on the wire 50cm high from the floor. The diameter of the wire was 1mm. The body of the rat was maintained parallel to the floor by hanging the tail by experimenter’s hand and the tail was then released. Its falling latency was measured as an index of forelimb strength.

The animal was lifted by its tail and allowed to grasp the horizontal wire with its forepaws and was then rotated partially downward and released. The ability of the animal to grasp to the wire also with its hind limbs was scored.

**Righting reflex**

For righting reflex, rat was held in the lumbosacral region. When the body was tilted to the left and right, the head was moved in the opposite direction in order to maintain the original position in the normal reflex.

**Y-Maze test**

The Y-maze was made of grey wood, covered with black paper and consisted of three arms with an angle of 120 degrees between each of the arms. Each arm was 8 cm width \( \times 30 \) cm length \( \times 15 \) cm height. The three identical arms were randomly designated: Start arm, in which the rat started to explore (always
open); Novel arm, which was blocked at the 1st trial, but open at the 2nd trial and the other arm (always open). The maze was placed in a separate room with enough light. The floor of the maze was covered with saw dust, which was mixed after each individual trial in order to eliminate olfactory stimuli. Visual cues were placed on the walls of the maze.

The Y-maze test consisted of two trials separated by an inter-trial interval (ITI). The first trial (training) was of 10 minutes duration and allowed the rat to explore only two arms (start arm and the other arm) of the maze, with the third arm (novel arm) blocked. After a 1 hour ITI (Ma et al., 2007), the second trial (retention) was conducted, during which all three arms were accessible and novelty vs familiarity was analyzed through comparing behaviour in all three arms. For the second trial, the rat was placed back in the maze in the same starting arm, with free access to all three arms for 5 minutes. The time spent in each arm was analyzed. Data was expressed as percentage of performance in all three arms during the five minutes of test (Akwa et al., 2001; Jobin, et al., 2010).

**Radial arm maze Test**

Radial maze behavioral testing was conducted under normal room lighting using an eight armed radial maze elevated 100 cm from the floor. Each arm of the maze (11.5 cm wide) extended 68.5cm from an octagonally shaped central platform (40 cm across). Black Plexiglas walls (11.5 cm high) were present for the first 20 cm of each arm to prevent the rat crossing from one arm to another without returning to the central platform. Circular food wells (1.3 cm deep, 3.2 cm diameter) were located 2.5cm from the end of each arm. The maze was centered in an enclosed room where lighting and spatial cues (e.g., posters, door, and boxes) remained constant throughout the course of the experiment. Arms were baited by placing one raisin in each food well.

Rats were placed on the maze 3 days prior to the start of formal acquisition testing in order to habituate them to the apparatus. On the first day of
habituation, 4 food pellets were scattered along the length of each arm. The rats were then systematically confined to each arm for 1 min to ensure their exposure to the entire maze. On the second day of habituation, the previous day’s procedure was repeated except that the animals were not confined to each arm following 5 min of exploration. On the third day, one food pellet was placed in the food well at the end of each arm and a second was placed halfway down each arm. Once the rats were habituated to the maze, testing began. Trials began by placing a single rat in the center of the maze facing away from the experimenter. The trial ended when the rat had obtained all 4 pellets or 5 min had elapsed, whichever occurred first. Rats were run until they achieved criterion performance for task acquisition. Criterion was attained when the rat collected 3 out of the 4 food pellets within their first 4 arm entries within a trial (while still completing the trial) with this level of performance being maintained for 5 consecutive criterion performance. The number of trials up to and including the last of these 5 criterion performance formed the “number of trials to criterion” measure. Experimental subjects were tested under blind conditions. The time of testing was consistent from day to day for each subject but testing of the various treatment groups was distributed randomly throughout the day.

**Morris water maze Test**

Water maze experiment was conducted during post-treatment. The custom-constructed water maze pool measured 100 cm in diameter by 50 cm in depth and was filled with water to a depth of 35 cm. A 10-cm-diameter white platform was located 1.5 cm below the surface of the water. Nontoxic white paint was added to the water to visually obscure the location of the platform. The pool had been divided into four quadrants of homogeneous size and the platform was located in the center of one of the quadrants, halfway between the center and the wall of the pool. All swim latencies were recorded with a manual stopwatch, a technique routinely employed by others (Hort *et al.*, 1999). The water maze task
Materials and Methods

consisted of 15 sessions conducted once daily over 15 successive days. Each session consisted of four trials separated by approximately 60 seconds. Rats were placed manually into the pool, facing the pool wall in the center of one of the quadrants that did not contain the platform. For any given rat, the location of the platform remained fixed across all trials and all sessions. The latency to find the platform was recorded as the time from release into the pool until the rat had reached the platform. A maximum of 60 seconds was allowed for each trial. Rats not reaching the platform within 60 seconds were guided to the platform and a score of 60 seconds was recorded for each of these experimenter-terminated trials. The rat was allowed to remain on the platform for the duration of the inter-trial interval. A 60-seconds probe test to determine the time spent in the platform quadrant after removing the platform from pool was conducted on the 13th, 14th and 15th day of the study (24 h after the last hidden platform session). Rats were released into the pool in the quadrant opposite to that previously associated with the escape platform. A manual time-sampling procedure (one measurement per second) was utilized to record the swimming bias of the rat in each of the four quadrants of the pool.

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). Sigma Plot software (version 2.0, Jandel GmbH, Erkrath, Germany). Competitive binding data were analysed using non-linear regression curve-fitting procedure (GraphPad PRISM™, San Diego, USA). Empower software were used for HPLC analysis. Relative Quantification Software was used for analyzing Real-Time PCR results.