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CHEMICALS USED IN THE STUDY AND THEIR SOURCES

Biochemicals

Serotonin creatinine sulfate, γ-aminobutyric acid, ketanserin, baclofen, bovine serum albumin fraction V, SOD, sodium octyl sulfonic acid, ethylene diamine tetra acetic acid (EDTA), Tris HCl, sucrose, magnesium chloride, calcium chloride, bromodeoxyuridine (BrdU) and paraformaldehyde (PFA) were purchased from Sigma Chemical Co., St. Louis, USA. All other reagents were of analytical grade purchased locally from SRL, India. HPLC solvents were of HPLC grade obtained from SRL and MERCK, India. Tissue freezing medium Jung was purchased from Leica Microsystems Nussloch GmbH, Germany. Chitosan (MW-25KDa) was a gift from Central Institute of Fisheries Technology, Cochin, India.

Radiochemicals

[Ethylene-\(^3\)H]-ketanserin hydrochloride (Sp. Activity 63.3Ci/mmol), [\(^3\)H] baclofen (Sp. Activity 42.9 Ci/mmol), [\(^3\)H] Gamma aminobutyric acid (Sp. Activity 76.2 Ci/mmol), [\(^3\)H] thymidine (Sp. Activity 18.0 Ci/mmol) and [\(^3\)H] leucine (Sp. Activity 63.0 Ci/mmol) were purchased from Amersham Bioscience, USA. [\(^3\)H] methyl S-adenosylmethionine (Sp. Activity 80 Ci/mmol), [\(^3\)H] IP\(_3\), [\(^3\)H] cAMP and [\(^3\)H] cGMP kits 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, Primers, endogenous control (β-actin) and Taqman probes for Real-Time PCR were purchased from Applied Biosystems, Foster City, CA, USA. GABA\(_{\beta}\) (Rn_00578911), 5HT\(_{2}\)A (Rn_01468302), CREB (Rn_00561126), phospholipase C (Rn_01647142), IGF-1 (Rn_99999087), SOD (Rn01477289), Bax (Rn_01480160) Akt-1 (Rn_00583646), NF-κB (Rn_01399583), TNF-α (Rn_99999017), Caspase-8 (Rn_00574069),
hepatocyte growth factor (Rn_00690368), Mat2A (Rn_01643368), BDNF (Rn_01484924), and GDNF (Rn_00569510) primers were used for the gene expression studies.

Confocal Dyes

Rat primary antibody for 5-HT2A (No: ab16028, Abcam), GABA_B (No: ab68426), Bromo deoxyuridine (Cat. No. B8434, Sigma Aldrich, USA) and secondary antibody of Alexa Fluor 594 (No: A11012 and No: A11005, Invitrogen) were used for the immunohistochemistry studies using confocal microscope.

ANIMALS

Adult male Wistar rats of 250-300g body weight were purchased from Kerala Agriculture University, Mannuthy, India and Amrita Institute of Medical Sciences, Kochi, India and used for all experiments. They were housed in separate cages under 12 hours light and 12 hours dark periods and were maintained on standard food pellets and water ad libitum. Adequate measures were also taken to minimize pain and discomfort of the animals. All animal care and procedures were taken in accordance with the Institutional, National Institute of Health and CPCSEA guidelines.

PREPARATION OF GABA AND 5-HT CHITOSAN NANOPARTICLES

The chitosan nanoparticles were prepared by ionic gelation method (Calvo et al., 1997). Chitosan was dissolved in 2% acetic acid to get chitosan solution of concentration 1mg/mL. Chitosan nanoparticles from 50 mL chitosan solution were precipitated by the addition of 33 mL of 1 mg / mL penta sodium tri polyphosphate (TPP) solution with rapid stirring. To incorporate GABA in to chitosan, a solution of concentration, 8.824 µg of GABA / mL of chitosan solution was prepared and the precipitation of GABA - chitosan nanoparticles were done by the above method. To incorporate serotonin creatinine sulphate (5-HT) to chitosan, 300 µg of 5-HT / mL of chitosan solution was prepared and the precipitation of 5-HT - chitosan nanoparticles were done. To prepare a
combination of GABA and 5-HT chitosan nanoparticles, 400 µg of 5-HT and 20 µg of GABA were dissolved in chitosan solution and the nanoparticles were precipitated by the addition of TPP. The precipitated nanoparticles were centrifuged for 20 minutes at 16,000xg. The pellet was washed thoroughly with distilled water and then resuspended in saline. The SEM image of the nanoparticles was taken with a magnification of 10000X by scanning electron microscope (JEOL Model JSM - 6390LV).

FT-IR spectroscopy

The FT-IR spectrum of chitosan, chitosan nanoparticles, GABA, GABA incorporated chitosan nanoparticles, 5-HT, 5-HT incorporated chitosan nanoparticles and GABA and 5-HT incorporated chitosan nanoparticles were taken using Fourier Transform Infra Red spectrometer (Thermo Nicolet, Avatar 370) with a spectral range of 4000-400 cm⁻¹.

Determination of encapsulation efficiency and in vitro release studies

The maximum encapsulation efficiency (Rao et al., 2010) of GABA with chitosan nanoparticles was obtained by giving emphasis to concentration of GABA added to the chitosan solution. The encapsulation efficiency was calculated by incorporating [³H] GABA with chitosan and the radioactivity of the GABA, which was bound on the chitosan nanoparticles, were related to its concentration (Motulsky & Christopaulos, 2004; Shilpa et al., 2012). Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The same method is adopted for determining the encapsulation efficiency of GABA in GABA and 5-HT chitosan nanoparticles.

The maximum encapsulation efficiency of 5-HT in both 5-HT chitosan nanoparticles and GABA and 5-HT chitosan nanoparticles was obtained by considering the 5-HT concentration that was added to the chitosan solution. The concentration of 5-HT, which was bound to the nanoparticles, were found by HPLC with electrochemical detector (Waters, USA) fitted with CLS-ODS reverse
phase column of 5 µm particle size. After centrifugation of nanoparticle suspension, the supernatant with unbound 5-HT was filtered through 0.22 µm HPLC grade filters and injected to the column. The mobile phase consisted of 50mM sodium phosphate dibasic, 0.03M citric acid, 0.1 mM EDTA, 0.6 mM sodium octylsulfonate and 15% methanol. The pH was adjusted to 3.25 with orthophosphoric acid, filtered through 0.22 µm (Millipore) and degassed. A Waters model 515, Milford, USA, pump was used to deliver the solvent at a rate of 1 mL/minute. The 5-HT was identified by amperometric detection using an electrochemical detector (Waters model 2465) with a reduction potential of +0.80V. The peaks obtained were compared with standard creatinine sulphate and quantitatively estimated using an integrator (Empower software) interfaced detector.

% Encapsulation = (Concentration of GABA or 5-HT bound to chitosan nanoparticles / Concentration of GABA or 5-HT added initially) x 100.

In in vitro release studies, the different nanoparticles were suspended in the respective PBS solution, pH 7.4. All were gently stirred at different time intervals from 0 to 40 hours. The concentration of released GABA and 5-HT at each time from the nanoparticles was calculated using the above mentioned methods to get a release profile in vitro.

CELL UPTAKE OF GABA AND 5-HT CHITOSAN NANOPARTICLES
Preparation of FITC labeled chitosan nanoparticles

Chitosan solution (1mg/mL), of volume 50mL was prepared and the nanoparticles were precipitated by adding TPP. The nanoparticles were centrifuged and the pellet was resuspended in 5 mL DMSO and sonicated for 1 minute. Then a solution of 10 mg/ mL FITC in DMSO was added to the nanoparticle suspension. Stirred the solution gently and kept overnight at dark. After stirring, particles were washed with DMSO several times until the non conjugated FITC was eliminated completely (Min et al., 2004). The liver was perfused initially with Ca$^{2+}$ buffer, pH 7.4 (142 mM NaCl, 6.7 mM KCl, 10 mM HEPES and 5.5 mM NaOH) and then with collagenase buffer, pH 7.6 (67 mM
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NaCl, 6.7 mM KCl, 100 mM HEPES, 4.76 mM CaCl$_2$, 2H$_2$O, 0.66 mM NaOH and collagenase). The perfused liver was minced in PBS, pH 7.4 and kept for collagenase digestion. The cells were filtered and washed. Resuspended the cells in William’s media and 150 µL of cell suspension (cell density of 1.6 $\times$ 10$^5$ cells/cm$^2$) was added to a four well glass slide. Then the cells were incubated in 5% CO$_2$ atmosphere for 24 hours at 37°C.

**Uptake of FITC labeled nanoparticles by liver cells**

50 µL of FITC labeled and unlabelled nanoparticles were added to the corresponding cell suspension in each well and incubated for 2 hours. After the incubation, the fluorescent images were captured using confocal microscope with an excitation at 488 nm (Yuqing et al., 2009).

**EXPERIMENTAL DESIGN**

The experimental rats were divided into the following five groups

1. Sham operated control (C)
2. Partially hepatectomised group without any treatment (PHNT)
3. Partially hepatectomised group with GABA chitosan nanoparticle treatment (GCNP)
4. Partially hepatectomised group with 5-HT chitosan nanoparticle treatment (SCNP)
5. Partially hepatectomised group with GABA and 5-HT chitosan nanoparticle treatment (GSCNP)

Each group consisted of 4-6 animals.

Chitosan nanoparticles modified with drugs, could be recognized by their respective receptors on cells and were transferred to liver cells through receptor mediated endocytosis. This enhanced their ability to target to the liver, in which receptor mediated cell signalling was activated and enabled the longevity of these nanoparticles in the liver. In contrast, chitosan nanoparticles without modification, targeted sparely to the liver, and a large part of these nanoparticles were cleared.
from the body in urine (Tian et al., 2010; Park et al., 2007). So giving importance to the above fact and based on our previous observations, a control group treated with chitosan nanoparticle alone was not included.

Two – thirds of the liver constituting the median and left lateral lobes were surgically excised under anaesthesia, following a 16 hour fast (Higgins & Anderson, 1931). Sham operations involved median excision of the body wall followed by all manipulations except removal of lobes. All rats were undergone surgeries between 7 and 9 A.M to avoid diurnal variations in responses. After the surgical excision of median and left lateral lobes of liver, 1 mL of 30 µg/µL GABA chitosan nanoparticles, 5-HT chitosan nanoparticles and GABA and 5-HT chitosan nanoparticles suspended in saline were injected intra peritoneal to the respective rats.

Sacrifice and tissue preparation

All the molecular level changes in liver including the DNA synthesis prior to the first mitotic phase occur between 20 and 24 h post hepatectomy (Bucher, 1963). The assays and experiments performed during this period provided a significant comparison among the experimental groups. Thus the experimental rats were sacrificed by decapitation 24 hours post hepatectomy. The liver, corpus striatum, cerebral cortex and brain stem were dissected out quickly over ice according to the procedure of Glowinski & Iversen (1966). The blood samples were collected and plasma was separated by centrifugation. The tissue samples and plasma were kept at -80°C until assay. All animal care and procedures were in accordance with Institutional and National Institute of Health guidelines.

EFFECT OF GABA AND 5-HT ENCAPSULATED CHITOSAN NANOPARTICLES ON DNA AND PROTEIN SYNTHESSES IN LIVER CELLS

After partial hepatectomy and treatments, liver from all the experimental groups were perfused first with Ca²⁺ buffer and then with collagenase buffer. Cell suspension of 150 µL (cell density of $1.6 \times 10^5$ cells/cm²) was added to a four well
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poly L-lysine coated glass slide. Then the cells were incubated for 24 hours at 37°C in 5% CO₂ atmosphere. Before incubation, [³H] leucine of specific activity 63 Ci/mmol was added to one set of culture plates for all the five experimental groups to determine the protein synthesis and [³H] thymidine of specific activity 18 Ci/mmol to the next set of plates to determine the measurement of DNA synthesis. All the experiments were done in triplicates. The cells were scrapped off from the culture plates and centrifuged at 2000xg for 20 minutes. The supernatant was discarded and the pellet was resuspended in 50 µL, 1M NaOH and kept overnight. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

DNA synthesis was further determined by analyzing the activity of thymidine kinase (TK) in all the experimental groups. A 10% liver homogenate was prepared in 50 mM Tris HCl buffer, pH 7.5. It was centrifuged at 36000xg for 30 minutes and the supernatant was collected. TK was assayed by determining the conversion of [³H] thymidine in the presence of ATP to [³H] thymidine monophosphate (TMP) by the binding of latter nucleotide to DEAE cellulose discs (Maliekal et al., 1997). The reaction mixture contained 5mM [³H] thymidine (0.5µCi), 10mM ATP, 100 mM NaF, 10 mM MgCl₂, 0.1 M Tris· HCl buffer, pH 8.0 and the liver supernatant fraction. After incubation at 37°C for 15 minutes the reaction mixture was spotted in DE 81 paper discs. The bound radioactivity of [³H] thymidine monophosphate was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

BEHAVIOURAL STUDIES

Animals were observed everyday for any overt abnormal activity.

Beam-walk test

After three days post hepatectomy, the animals were tested for the balance and motor coordination on a narrow beam maze (Allbutt & Henderson, 2007). This has a smooth wooden narrow beam of 105cm long, 4cm in width and thickness of 3cm. The beam was elevated from the ground by 1m with additional
supports. It has a start platform of 20cm in dimension from the start of the beam and an end platform of 20cm dimension at the end of 105cm long beam. There was food on the end platform for the reward of the animals. The journey time between start and end goal was measured. The time was recorded when the animal placed a weight bearing step entirely over the start line. The stopwatch was then stopped when all four feet were placed entirely upon the finishing platform at the opposite end of the beam. The maximum time allowed for the task was 2 min.

**Rotarod Test:**

Rotarod has been used to evaluate motor coordination by testing the ability of rats to remain on revolving rod (Dunham & Miya, 1957). The apparatus has a horizontal rough metal rod of 3 cm diameter attached to a motor with variable speed. This 70 cm long rod was divided into four sections by wooden partitions. The rod was placed at a height of 50 cm to discourage the animals to jump from the rotating rod. The rate of rotation was adjusted in such a manner that it allowed the normal rats to stay on it for five minutes. Each rat was given five trials before the actual reading was taken. The readings were taken at 10, 15 and 25 rpm after three days of hepatectomy in all groups of rats.

**Grid Walk Test**

Deficits in descending motor control were examined by assessing the ability to navigate across a 1 m long runway with irregularly assigned gaps (0.5–5 cm) between round metal bars. Crossing this runway requires that animals accurately place their limbs on the bars. In baseline training and postoperative testing, every animal had to cross the grid for at least three times. The number of footfalls (errors) was counted in each crossing for 3 minute and a mean error rate was calculated (Z'Graggen et al., 1998).
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GABA\textsubscript{B} AND 5-HT\textsubscript{2A} RECEPTORS BINDING STUDIES USING \[^{3}H\] BACLOFEN AND \[^{3}H\] KETANSERIN

\[^{3}H\] Baclofen binding to GABA\textsubscript{B} receptor in the membrane preparations were assayed (Hills et al., 1987). Crude membrane preparation was suspended in 50 mM Tris sulphate buffer, pH 7.4 containing 2 mM CaCl\textsubscript{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 100 µM unlabelled baclofen. The incubations were carried out at 20°C for 20 minutes. The binding reactions were terminated by centrifugation at 14000xg for 10 minutes. The dried pellet was resuspended and counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

\[^{3}H\] Ketanserin binding to 5-HT\textsubscript{2A} receptor in the crude synaptic membrane preparation was done according to the modified procedure of Leysen et al. (1982). Crude membrane preparation was suspended in 50 mM Tris sulphate buffer, pH 7.6 containing 0.3 - 0.4 mg protein. In saturation binding experiments, assays were done using different concentrations of 0.5-10nM of \[^{3}H\] ketanserin which was incubated with and without excess of unlabelled 10 µM 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 50 mM Tris sulphate buffer, pH 7.6. The bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter.

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 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.

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

Preparation of RNA

RNA was isolated from the liver and different brain regions - brain stem, corpus striatum and cerebral cortex 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
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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 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. The thermocycling profile conditions were as follows:

50ºC -- 2 minutes --- Activation
95ºC -- 10 minutes --- Initial Denaturation
95ºC -- 15 seconds --- Denaturation 40 cycles
50ºC -- 30 seconds --- Annealing
60ºC -- 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}).

**DETERMINATION OF SOD ACTIVITY**

The liver, brain stem, cerebral cortex and corpus striatum were homogenized in 0.1M potassium phosphate buffer, pH 7.8 and centrifuged at 100,000 x g for 60 min at 4°C. The supernatant corresponds to the cytosolic fraction containing CuZn-SOD. The pellets were resuspended in the buffer, freeze-thawed three times and centrifuged at 100,000 x g for 60 min at 4°C. The supernatant, the particulate fraction containing Mn-SOD, was mixed with the cytosolic fraction to obtain the total enzyme fraction. SOD was analyzed after inhibition by SOD of the pyrogallol autoxidation (Marklund & Marklund, 1974) at
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pH 8.2 in the presence of EDTA. A 3ml assay mixture contained 0.2 mM pyrogallol, 1 mM EDTA and 50 mM Tris-HCl buffer. Pyrogallol autooxidation was monitored at 420 nm for 3 min in a spectrophotometer (Shimadzu UV-1700) with or without the enzyme. The inhibition of pyrogallol oxidation was linear with the activity of the enzyme present. Fifty percent inhibition/mg protein/min was taken as one unit of the enzyme activity.

DNA METHYLATION STUDY

The DNA was isolated from the liver of experimental rats using TRI reagent according to the procedure of Chomczynski (1993). DNA concentration was determined by ultraviolet spectrophotometry (UV-1700 Pharma Spec, Shimadzu) with absorbance at 260 and 280 nm. All DNA samples had 260 to 280 absorbance ratios ≥ 1.7. DNA methylation was determined by using the modified method of Balaghi and Wagner (1993), in which DNA is incubated with [³H] methyl S-adenosylmethionine in the presence of the CpG Methyl transferase. The reaction mixture contained 0.25 µg DNA, 0.015 U CpG Methyl transferase enzyme (product no. M0226S; New England Biolabs, Beverly, MA), [³H] methyl S-adenosylmethionine (80 Ci/mmol, American radiolabeled chemicals, Inc., Saint Louis, USA), 1.5 µl NEB buffer (New England Biolabs, Beverly, MA), and sterile-filtered water to a total reaction volume of 15 µl. The mixture was incubated at 30 °C for 1 h and placed on ice for 5 min. The reaction mixture were loaded onto a 2.5-cm, round, Whatman DE81 ion-exchange paper filter. The filter was washed successively 3 times with 7.5 ml of 0.5 M sodium phosphate buffer (pH 8.0), then with 1 ml 70% ethanol, and finally with 1mL 100% ethanol. The filter was dried at room temperature and the radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The ability of DNA to incorporate [³H] methyl groups in vitro is inversely related to endogenous DNA methylation.
cAMP CONTENT IN THE LIVER AND BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS IN VIVO

Brain tissues - (cerebral cortex, brain stem and corpus striatum) and liver 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 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 } [\(^3\)H] \text{ cAMP} + \text{Binding protein} = \text{Bound } [\(^3\)H] \text{ cAMP-binding protein} + \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, [\(^3\)H] cAMP and binding protein in case of standards; buffer, [\(^3\)H] cAMP and binding protein for zero blank and unknown samples, [\(^3\)H] cAMP and binding protein for determination of unknown
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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).

\[ C_o/C_x \]

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 \( C_o/C_x \) value for the sample, the number of picomoles of unknown cAMP was calculated.

**IP\(_3\)** CONTENT IN THE LIVER AND BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS IN VIVO

Brain tissues-(cerebral cortex, brain stem and corpus striatum) and liver 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 IP\(_3\) assay using \([^3H]\) IP\(_3\) Biotrak Assay System kit.

**Principle of the assay**

The assay was based on competition between \([^3H]\) IP\(_3\) and unlabelled IP\(_3\) in the standard or samples for binding to a binding protein prepared from bovine adrenal cortex. The bound IP\(_3\) was then separated from the free IP\(_3\) by centrifugation. The free IP\(_3\) 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 IP\(_3\) 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. 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:

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

NSB- non specific binding and B₀ - 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 tissues- (cerebral cortex, brain stem and corpus striatum) 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 cGMP assay using [³H] cGMP Biotrak Assay System kit.
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**Principle of the assay**

The assay is based on the competition between unlabelled cGMP and a fixed quantity of the \[^3H\] cGMP for binding to an antiserum, which has a high specificity and affinity for cGMP. The amount of \[^3H\] 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 100 µl 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 90 min at 2 - 8°C. Ammonium sulphate was added to all tubes, mixed and allowed to stand for 5 min in ice bath. The tubes were centrifuged at 12000 x g for 2 min 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.
IMMUNOCYTOCHEMISTRY OF GABA<sub>B</sub> AND 5-HT<sub>2A</sub> RECEPTORS IN LIVER AND BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS USING CONFOCAL MICROSCOPE

The experimental rats were deeply anesthetized and was transcardially perfused with PBS (pH 7.4) followed by 4% paraformaldehyde in PBS. After perfusion the liver from each experimental group was dissected out and fixed in 4% paraformaldehyde for 1 hour and then equilibrated with 30% sucrose solution in PBS (0.1 M). 10 μm liver sections were cut using Cryostat (Leica, CM1510 S). The sections were washed with PBS and then blocked for 1 hour with PBS containing 5% normal goat serum and 0.1% triton X-100. The primary antibodies of GABA<sub>B</sub> (1:500 dilution in PBS with 5% normal goat serum and 0.1% triton X-100) and 5-HT<sub>2A</sub> (1:1000 dilution in PBS with 5% normal goat serum and 0.1% triton X-100) were added to the respective sections and incubated overnight at 4 °C. After overnight incubation, the liver slices were rinsed with PBS and then incubated with fluorescent labelled secondary antibody (Alexa Fluor 594, code-A11012) prepared in PBS with 5% normal goat serum and 0.1% triton X-100 at 1:1000 dilution. The sections were washed with PBS thoroughly and then observed and photographed using confocal imaging system (Leica SP 5).

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.

IMMUNOCYTOCHEMISTRY OF BROMODEOXYURIDINE IN THE LIVER OF CONTROL AND EXPERIMENTAL RATS USING CONFOCAL MICROSCOPE

Liver cell replication was evaluated based on the incorporation of BrdU (Sigma-Aldrich, St. Louis, Mo), a thymidine analog that incorporates into DNA in
Materials and Methods

the S phase. All experimental groups of rats were intraperitoneally injected with BrdU, two hours prior to sacrifice with a dosage of 50 mg/kg body weight, dissolved in saline (Masson et al., 2009). Anaesthetized animals were transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (pH 7.4). After perfusion the liver was dissected and immersion fixed in 4% paraformaldehyde for 1 hr and then equilibrated with 30% sucrose solution in 0.1M PBS. 6 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. Liver sections were blocked with 5% normal goat serum for 4 hours. The sections were then incubated overnight at 4 °C with mouse primary antibody for Bromo deoxyuridine (BrdU) (Cat. No. B8434, Sigma-aldrich, St. Louis, USA, 1: 500 dilution in a 1X PBS solution containing 5% normal goat serum). After overnight incubation the sections were washed with PBS and then incubated for 1 hour with secondary antibody conjugated with Alexa Fluor 594 (Cat. No- A11005, 1:500 dilution in a 1X PBS solution containing 5% normal goat serum). After the incubation, the sections were washed with PBS. Tap excess PBS off, the slides and mount cover glass with Prolong Gold anti-fade mounting media. The sections were observed and photographed using confocal imaging system (Leica SP 5). 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 specific binding sites 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). Competitive binding
data were analysed using non-linear regression curve-fitting procedure (GraphPad
PRISM™, San Diego, USA). Empower software was used for HPLC analysis.
Relative Quantification Software was used for analyzing Real-Time PCR results.