Materials and Methods

Chemicals used and their sources

Biochemicals used in the present study were purchased from Sigma Chemical Co., St. Louis, USA. All other reagents were of analytical grade purchased locally.

Biochemicals

Mesulergine, ethylene diamine tetra acetic acid (EDTA), Tris HCl, calcium chloride, atropine, Carbamazepine, Pilocarpine, and MK-801 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, India and Sigma Chemical Co., St. Louis, USA.

Radiochemicals

\[^{[N^6-methyl-^3H]}\] mesulergine (Sp. Activity \(79.0\) Ci/mmol) was purchased from Amersham Life Science, UK. \((+)-[^{3-^3H}]\) MK-801 (Sp. Activity \(27.5\) Ci/mmol) was purchased from Perkin Elmer Nen Life and Analytical Sciences, Boston, MA, USA. The \[^{[H]}\] IP3, \[^{[H]}\] cGMP and \[^{[H]}\] 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. 5-HT\(_2\C\) (Rn00562748_m1), NMDA2b (Rn00561352_m1), mGlu5 (Rn00566628_m1) and GLAST (Rn00570130_m1) primers were used for the study.
Confocal Dyes

Immunofluorescent antibodies of 5-HT\textsubscript{2C} was purchased from Neuromics, USA. Fluorescently labeled NMDA2b and mGlu5 receptor specific antibodies were obtained from Becton and Dickinson, USA. FITC and Rhodamine were purchased from Chemicon, USA.

Animals

Adult male Wistar rats of 250-300g body weight purchased from Amrita Institute of Medical Sciences, Cochin were used for all experiments. They were housed in separate cages under 12 hrs light and 12 hrs dark periods and were maintained on standard food pellets and water ad libitum.

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, Kolenchery and voucher specimens are deposited at the herbarium of the Centre for Neuroscience, Dept. of Biotechnology, Cochin University of Science and Technology, Cochin, Kerala (No. MNCB3).

Preparation of Bacopa monnieri Plant Extract

Crude whole plant extract was used to study the anti-epileptic effect in pilocarpine induced temporal lobe epilepsy. *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-epileptic effect in pilocarpine induced temporal lobe epilepsy.
Epilepsy Induction

Adult male Wistar rats, weighing 250 to 300 g, were housed for 1 to 2 weeks before experiments were performed. Experimental animals were injected with pilocarpine (350 mg/Kg i.p.), preceded by 30 min with atropine (1 mg/Kg i.p.) to reduce peripheral pilocarpine effects. Within 20 to 40 min after the pilocarpine injection, essentially all of the animals developed status epilepticus (SE). Behavioural observation continued for 5 hrs after pilocarpine injection. Pilocarpine induced seizures were graded according to the Racine scale using stage 1-5: Stage 0- in which the rats showed no convulsion; stage 1- in which rats showed Facial Automatism; stage 2- Head nodding, stage 3- unilateral forelimb clonus, stage 4- bilateral forelimb clonus, stage 5, rearing, falling and generalized convulsions. The occurrence of stage 3-5 was considered as one complete seizure. SE was allowed to continue for 1 hr and then control and experimental animals were treated with diazepam (4 mg/Kg i.p.). Animals recovered from this initial treatment within 2 to 3 days and were observed for the next 3 weeks. Animals were monitored by video recording and by clinical observation to evaluate the development of seizure discharge. Seizures were scored on a scale from 1 to 5, as used for the scoring of kindled seizures as described by Racine, (1972). Over 80% of the animals were found to have recurrent partial and generalized seizures after 3-4 weeks after the initial pilocarpine injection. No seizures were observed in control animals. 24 days after pilocarpine treatment, the rats were continuously video monitored for 72 hrs. The behaviour and seizures were captured with a CCD camera and a Pinnacle PCTV capturing software card and stored in the hard disk of the computer. One trained technician, blind to all experimental conditions, viewed all videos. Seizure activity was rated as previously mentioned by Racine (1972). Seizures were assessed by viewing behavioural postures (i.e. lordosis, straight tail, jumping/ running, forelimb clonus and/or rearing during fast forward observation of the videos. Once a behavioural posture was observed the video was rewound to the beginning of the behaviour and examined at real-time speed.
Determination of Anti-Epileptic Potential of *Bacopa monnieri*

Experimental animals were divided into following groups

a) Group 1 Control (given saline injection)
b) Group 2 Epileptic
c) Group 3 Control rats treated with *Bacopa monnieri*
d) Group 4 Epileptic rats treated with *Bacopa monnieri*
e) Group 5 Epileptic rats treated with Carbamazepine

Animal Groups

The rats were initially divided into two groups- Control and Epileptic. The epileptic group was injected with pilocarpine according to the previously established protocols as described earlier (Reas *et al.*, 2008). The control group received saline instead of pilocarpine. The epileptic group showed spontaneous recurrent seizures approximately 20 minutes after pilocarpine injection. Those rats that did not show spontaneous seizures after pilocarpine treatment were excluded from the study group. The rats were singly housed and maintained for 24 days with standard food and water *ad libitum* after pilocarpine treatment. After 24 days the rats were subjected continuous video monitoring for 72 hrs. The behaviour and seizures were observed. Those experimental rats that did not show seizures were excluded from the study group. The experimental group was again divided into four. The first group that did not receive the treatment was epileptic. Carbamazepine was given orally to the third group of epileptic rats (150 mg/ Kg body weight/day). Extract of *Bacopa monnieri* was given orally to the third and fourth group of epileptic rats in the dosage of 300 mg fresh plant/Kg body weight/day for 15 days. After 15 days of treatment the rats were again subjected to continuous video monitoring for 72 hrs. The rats were sacrificed after the video observation.
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Estimation of Blood Glucose

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

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

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

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

The hydrogen peroxide formed in this reaction reacts with 4-aminoantipyrine and 4-hydroxybenzoic acid in the presence of peroxidase (POD) to form N-(4-antipyryl)-p-benzo quinoneimine. The addition of mutarotase accelerates the reactions. The amount of dye formed is proportional to the glucose concentration. The absorbance was read at 505nm in (Shimadzu UV-1700 pharmaSPEC) spectrophotometer.

Tissue preparation

Control and experimental rats were sacrificed by decapitation. The brain regions (cerebral cortex, cerebellum and brainstem) and body parts were dissected out quickly over ice according to the procedure of Glowinski & Iversen (1966). Hippocampus was dissected out quickly over ice according to the procedure of Heffner et al., (1980). 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.
Quantification of brain monoamines and their metabolites in the experimental groups of rats.

The monoamines were assayed according to the modified procedure of Paulose et al., (1988). The cerebral cortex, hippocampus, brain stem and Cerebellum (CB) 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 serotonin (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, 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.
5-HT$_{2C}$ RECEPTOR BINDING STUDIES USING [$^3$H] RADIOLIGANDS IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

**5-HT$_{2C}$ Receptor Binding Studies Using [$^3$H]mesulergine**

5-HT$_{2C}$ receptor assay was done using [$^3$H] mesulergine in the synaptic membrane preparations of brain regions were done as previously described by Herrick-Davis *et al.*, (1999). Crude synaptic membrane preparation was suspended in 50 mM Tris-HCl buffer, pH 7.4 and used for assay. In the saturation binding experiments, assays were done using different concentrations i.e., 0.05nM-3nM of [$^3$H] mesulergine was incubated with and without excess of unlabelled 100µM 5-HT. Tubes were incubated at 25°C for 60 min. and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing 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.

**NMDA RECEPTOR BINDING STUDIES USING [$^3$H] RADIOLIGANDS IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS**

**NMDA Receptor Binding Studies Using [$^3$H] MK-801**

The membrane fractions were prepared by a modification of the method described by Hoffman *et al.*, (1996). Cerebral cortex was homogenized in a 0.32 M sucrose buffer solution containing 10 mM HEPES/1 mM EDTA buffer, pH 7.0. The homogenate was centrifuged at 1,000 × g for 10 min and the supernatant was centrifuged at 40,000 × g for 1 h. The pellet was resuspended and homogenized in 10 mM HEPES buffer containing 1.0 mM EDTA, pH 7.0 and centrifuged at 40,000 × g for 1 h. The final pellet was suspended in HEPES/EDTA buffer and stored at -80°C until binding assays were performed. The [$^3$H] MK-801 binding saturation assay was performed in a concentration range of 0.25 to 50 nM at 23°C in an assay medium containing 10 mM HEPES, pH 7.0, 200 - 250 µg of protein, 100 µM glycine and 100 µM glutamate. After 1 h of incubation, the reaction was stopped by filtration through GF/B filters and washed with HEPES
buffer pH 7.0. Specific [³H] MK-801 binding was obtained by subtracting nonspecific binding in the presence of 100 µM unlabeled MK-801 from the total binding. Bound radioactivity was counted with cocktail-T in a Wallac 1409 a 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 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.

**GENE EXPRESSION STUDIES OF 5-HT2c, NMDA2b, mGlu5 and GLAST RECEPTOR INDIFFERENT BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS.**

**Preparation of RNA**

RNA was isolated from the different brain regions (cerebral cortex, hippocampus, cerebellum and brainstem) of control and experimental rats using the Tri reagent from Sigma Aldrich.
Materials and Methods

**Isolation of RNA**

Tissue (25-50 mg) homogenates were made in 0.5 ml Tri Reagent and was centrifuged at 12,000xg for 10 minutes at 4°C. The clear supernatant was transferred to a fresh tube and it was allowed to stand at room temperature for 5 minutes. 100µl of chloroform was added to it, mixed vigorously for 15 seconds and allowed to stand at room temperature for 15 minutes. The tubes were then centrifuged at 12,000xg for 15 minutes at 4°C. Three distinct phases appear after centrifugation. The bottom red organic phase contained protein, interphase contained DNA and a colourless upper aqueous phase contained RNA. The upper aqueous phase was transferred to a fresh tube and 250µl of isopropanol was added and the tubes were allowed to stand at room temperature for 10 minutes. The tubes were centrifuged at 12,000xg for 10 min at 4°C. RNA precipitate forms a pellet on the sides and bottom of the tube. The supernatants were removed and the RNA pellet was washed with 500µl of 75% ethanol, vortexed and centrifuged at 12,000xg for 5 min at 4°C. The pellets were semi dried and dissolved in minimum volume of DEPC-treated water. 2 µl of RNA was made up to 1 ml and absorbance were measured at 260nm and 280nm in spectrophotometer (Shimadzu UV-1700 pharmaSPEC). 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 an 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 PCR analyses were conducted with gene-specific primers and fluorescently labelled Taqman probes of 5-HT2C (Rn00562748_m1), NMDA2b (Rn00561352_m1), mGlu5 (Rn00566628_m1) and GLAST (Rn00570130_m1). Endogenous control (β-actin) was labeled with a reporter dye (VIC). 12.5µl of TaqMan 2X Universal PCR Master Mix was taken and 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:

- 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 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 $\Delta\Delta CT$ 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 $\beta$-actin in the same samples ($\Delta CT = CT_{Target} - CT_{\beta\text{-actin}}$). It was further normalized with the control ($\Delta\Delta CT = \Delta CT - CT_{Control}$). The fold change in expression was then obtained ($2^{\Delta\Delta CT}$).

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

Brain tissues – cerebral cortex, hippocampus, cerebellum and brainstem 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 40,000xg for 15 min. 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 15 minutes and they were centrifuged at 2000 x g for 10 minutes at $4^\circ C$. The supernatant was aspirated.
out and the pellet was resuspended in water and incubated at room temperature for 10 minutes. 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, hippocampus, cerebellum and brainstem 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 40,000xg for 15 min and the supernatant was transferred to fresh tubes for cGMP assay using [3H]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 [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 resuspended 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 \[^{3}H\]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 90 minutes at 2 - 8°C. Ammonium sulphate was added to all tubes, mixed and allowed to stand for 5 minutes in ice bath. The tubes were centrifuged at 12000xg for 2 minutes at room temperature. The supernatant was aspirated out and the pellet was resuspended 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 Co/Cx on the Y-axis and cGMP concentration (pmoles/tube) on the X-axis of a linear graph paper. Co - the cpm bound in the absence of unlabelled cGMP; Cx - 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 - cerebral cortex, hippocampus, cerebellum and brainstem 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 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.

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

Separation of the protein bound cAMP from unbound nucleotide was 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 was 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, \([ ^3 \text{H} \text{cAMP} \text{ and binding protein} \) in case of standards; buffer, \([ ^3 \text{H} \text{cAMP} \text{ and binding protein for zero blank and unknown samples,} [ ^3 \text{H} \text{cAMP} \text{ and binding protein for determination of unknown samples. The mixture was incubated at 2°C for 2h. Cold charcoal reagent was added to the tubes and the tubes were immediately centrifuged at 12,000 \text{x g} \text{ for 2min at 2°C. Aliquots of the supernatant was immediately transferred to scintillation vials and mixed with cocktail-T and counted in a liquid scintillation counter (Wallac, 1409).} \)

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C₀/Cₓ is plotted on the Y-axis against picomoles of inactive cAMP on the X-axis of a linear graph paper, where C₀ is the counts per minute bound in the absence of unlabelled cAMP and Cₓ is the counts per minute bound in the presence of standard or unknown unlabelled cAMP. From the C₀/Cₓ value for the sample, the number of picomoles of unknown cAMP was calculated.

**Elevated Plus Maze**

The elevated plus-maze is a widely used animal model of anxiety that is based on two conflicting tendencies; the rodent’s drive to explore a novel environment and it’s aversion to heights and open spaces. Four arms were arranged in the shape of a cross. Two arms had side walls and an end wall ("closed arms") - the two other arms had no walls ("open arms"). The open arms were surrounded by small ledges to prevent the animal from falling from the maze. The maze was fastened to a light-weight support frame. Thus “anxious” animals spent most of the time in the closed arms while less anxious animals explored open areas longer. The control and experimental groups of rats were subjected to social interaction test during post-treatment period once daily for 15 days.

Rats were placed individually into the centre of elevated plus-maze consisting of two open arms (38L x 5W cm) and two closed arms (38L x 5W x 15H cm), with a central intersection (5cm x 5cm) elevated 50 cm above the floor. Behaviour was tested in a dimly lit room with a 40W bulb hung 60 cm above the central part of the maze. The investigator sitting approximately 2 metre apart from the apparatus observes and detects the movements of the rats for a total of 5 minutes. The experimental procedure was similar to that described by Pellow *et al.*, (1985). During the 5 minutes test period the following parameters were measured to analyze the behavioural changes of the experimental rats using elevated plus-maze: open arm entry, closed arm entry, percentage arm entry, total arm entry, time spent in open arm, time spent in closed arm, percentage of time spent in open arm, head dipping, stretched attend posture and grooming (Holmes & Rodgers, 1998). An entry was defined as entering with all four feet into one arm.
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 control and experimental groups of rats were subjected to social interaction test during post-treatment period once daily for 15 days. 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 to allow 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 15 days of treatment in all groups of rats.

Social Interaction

The control and experimental groups of rats were subjected to social interaction test during post-treatment period once daily for 15 days. In the social interaction test, as previously (Millan et al. 2005), described by two unfamiliar, weight-matched rats receiving the same treatment were placed in opposite corners for 10 min into a brightly-lit chamber (30 × 30 × 60 cm; width, length and height, respectively) with floor covered with wood shavings. The total time spent in active social behaviour - allogrooming, sniffing the partner, crawling under and over, following was recorded, for each rat separately.

Forced Swim Test

The control and experimental groups of rats were subjected to forced swim test during post-treatment period once daily for 15 days as reported in Porsolt et al. (1977) and Millan et al. (2001). In short, cylindrical glass tanks (50 cm tall X18 cm diameter), filled to a depth of 30 cm with 22 (±1) °C water and were used in the forced swimming test. Testing consisted of two phases, the induction phase and the test phase. During the induction phase animals were placed in the water for 15 min. After 24 h the rats are placed in the same tanks for
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5 min. The movements of the rats were videotaped for off-line measurement of the duration of immobility (seconds). The behavioural variable ‘immobility’ was defined as follows: making no movements for at least 2 seconds or making only those movements that were necessary to keep the nose above the water. The rats were allowed to slightly move their forepaws or support themselves by pressing their paws against the wall of the cylinder. Active climbing, diving and swimming along the wall were scored as mobility and the time taken was recorded in seconds.

Confocal Studies

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 (Chen et al., 2007). After perfusion the brains were dissected and immersion fixed in 4% paraformaldehyde for 1 hr and then equilibrated with 30% sucrose solution in PBS (0.1 M). 40 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 slices were were incubated overnight at 4 °C with either rat primary antibody for 5-HT2C (No: RA24505 Neuromics, diluted in PBST at 1: 500 dilution), NMDA2b (No: 610416 BD Biosciences, diluted in PBST at 1: 500 dilution), mGlu5 (No: AB7130F, Chemicon, diluted in PBST at 1: 500 dilution) (polyclonal or monoclonal). After overnight incubation, the brain slices were rinsed with PBST and then incubated with appropriate secondary antibody of either FITC (No: AB7130F, Chemicon, diluted in PBST at 1: 1000 dilution) or Rhodamine dye (No:AP307R Chemicon, diluted in PBST at 1: 1000 dilution). The sections were observed and photographed using confocal imaging system (Leica SP 5).

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.