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

6-hydroxydopamine, serotonin, γ-aminobutyric acid, glutamate, (+)MK-801[(+)-5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine maleate, apomorphine, amphetamine, bovine serum albumin fraction V, ethylene diamine tetraacetic acid (EDTA), Tris HCl, sucrose, Magnesium chloride, calcium chloride, HEPES - [n’ (2-hydroxy ethyl)] piperazine-n’-[2-ethanesulfonic acid], glycine, ascorbic acid, sodium dodecyl sulfate and paraformaldehyde were purchased from Sigma Chemical Co., St. Louis, USA. All other reagents were of analytical grade purchased locally from SRL, India.

Radiochemicals

L-[G-3H]Glutamic acid (Sp. Activity 49.0 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 [3H] IP3, [3H] cGMP and [3H] 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. NMDAR1 (Rn_00433800), NMDA2B (Rn00561352_m1), mGluR5 (Rn00566628_m1), GLAST (Rn00570130_m1), Bax (Rn 01480160_g1), CREB (Rn 00578826_m1), tumor necrosis factor-α (Rn99999017_m1), α-synuclein (Rn00569821_m1), tyrosine hydroxylase (Rn00562500_m1), Nestin...
(Rn00564394_m1), and Glial fibrillary acidic protein (Rn00566603_m1) primers were used for the gene expression studies using real time PCR.

**Confocal Dyes**

Rat primary antibody for NMDAR1 (No: 556308, BD Pharmengin™), NMAD2B (No: 610416, BD Pharmengin™), mGlur5 (No: AB7130F, Chemicon), Nestin (No. MAB353, Chemicon), Glial fibrillary acidic protein (No. MAB360, Chemicon), tyrosine hydroxylase (No. 106K4865, Chemicon) and secondary antibody of either FITC (No: AB7130F, Chemicon), Rhodamine dye (No: AP307R Chemicon) and CY5 (No: AP124S, Chemicon) were used for the immunohistochemistry studies using confocal microscope. PKH2GL cell linker kit (No. 019K0671) from Sigma Chemical Co., St. Louis, USA used for tagging the bone marrow cells.

**Animals**

Adult male Wistar rats of 250-300g body weight were purchased from Kerala Agriculture University, Mannuthy and Amrita Institute of Medical Sciences, Kochi 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 guidelines and CPCSEA guidelines.

**Experimental design**

The experimental rats were divided into the following groups i) Control ii) 6-OHDA infused (6-OHDA) iii) 6-OHDA infused supplemented with Serotonin (6-OHDA + 5-HT) and iv) 6-OHDA infused supplemented with GABA (6-OHDA + GABA) v) 6-OHDA infused supplemented with BMC (isolated from rats on femur) (6-OHDA + BMC) vi) 6-OHDA infused supplemented with 5-HT and BMC (6-OHDA+5-HT+BMC) vii) 6-OHDA infused supplemented with GABA and BMC (6-OHDA+ GABA+BMC) viii) 6-OHDA infused supplemented with 5-HT, GABA and BMC (6-OHDA+5-HT+GABA+BMC). Each group consisted of 6-8 animals.
Rats were anesthetized with Chloryl Hydrate (400 mg/kg body weight, i.p.). The animal was placed in the flat skull position on a cotton bed on a stereotaxic frame (BenchmarkTM, USA) with incisor bar fixed at 3.5 mm below the interaural line and the coordinates of the striatum (Paxinos & Watson, 1982) were measured accurately as anteroposterior -4.68mm, lateral 3.10mm and dorsoventral 7.8mm relative to bregma and ventral from dura with the tooth bar set at 0 mm. 6-OHDA, 8μg in 1μl in 0.2% ascorbic acid, was infused into the right SNpc at a flow rate of 0.2μl/min. After stopping the infusion of the 6-OHDA, the probe was kept in the same position for a further 5 min for complete diffusion of the drug and then slowly retracted. All the groups except Control group were infused with 6-OHDA and in control animals, 1 μl of the vehicle, 0.2% ascorbic acid, was infused into the right SNpc. Proper postoperative care was provided till the animals recovered completely.

**Rotational behaviour**

Amphetamine-induced (5 mg/kg, i.p.) rotational behaviour was assessed as described earlier (Ungerstedt, 1971). Rats were tested with amphetamine on the 14th day after intranigral injection of 6-OHDA and with apomorphine (1 mg/kg, s.c.) on the 16th day. Animals that had completed a 360° circle towards the intact (contralateral) and the lesioned (ipsilateral) sides were counted for 60 min continuously and recorded separately. Animals that showed no significant contralateral rotations were excluded from the study.

**Treatment**

On the 18th day and Stereotaxic single dose of 1μl of 5-HT (10μg/μl), GABA(10μg/μl) and 10μl of Bone marrow cell (BMC) (10⁶ Cells/10 μl) suspension individually and in combinations were infused into the right SNpc at a flow rate of 0.2 μl/min into the respective groups. Bone marrow cells were collected from femurs with saline using a syringe with a No. 18 G needle. Cells were disaggregated by gentle pipetting several times. Cells were passed through 30-μm nylon mesh to remove
remaining clumps of tissue. Cells were washed by adding fresh saline, centrifuging for 10 min at 200g and removing supernatant. The cell pellet was resuspended in 1 ml of saline. Cell counting was done using haemocytometer.

**Tissue preparation**

All the control and experimental rats were sacrificed on the 30\textsuperscript{th} day by decapitation. The brain regions – corpus striatum, cerebral cortex, cerebellum and brain stem were dissected out quickly over ice according to the procedure of Glowinski and Iversen, (1966) and SNpc were micropunched according to Palkovits and Brownstein (1983). 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.

**Behavioural studies**

Animals were observed everyday for any overt abnormal activity. Body weight was recorded on the 18\textsuperscript{th} day before the treatment and on the 30\textsuperscript{th} day before decapitation. Apomorphine-induced (1 mg/kg, s.c.) rotational behaviour was accessed once again on the 30\textsuperscript{th} day.

**Forelimb use asymmetry test (cylinder test).**

The animals were evaluated in the forelimb use asymmetry test (cylinder test) (Schallert et al. 2000) 12 days after treatment. At each time point, the animals were placed in a Plexiglas cylinder (20-cm diameter × 30-cm high) elevated on a glass plate for a 3-minute period on 2 consecutive days. Testing was done during the dark phase of the cycle and under red lighting. The trials were videotaped from below and scored at a later date by an investigator blind to the animal’s treatment. Forelimb placements on the walls of the cylinder were categorized as left independent, right independent or simultaneous movements and a forelimb use asymmetry score was calculated as:

\[
\frac{\text{ipsi} + \frac{1}{2} \text{both}}{\text{ipsi} + \text{contra} + \text{both}} \times 100
\]
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Where ipsi and contra refer to the forelimbs, ipsilateral and contralateral refer to the 6-OHDA-induced lesion, respectively. Animals had to make greater than 20 movements at any given time point for their data to be included in the analysis.

Rotarod Test

Rotarod has been used to evaluate motor coordination by testing the ability of rats to remain on revolving rod (Dunham and 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 12 days of treatment in all groups of rats.

Swim-test

Swim-test was carried on the 30th day in water tubs (40 cm length×25 cm width×16 cm height). The depth of water was kept at 12 cm and the temperature was maintained at 27±2°C. The animals were wiped dry immediately after the experiment using a dry towel and returned to cages kept at 27±2°C. Swim-score scales were: 0, hind part sinks with head floating; 1, occasional swimming using hind limbs while floating on one side; 2, occasional floating/swimming only; 3, continuous swimming (Haobam et al., 2005).

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 ×30 cm length ×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 sawdust, 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. Data was calculated according to the number of visit to the novel arm during the five minutes of test (Akwa et al., 2001).

**Radial arm maze Test**

Radial maze behavioural 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
Materials and Methods

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. Performance was recorded during daily behavioural trials according to the terminology in previous studies (Leung et al., 1990). Entry into an unbaited arm was scored as a reference error and reentry into a baited arm was scored as a working error.

Quantification of Glutamate

Glutamate content in the brain regions – corpus striatum, cerebral cortex, hippocampus, cerebellum and brain stem of control and experimental rats were quantified by displacement method using modified procedure of Enna and Snyder, (1976). Tissues were homogenized in 20 volumes of 0.32 M sucrose, 10 mM Tris/HCl and 1 mM MgCl₂ buffer, pH 7.4, with a polytron homogenizer. The homogenate was centrifuged twice at 27,000 x g for 15 min. The supernatant was pooled and used for the assay. The incubation mixture contained 1 nM [³H] glutamate with and without glutamate at a concentration range of 10⁻⁹ M to 10⁻⁴ M. The unknown concentrations
were determined from the standard displacement curve using appropriate dilutions and calculated for nmols/g wt. of the tissue.

**Quantification of Dopamine**

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

DA contents was 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 0.22μm 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 detection. The peaks were identified by relative retention times compared with external standards and quantitatively estimated using an integrator (Empower software) interfaced with the detector.

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

**Glutamate Receptor Binding Studies Using [3H]Glutamate**

Membranes were prepared according to the modified method of Timothy et al., (1984). The brain regions - corpus striatum, cerebral cortex, hippocampus,
cerebellum and brain stem were homogenized in 20 volumes of 0.32 M sucrose, 10 mM Tris-HCl and 1 mM MgCl₂ buffer, pH 7.4, with a polytron homogenizer. The homogenate was centrifuged twice at 1,000 x g for 15 min at 4°C and the pellets were discarded. The supernatants were pooled and centrifuged at 27,000 x g for 15 min. The resulting pellet was lysed in a 10 mM Tris-HCl buffer, pH 7.4, for 30 min and centrifuged at 27,000 x g for 15 min. The resultant pellet was washed three times in 10 mM Tris/HCl buffer, pH 7.4, and centrifuged at 27,000 x g for 15 min. All steps were carried out at 4°C.

Membranes were incubated in 0.25 ml reaction mixture containing 25 mM Tris-HCl, pH 7.4, 5 mM MgCl₂ and 20 nM to 350 nM of [³H]Glutamate containing 0.2 mg to 0.3 mg protein concentrations. Incubation was carried out at 30°C for 15 min and the reaction was stopped by centrifugation at 27,000 x g for 15 min. The pellet and the wall of the tube were quickly and carefully washed with ice-cold distilled water. 0.1% SDS and scintillation fluid were added to the dry pellet and radioactivity incorporated was determined with a Wallac scintillation counter. All the assays were carried out in triplicate. Nonspecific binding was determined by adding 350 µM nonradioactive glutamate to the reaction mixture in a parallel assay. Specific binding was considered to be the difference between total and nonspecific binding.

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

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

The membrane fractions were prepared by a modification of the method described by Hoffman et al., (1996). The brain regions - corpus striatum, cerebral cortex, hippocampus, cerebellum and brain stem were 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 x g for 10 min and the supernatant was centrifuged at 40,000 x 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 10 mM HEPES, 1 mM EDTA buffer, pH 7.0 and stored at -80°C until binding assays were performed. The [³H] 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 thrice 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 ($B_{max}$) 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 DIFFERENT BRAIN REGIONS OF
CONTROL AND EXPERIMENTAL RATS

Preparation of RNA

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

Isolation of RNA

Tissue (25-50 mg) homogenates were made in 0.5 ml Tri Reagent and was
centrifuged at 12,000 x g for 10 minutes at 4°C. The clear supernatant was transferred
to a fresh tube and it was allowed to stand at room temperature for 5 minutes. 100μl
of chloroform was added to it, mixed vigorously for 15 seconds and allowed to stand
at room temperature for 15 minutes. The tubes were then centrifuged at 12,000 x g
for 15 minutes at 4°C. Three distinct phases appear after centrifugation. The bottom
red organic phase contained protein, interphase contained DNA and a colourless upper
aqueous phase contained RNA. The upper aqueous phase was transferred to a fresh
tube and 250 μl of isopropanol was added and the tubes were allowed to stand at room
temperature for 10 minutes. The tubes were centrifuged at 12,000 x g for 10 min at
4°C. RNA precipitated as a pellet on the sides and bottom of the tube. The
supernatants were removed and the RNA pellet was washed with 500 μl 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. 2 μl of RNA
was made up to 1 ml and absorbance was measured at 260 nm and 280 nm 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 1 OD at 260 = 42μg.
cDNA Synthesis

Total cDNA synthesis was performed using ABI PRISM cDNA Archive kit in 0.2 ml microfuge tubes. The reaction mixture of 20 μl contained 0.2 μg 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 MasterMIX (Applied Biosystems). The volume was made up with RNAselite free water. Each run contained both negative (no template) and positive controls. The thermocycling profile conditions were as follows:
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50ºC -- 2 minutes ---- Activation
95ºC -- 10 minutes ---- Initial Denaturation
95ºC -- 15 seconds ---- Denaturation 40 cycles
50ºC -- 30 seconds --- Annealing
60ºC -- 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 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}).

IP3 CONTENT IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

The brain regions - corpus striatum, cerebral cortex, hippocampus, cerebellum and brain stem were homogenised in a polytron homogeniser in 50 mM Tris-HCl buffer, pH.7.4, containing 1 mM EDTA to obtain a 15% homogenate. The homogenate was then centrifuged at 40,000 x g 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 100 µl 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°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:

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

NSB- non specific binding and Bo - zero binding. IP3 concentrations in the samples were determined by interpolation from the plotted standard curve.

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

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

**Principle of the assay**

cAMP assay kit was used. The assay was 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 was 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.

\[
\begin{align*}
\text{Free }[^3\text{H}]\text{ cAMP} & \quad + \quad \text{Binding protein} \\
\text{cAMP} & \quad = \quad + \\
\text{Bound }[^3\text{H}]\text{ cAMP-binding protein} & \quad \text{cAMP-binding protein}
\end{align*}
\]

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 samples were 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 and binding protein in case of standards; buffer, }[^3\text{H}]\text{cAMP and binding protein for zero blank and unknown samples, }[^3\text{H}]\text{cAMP and binding protein for determination of unknown samples. The mixture was incubated at 2°C for 2 hours. Cold charcoal reagent was added to the tubes and the tubes were immediately centrifuged at 12,000 x g for 2 min 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.

cGMP CONTENT IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

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

**Principle of the assay**

The assay was based on the competition between unlabelled cGMP and a fixed quantity of the \([^{3}H]cGMP\) for binding to an antiserum, which has a high specificity and affinity for cGMP. The amount of \([^{3}H]cGMP\) 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 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.
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**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 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 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 12000 x g 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 were determined by interpolation from the plotted standard curve.

**Bone marrow cell differentiation pattern studies using PKH2GL cell linker dye.**

BMC were tagged with PKH2GL cell linker dye according to the kit protocol Sigma Chemical Co., St. Louis, USA. Tagged BMC (10⁶ Cells/ 10µl) suspension was infused individually and in combination stereotactically into the right SNpc at a flow rate 0.2 µl/min to the respective groups. 10 µm brain sections were cut using Cryostat (Leica, CM1510 S). Brain slices were incubated overnight at 4°C with primary antibody for nestin (No. MAB353, Chemicon, diluted in PBST at 1: 500 dilution) and Glial fibrillary acidic protein (No. MAB360, Chemicon, diluted in PBST at 1: 500 dilution). After overnight incubation brain slices were incubated with the secondary antibody with CY5 (No: AP124S, Chemicon), diluted in PBST at 1: 1000 dilution). The sections were observed and photographed using confocal imaging system (Leica SP 5).
NMDAR1, NMDA2B, mGluR5 AND TYROSINE HYDROXYLASE
EXPRESSION STUDIES IN THE BRAIN REGIONS OF CONTROL AND
EXPERIMENTAL RATS USING CONFOCAL MICROSCOPE

Control and experimental rats were deeply anesthetized with ether. The rat was transcardially perfused with Phosphate buffered saline (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 0.1 M PBS, pH- 7.0. 20 µm 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 either rat primary antibody for NMDAR1 (No: 556308 BD Pharmengin™, diluted in Phosphate buffered saline Triton X- 100 (PBST) at 1: 500 dilution), NMDA2B (No: 610416 BD Pharmengin™, diluted in PBST at 1: 500 dilution), mGluR5 (No: AB7130F, Chemicon, diluted in PBST at 1: 500 dilution) and tyrosine hydroxylase (No. 106K4865, Chemicon, diluted in PBST at 1: 500). 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, Sigma Plot software (version 2.0, Jandel GmbH, Erkrath, Germany). Competitive binding data were analysed using non-linear regression curve-fitting
procedure (GraphPad PRISM\textsuperscript{TM}, San Diego, USA). Relative Quantification Software was used for analyzing Real-Time PCR results.