4.1 Animals

The studies were carried out on male Swiss Albino mice (22-28g weight) and were obtained from the breeder (Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana, India). Swiss Albino mice were housed in groups of 10 mice/cage at room temperature of 22±2 °C, under natural light/dark cycle and had free access to water and food (standard laboratory pellets) before the experiments. The mice were acclimatized at lab conditions for 5 days before the start of the experiment. Each experimental group consisted of 10 animals. All the experimental work had been carried out from 08:00 to 16:00 h. The experimental protocol was duly approved by the Institutional Animal Ethics Committee (IAEC) and the care of the animals was carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India vide protocol approval no. 107/99/CPCSEA/-2009-4.2.

4.2 Drugs and chemicals

Pentylenetetrazole, 5-HT1A receptor agonist: 8-OH-DPAT (8-Hydroxy-N,N-dipropyl-2-aminotetralin), 5-HT1A receptor antagonist: WAY-100,635; 5-HT2A/2C receptor agonist: m-CPBG [1-(3-Chlorophenyl)biguanide hydrochloride], DTNB, Griess reagent, 5-Hydroxytriptamine, Tacrine and Methanol, HPLC grade were procured from Sigma-Aldrich, Co. (St. Louis, MO, USA). Glutamate and GABA was purchased from Loba Chemie, Mumbai. Dopamine, β-mercaptoethanol, and o-phthalaldehyde were purchased from HiMedia, Mumbai. Norepinephrine and phenytoin, were received as gift samples from Troikaa Pharmaceuticals, Dehradun, Uttarakhand, India and Jackson Laboratories Ltd., India, respectively. 5-HT3 receptor antagonist: Ondansetron, sodium valproate and atropine were received as gift samples from Q. P. Pharmachem, Derabassi, India.

All other reagents were of the highest purity available and HPLC grade water was procured from our laboratory (Lab Pure, Bio Age, India) and filtered through Millipore Membrane (0.45 μm) filtered (Millipore, USA).

4.3 Induction of kindling

Pentylenetetrazole, dissolved in warm saline was injected intraperitoneally (i.p.), at a sub-convulsive dose of 35 mg/kg at 48±2 h intervals. After each pentylenetetrazole
injection the mice were placed in isolated transparent Plexiglas observation chamber, and their behavior was video monitored for 1 h. The intensity of the convulsions was registered according to a seven point scale (modified Racin’s Scale): Stage 0: no response; Stage 1: hyperactivity, restlessness and vibrissae twitching; Stage 2: head nodding, head clonus and myoclonic jerks; Stage 3: unilateral or bilateral limb clonus; Stage 4: forelimb clonic seizures; Stage 5: generalized clonic seizures with falling; Stage 6: hind limb extensor and death was considered as Stage 7.

Gradual transition in stages of convulsions appears with increasing number of pentylenetetrazole injections. The transition of the convulsion intensity from the 4th to 5th degree reflected the generalization of the convulsive activity, manifested by the clonic-tonic convulsion. Regular pentylenetetrazole administration was withdrawn after establishment of pentylenetetrazole kindling; appearance of clonic-tonic convulsions after three successive pentylenetetrazole administrations. Kindled mice retain their convulsive behavior for a long time (21 days after withdrawal of pentylenetetrazole injections). Later a single subconvulsive dose of pentylenetetrazole (35 mg/kg) can provoke clonic-tonic convulsion within this period.

### 4.4 Experimental Groups

In this study, group 1, 3, 5 and 7 served as naïve animals and were not kindled. While animals belonging to other groups were kindled using pentylenetetrazole and successfully kindled animals were included in the study. Animals showing resistance to kindling were excluded from the study. This study comprises of following groups belonging to different experiments.

**Group 1:** Naive (2 month age) (n = 10)

**Group 2:** PTZ-kindled (2 month age) (n = 10)

**Group 3:** Naive (6 month age) (n = 10)

**Group 4:** PTZ-kindled (6 month age) (n = 10)

**Group 5:** Naive (12 month age) (n = 10)

**Group 6:** PTZ-kindled (12 month age) (n = 10)

**Group 7:** Naive group (n = 10)

**Group 8:** Postictal PTZ-kindled group (10 ml/kg/day; i.p.; n = 10) [Vehicle Control]
Group 9: Interictal PTZ-Kindled group (10 ml/kg/day; i.p.; n = 10)

Group 10: Phenytoin *per se* treatment group (30 mg/kg/day; i.p.; n = 7)

Group 11: Sodium valproate *per se* treatment group (300 mg/kg/day; i.p.; n=8)

Group 12: Phenytoin + tacrine treatment group (0.3 mg/kg/day; i.p.; n=8)

Group 13: Phenytoin + atropine treatment group (10 mg/kg/day; i.p.; n=7)

Group 14: Phenytoin + tacrine + atropine treatment group (n=8)

Group 15: 8-OH-DPAT treatment group (1 mg/kg/day; s.c.; n=7)

Group 16: WAY-100635 treatment group (0.3 mg/kg/day; s.c.; n=8)

Group 17: WAY-100635 + 8-OH-DPAT treatment group (n=7)

Group 18: (-) DOI treatment group (1 mg/kg/day; s.c.; n=7)

Group 19: Olanzapine treatment group (2.5 mg/kg/day; s.c.; n=8)

Group 20: Olanzapine + DOI treatment group (n=7)

Group 21: m-CPBG treatment group (1 mg/kg/day; i.p.; n=7)

Group 22: Ondansetron treatment group (1 mg/kg/day; i.p.; n=8)

Group 23: Ondansetron + m-CPBG treatment group (n=7)

Table 4.1 Composition of different groups in different experiments

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Group Numbers</th>
<th>PTZ Challenged groups (after kindling)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment I</td>
<td>1-6</td>
<td>---</td>
</tr>
<tr>
<td>Experiment II</td>
<td>7-9</td>
<td>8</td>
</tr>
<tr>
<td>Experiment III</td>
<td>7, 8, 10, 11</td>
<td>8, 10, 11</td>
</tr>
<tr>
<td>Experiment IV</td>
<td>7, 8, 10, 12-14</td>
<td>8, 10, 12-14</td>
</tr>
<tr>
<td>Experiment V</td>
<td>7, 8, 15-23</td>
<td>8, 15-23</td>
</tr>
</tbody>
</table>

All the treatment groups were followed up to 20 days. Experimental design of each experiment has been detailed out in different chapters.

4.5 Behavioral Evaluations

The animals belonging to group 8-23 were challenged with subconvulsive dose of pentylenetetrazole (35 mg/kg; i.p.) on day 5, 10, 15 and 20 and their seizure severity
score was recorded. Once the locomotor activity of animal become normalized (our preliminary observations in actophotometer and open field test, suggested that locomotor activity of PTZ challenged animals become normalized after 2 h) the effect on learning and memory was evaluated using elevated plus maze and passive shock avoidance paradigm on day 0, 5, 10, 15 and 20.

4.5.1 Seizure Severity Score

After each PTZ challenging dose animals were observed for 30 minutes and scored for according to the seven point scale (modified Racin’s Scale) discussed in 4.3.

4.5.2 Learning and Memory

Learning and memory was evaluated using elevated plus maze model and passive shock avoidance paradigm.

A. Transfer Latency in Elevated Plus Maze Model

Spatial memory was evaluated using elevated plus maze on days 0, 10, 15 and 20 following the procedure previously standardized in our laboratory. The elevated plus maze for mice consisted of two open arms (16 cm × 5 cm) and two closed arms (16 cm × 5 cm × 12 cm) and maze was elevated to a height of 25 cm from floor.

Each mouse was gently placed at the distal end of an open arm of the apparatus facing away from the central platform and the time it took for the mice to move from the open arm to either of the enclosed arms (transfer latency) was recorded. The criterion of an animal’s entry into the enclosed arm was crossing with all four legs of an imaginary line separating the enclosed arm from the central space. After entering the enclosed arm, the mice were allowed to move freely in the maze regardless of open and enclosed arms for 20s. Then, the rat was returned to its home cage.

Retention was examined on day 5, 10, 15 and 20. To remove any confounding olfactory cues the maze was cleaned with alcohol–water solution after each mice.

B. Number of Mistakes and Step Down Latency in Passive Shock Avoidance Paradigm

For the evaluation of contextual fear memory, modified passive shock avoidance paradigm previously standardized in our laboratory was used. The apparatus consisted of a Plexiglas box (27 cm × 27 cm × 27 cm) with a grid floor (3 mm stainless steel rods set 8 mm apart), having a shock free zone (non-conducting platform 10 cm × 7
cm × 1.7 cm) in the center of the grid floor. Electric shock (20 V AC) was delivered to the grid floor.

Each mouse was trained to stay on the shock free zone for at least 120s, for this the animals were gently placed on the shock free zone, when the mouse stepped down placing all its paws on the grid floor, shocks were delivered for 15s. The process was repeated till the animal learned to stay on shock free zone for at least 120s and number of trials was counted on day 0.

The retrieval of learned task was evaluated by recording the changes in the number of mistakes and step down latency on day 5, 10, 15 and 20.

4.6 Neurochemical Estimations

In this study we analyzed amino acids (GABA and glutamate) and monoamines (noradrenaline, dopamine and serotonin) using HPLC-FD method. Total nitrite and acetylcholinesterase were estimated in brain using microplate reader method. These methods were first standardized in our laboratory and then it was used to analyze the neurochemical status of brain in various experimental models.

After behavioral evaluation on day 20 (4h after last pentylenetetrazole injection in postictal group) all animals were sacrificed by cervical dislocation and their brains were dissected to isolate different brain regions (cortex, hippocampus, cerebellum and brain stem). Isolated brain parts were weighed and subdivided into two equal portions. One portion was homogenized in ice cold 10% w/v (0.1M) perchloric acid and centrifuged at 14,000 g for 30 minutes at 4°C (REMI C-24BL, Cooling Centrifuge, REMI, India) and clear supernatants were used for estimation of amino acids (glutamate and GABA), monoamines (noradrenaline, dopamine and serotonin). While second portion was homogenized in ice cold 10% w/v (0.05M, pH 7.4) phosphate buffer and centrifuged at 6000 g for 20 minutes at 4°C and clear supernatant was used for estimation of total nitrite level and acetylcholinesterase activity.

4.6.1 Estimation of Glutamate and GABA by HPLC-FD Method

In this study the rapid estimation method of glutamate and GABA was developed. The method is based on the conventional derivatization technique which involves most commonly used derivatizing agent o-phthalaldehyde. o-phthalaldehyde reacts
with primary amines in the presence of thiol and generates derivatives which are both electroactive and fluorescent. The methodology is explained in detail as follows.

**HPLC Equipment and Conditions:** The Waters HPLC system (Milford, USA) consisted of 515 binary pumps (Waters, USA), 2475 Fluorescent detector (Waters, USA) and Rheodyne manual injector (20 μL). The chromatographic separation was achieved on reversed-phase analytical column (150 mm x 4.6 mm i.d., 5 μm; Agilent, USA). Chromatographic analyses were performed at room temperature. The data were acquired and processed in the Empower Pro® Operating System (Waters®, Milford, USA).

**Mobile Phase:** The mobile phase consisted of 100 mM disodium hydrogen phosphate and methanol in 60: 40 ratio. The pH of the mobile phase was maintained to 6.70 ± 0.01 using o-phosphoric acid and then filtered through a 0.45 μm membrane (Millipore, USA) and degassed (Transsonic T 570/H, Elma, Germany). The flow rate was set to 1 mL/min.

The stock derivatizing solution was prepared by dissolving 27 mg of o-phthalaldehyde in 1 ml of HPLC grade methanol. 5 μL of β-mercapto ethanol was added to this solution and volume made up to 10 ml using 0.1M sodium tetraborate (pH 9.3). For the preparation of working derivatizing solution, 2.5 mL of the stock derivatizing solution was mixed with 7.5 ml of 0.1M sodium tetraborate buffer. This solution was prepared and utilized freshly. The derivatization was performed by mixing 100 μL sample or standard solutions with 100 μL of working derivatizing solution and was vortex for a minute. The resulting solution was filtered through a sample filter (0.45 μm, Millipore, USA) and injected to HPLC.

One milligram per milliliter stock solutions of GABA/glutamate standards were prepared in HPLC grade water, aliquoted out and stored at -20°C. The working standard solutions (10, 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 ng/mL) were prepared by further dilution of stock solution and used for the preparation of the standard glutamate and GABA curve plot. The standard curve of glutamate and GABA was plotted using these concentrations and corresponding area under the curve.
4.6.2 Estimation of Noradrenalin, Dopamine and Serotonin by HPLC-FD Method

Estimation of the monoamines in the brain samples was done using HPLC-FD method standardized in our laboratory. The modified methodology is explained in detail as follows.

**HPLC Equipment and Conditions:** The Waters HPLC system (Milford, USA) consisted of 515 binary pumps (Waters, USA), 2475 Fluorescent detector (Waters, USA) and Rheodyne manual injector (20μL). The chromatographic separation was achieved on reversed-phase analytical column (150 mm x 4.6 mm i.d., 5 μm; Agilent, USA). Chromatographic analyses were performed at room temperature. The data were acquired and processed in the Empower Pro® Operating System (Waters®, Milford, USA).

**Mobile Phase:** The mobile phase consisted of sodium acetate (0.02 M), methanol (16%), heptane sulfonic acid (0.055%), EDTA (0.2 mM), and dibutylamine (0.1%, v/v). The solution was adjusted to pH 3.92 ± 0.01 with o-phosphoric acid and filtered through a 0.45 μm membrane (Millipore, USA) and degassed (Transsonic T 570/H, Elma, Germany). The flow rate was set to 1 ml/min.

Stock solutions of standards (1 mg/mL) were prepared in 0.1N hydrochloric acid and was stored at -20°C and utilized within one month of preparation. The working standard solutions (10, 20, 30, 40, 50, 60, 70, 80 and 90 ng/mL) were prepared freshly in 0.1 M Perchloric acid. For the sample preparation the supernatant of brain homogenates were filtered through 0.45 μm membranes and injected into HPLC column.

4.6.3 Estimation of Nitrite Level using Microplate Method

Nitrite is the stable end product of nitric oxide in vitro systems. Accumulation of nitrite was measured in cell-free supernatants from brain homogenate by microplate reader method. The method involves the use of the Griess diazotization reaction to spectrophotometrically detect nitrite formed. In this method 50μl of the brain homogenate (filtered)/standard (NaNO₂) was mixed with 50μl of the Griess reagent using 96 well plate. The plate was shaken at 150 shakes per minute for one minute to ensure the proper mixing of samples/standard with Griess reagent. The plate was incubated for 30 minutes at room temperature. The absorbance of nitrite containing
samples were measured at 540 nm using Microplate Reader (APR-4 Microplate Reader, Logotech, ISE Group, Germany) against the photometric reference (Blank: 50µl HPLC grade water + 50µl Griess Reagent).

**4.6.4 Estimation of Brain Acetylcholinesterase Activity**

For the estimation of brain acetylcholinesterase activity, the method previously described by Ellman and his colleagues (Ellman et al., 1961) with slight modification was adopted. Briefly, 40µl of filtered brain homogenate was mixed with 80µl of Ellman Reagent (39.6 mg DTNB and 15 mg NaHCO₃ dissolved in 10 ml 50mM phosphate buffer pH 7.4) and 200µl of 50mM phosphate buffer (pH 8) in 96 well plate and shaken properly. The absorbance of the reaction mixture was recorded prior to addition of substrate at 412nm. The reaction was initiated by adding 10µl of the enzyme substrate (10mM Acetylthiocholine iodide) to each well and was allowed to incubate for 15 minute. Yellow color developed and the solution was read at 412nm using Microplate Reader (APR-4 Microplate Reader, Logotech, ISE Group, Germany).

A molar extinction coefficient of 14150 M⁻¹Cm⁻¹ was used to calculate enzyme activity. Enzyme activity was expressed as μM of acetylcholine hydrolyzed per milligram of wet tissue.

**4.7 Results of Standardization of Neurochemical Estimation**

**4.7.1 Estimation of Amino Acids**

Glutamate/GABA was detected with at the excitation wavelength 345 nm and an emission wavelength of 442 nm and their peaks were identified by comparing their retention time in the sample. In this method glutamate eluted first at 2.7 min while GABA eluted later at 13.6 min. Application of different standard concentrations of glutamate/GABA provides a straight line equation and amount of glutamate/GABA was quantified using area under the peak of corresponding sample using their straight line equation. The straight line equation was derived (Glutamate: \( y = 44187x + 1E+06, R^2 = 0.9987 \) and GABA: \( y = 219217x - 6E+06; R^2 = 0.9964 \); where y: area under peak and x: concentration in ng/mL) and utilized further to determine the different concentration of glutamate and GABA in the samples. The results were expressed as ng/g of wet tissue.
4.7.2 Estimation of Monoamines

Norepinephrine, dopamine and serotonin were detected at the excitation wavelength 280 nm and an emission wavelength of 315 nm. Monoamine peaks were identified by comparing their retention time in the sample. Application of different standard concentrations of norepinephrine, dopamine and serotonin provides a straight line equation and amount of norepinephrine, dopamine and serotonin was quantified using area under the peak of corresponding sample using their straight line equation. The straight line equation was derived (NA: $y = 124373x + 255920$, $R^2 = 0.9981$; DA: $y = 70355x + 113535$; $R^2 = 0.9938$; 5-HT: $y = 151725x - 446462$; $R^2 = 0.997$; where $x$: concentration in ng/ml and $y$: area under peak) and utilized further to determine the different concentration of norepinephrine, dopamine and serotonin in the samples. The results were expressed as ng/g of wet tissue.

Figure 4.1 Chromatogram showing elution of Glutamate and GABA using HPLC-FD Method
Chapter 4

Psychoneuropharmacological Investigations to Reveal Pathobiology of Cognition Deficit in Epilepsy

Figure 4.2 Standard Plot of Glutamate

$y = 44187x + 1E+06$

$R^2 = 0.9987$

Figure 4.3 Standard Plot of GABA

$y = 219217x - 6E+06$

$R^2 = 0.9964$
Figure 4.4 Chromatogram showing elution of Norepinephrine, Dopamine and Serotonin using HPLC-FD Method

Figure 4.5 Standard Plot of Norepinephrine

\[
y = 124373x + 255920 \\
R^2 = 0.9981
\]
Chapter 4

Psychoneuropharmacological Investigations to Reveal Pathobiology of Cognition Deficit in Epilepsy

Figure 4.6 Standard Plot of Dopamine

$y = 70355x + 113535$
$R^2 = 0.9938$

Figure 4.7 Standard Plot of Serotonin

$y = 151725x - 446462$
$R^2 = 0.997$
4.7.3 Estimations of Total Nitrite Content

The concentration of total nitrite was estimated using the straight line equation of nitrite \( y = 0.0008x + 0.0041; \ R^2 = 0.9916; \) where \( x: \) concentration in ng/ml and \( y: \) area under peak \) drawn against standard solution of sodium nitrite at 5, 10, 15, 20, 25 and 30 ng/ml. The results were expressed as ng/g of wet tissue.

![Figure 4.8 Standard Plot for Total Nitrite](image)

4.7.4 Estimation of Acetylcholinesterase Activity

For the estimation of acetylcholinesterase activity in the brain samples the absorbance of sample before and after Ellman reaction was taken and acetylcholinesterase activity was calculated using following formula. Enzyme activity was expressed as \( \mu M \) of acetylcholine hydrolyzed per milligram of wet tissue.

\[
\text{AChE Activity} = \frac{\Delta OD \times \text{Concentration of homogenate}}{\text{Molar Extinction coefficient} \times \text{Volume of sample}}
\]

Where,

\( \Delta OD = \) difference in absorbance before and after adding substrate,

\( \text{Concentration of homogenate} = 10 \ \text{mg/ml} \)

\( \text{Molar Extinction Coefficient} = 14150 \ \text{M}^{-1}\text{cm}^{-1} \)
Volume of sample = 40 µl

Thus different methods were developed for the estimation of different neurotransmitter in small volume of brain samples obtained in further experiments. The preparation of different homogenate for the estimation of different neurotransmitters has been detailed further.

4.8 Preparation of Drugs and Solutions

**Preparation of Pentylentetrazole (35 mg/kg; 3.5 mg/ml)**

35 mg of Pentylentetrazole was dissolved warm saline and volume made up to 10 ml with normal saline (0.9% w/v).

**Preparation of Phenytoin (30 mg/kg; 3 mg/ml)**

30 mg of Phenytoin Sodium was dissolved and volume made up to 10 ml with normal saline.

**Preparation of Sodium Valproate (300 mg/kg; 30 mg/ml)**

300 mg of Sodium Valproate was dissolved and volume made up to 10 ml with normal saline.

**Preparation of Tacrine (0.3 mg/kg; 0.03 mg/ml)**

3 mg of Tacrine was dissolved and volume made up to 10 ml with normal saline and kept as stock. Further 1 ml of this stock was diluted up to 10 ml with normal saline.

**Preparation of Atropine (10 mg/kg; 1 mg/ml)**

10 mg of Atropine was dissolved and volume made up to 10 ml with normal saline.

**Preparation of 8-OH-DPAT (1 mg/kg; 0.1 mg/ml)**

1 mg of 8-OH-DPAT was dissolved and volume made up to 10 ml with HPLC grade water.

**Preparation of WAY-100,635 (0.3 mg/kg; 0.03 mg/ml)**

3 mg of WAY-100,635 was dissolved and volume made up to 10 ml with HPLC grade water and kept as stock. Further 1 ml of this stock was diluted up to 10 ml with HPLC grade water.
**Preparation of R (-) DOI (1 mg/kg; 0.1 mg/ml)**

1 mg of R (-) DOI was dissolved and volume made up to 10 ml with HPLC grade water.

**Preparation of Olanzapine (2.5 mg/kg; 0.25 mg/ml)**

2.5 mg of Olanzapine was dissolved in minimum amount of 0.1 N hydrochloric acid and volume made up to 10 ml with normal saline. pH of final solution was adjusted to 6.65 with sodium hydroxide.

**Preparation of m-CPBG (1 mg/kg; 0.1 mg/ml)**

1 mg of m-CPBG was dissolved and volume made up to 10 ml with HPLC grade water.

**Preparation of Ondansetron (1 mg/kg; 0.1 mg/ml)**

1 mg of Ondansetron was dissolved and volume made up to 10 ml with HPLC grade water.

**Preparation of NaNO₂/NaNO₃ Standard**

1 mg NaNO₂ and 1 mg NaNO₃ was dissolved and volume made up to 1 ml with HPLC grade water to produce 1mg/ml stock solution of standard and different dilutions of standard were made to plot standard curve.

**Preparation of 1% Phosphoric Acid (100ml)**

1 ml of Phosphoric Acid (H₃PO₄) was added to HPLC grade water and volume made up to 100ml.

**Preparation of 0.1M Phosphate Buffer (pH 7.4, 300ml)**

**Solution A:** 5.22 g of K₂HPO₄ and 4.68 g of NaH₂PO₄ was dissolved and volume made up to 150 ml with HPLC grade water.

**Solution B:** 6.2 g NaOH was dissolved and volume made up to 150 ml with HPLC grade water.

Solution B was added to solution A to get pH 7.4 and then finally volume was made up to 300 ml using HPLC grade water.
Preparation of Ellman Reagent (10 mM DTNB)

39.6 mg of 5, 5’-dithiobis-(2-nitrobenzoic acid) with 15 mg NaHCO₃ was dissolved and volume made up to 10 ml with 0.1 M Phosphate Buffer.

Preparation of Acetylthiocholine iodide (10 mM)

28.9 mg of Acetylthiocholine iodide was dissolved and volume made up to 10 ml with HPLC grade water.