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MATERIALS AND METHODS

3.1 Chemicals

All the chemicals used in present study were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Himedia (Mumbai, India), Merck (Mumbai, India), Sisco Research Laboratories (Mumbai, India), Bio-Rad Laboratories (Hercules, CA, USA), Rankem (Bangalore, India). Pentylenetetrazole (PTZ) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and curcumin was purchased from Himedia. Anti-GFAP (glial fibrillary acidic protein) antibody used for western blotting was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Iba-1 (ionized calcium-binding adapter molecule 1) antibody used for western blotting was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Iba-1 and GFAP antibody used for immunohistochemical staining were obtained from Wako chemicals (Osaka, Japan) and Dako Corp., (Carpinteria, CA, USA) respectively. ELISA kits (OptEIA Set with specific antibody) for TNF-α, IL-1β, IL-6 were purchased from BD Biosciences, (San Jose, CA, USA) and MCP-1 kit was purchased from Ray Biotech Inc., (Norcross, GA, USA). cDNA synthesis kit used was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Pre-stained protein markers and PVDF membrane were obtained from Bio-Rad Laboratories (Hercules, CA, USA) and oligonucleotide primers for GFAP, Iba-1, TNF-α, IL-1β, IL-6, MCP-1, MMP-9, MMP-2 were procured from Invitrogen Corporation, Carlsbad, CA, USA and Integrated DNA Technologies (Coralville, Iowa, USA).

3.2 Animals

Adult male Wistar rats weighing 220-250 g were procured from the Central Animal House facility of Panjab University. The animals were housed under standard housing conditions. They were given free access to standard pellet diet (Ashirwad Industries, Ropar, Punjab, India) and water ad libitum throughout the treatment paradigm. The procedures followed were approved by the Institutional Animal Ethics Committee (IAEC) and were in accordance with ethical Guidelines for humane use and care of laboratory animals (978/08-01-2010).
3.3 Drug and treatment schedule

Animals were randomly segregated into following four groups with each group comprising minimum of seven animals. The schematic representation of the treatment paradigm is provided in Figure 3.1.

**Control:** Animals were administered with vehicle (saline) daily for the duration of treatment.

**PTZ:** Animals were administered PTZ intraperitoneally (dissolved in saline) at a dose of 40 mg/kg every alternate day for a period of 30 days and a single dose of PTZ on day 40 (challenge dose) was administered to ensure kindling success.

**PTZ + Curcumin:** Animals were administered with curcumin daily 30 min before PTZ injection at a dose of 100 mg/kg orally for 40 days and PTZ was administered only for 30 days. A single dose of PTZ was administered on day 40 similarly as given in PTZ group to ensure kindling success.

**Curcumin:** Animals were administered with curcumin (suspended in 1% carboxymethyl cellulose in distilled water) daily at a dose of 100 mg/kg body weight, orally for 40 days.

![Figure 3.1: Schematic diagram indicating the treatment paradigm](image)

3.3.1 Kindling Procedure

PTZ, a GABA<sub>A</sub> antagonist is commonly used as a convulsant agent, administration (repeatedly) in sub-convulsive dose for a specific period of time, slowly results in development of kindled model of epilepsy, also referred to as chronic epilepsy (Hansen et al., 2004). Kindling was induced in rats with repeated administration of sub-convulsive
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dose of PTZ 40 mg/kg body weight in volume of 1 ml/kg every alternate day for 30 days. After every PTZ injection, each animal was placed separately in a cage and the occurrence of the seizures was observed for a period of 25 to 30 min. Seizures were classified according to the Racine scale where score 0 indicates no behavioral changes; score 1 indicates facial movements, ear and whisker twitching; score 2 indicates myoclonic convulsions without rearing; score 3 indicates myoclonic convulsions with rearing; score 4 indicates clonic-tonic convulsion; score 5 indicates generalized clonic-tonic seizures with loss of postural control (Racine, 1972).

The success of kindling was assessed by giving a challenge dose of PTZ (40 mg/kg), 10 days after the last PTZ injection (on day 40) and seizures were scored. Many investigators have used a similar model to evaluate the process of epileptogenesis and its associated cognitive deficits (Kumar et al., 2013). The PTZ + curcumin group was administered PTZ for 30 days followed by a single dose of PTZ to access the success of kindling as done in PTZ treated group. Rats that fulfilled the kindling criteria and had tonic-clonic seizures following challenge dose were included in the study. The cumulative kindling score was calculated for each group.

3.4 Neurobehavioral Studies

After the completion of treatment, animals in each group were subjected to various neurobehavioral tests for assessing the locomotor activity (Actophotometer), motor coordination (Rotarod), anxiety and spatial memory (Elevated plus maze), exploratory behavior, learning and memory functions (Active avoidance and passive avoidance tasks, Morris water maze, Y maze). Behaviors on mazes were recorded by computer-based video tracking system (ANY-Maze, Stoelting, Wood Dale, IL, USA).

3.4.1 Rotarod

The rotarod test was used to evaluate the motor co-ordination of rats according to the method of Costanzo et al., (2002). The apparatus consisted of a metal rod of 3 cm diameter and 75 cm length attached to a motor with speed adjustment knobs (Figure 3.2). The rats were trained to stay on the rotating rod (12 rpm speed) for 3 min. After 40 days of treatment each animal was taken individually to the rotarod treadmill rotating at 12 rpm and
the time spent on rotating rod was recorded for each animal in different groups. The apparatus records the time and stops counting when the rat falls off the rotating rod and the maximum time allocated to each animal was 180 s per trial. Latency (time) to fall off the rotating rod was recorded. Neurological deficits were evaluated by the inability of the animal to remain (stay) on the rotating roller for the test period i.e. 180 s.

![Rotarod Apparatus for assessing motor co-ordination](image)

**Figure 3.2: Rotarod Apparatus for assessing motor co-ordination**

### 3.4.2 Elevated plus maze (EPM) for spatial learning and memory

Spatial long term memory was assessed according to the method of Itoh et al., (1991). Transfer latency (TL) is the time taken by the animal to move from an open arm to the enclosed arm and was utilized as an index of learning and memory process. EPM apparatus consisted of two open arms and two closed arms extended from a central platform. The apparatus was kept at a height of 50 cm above the ground (Figure 3.3). Animals were trained for three days before starting experiments. The rats that failed to enter enclosed arm within 90 s after training were excluded from the study. After completion of training, i.e. on day first, each animal was placed individually at the end of either of the open arm and the transfer latency was recorded. The animal was allowed to explore the maze for 20 s and then returned to home cage. On day second, the rat was placed again on the maze and TL was recorded. The retention session or transfer latency was recorded for all the animals.
after 40 days treatment and before giving challenge dose of PTZ. Percentage retention was calculated using the formula

\[
\frac{\text{Transfer latency day 1st} - \text{transfer latency day 2nd}}{\text{Transfer latency day 1st}} \times 100
\]

3.4.3 Elevated plus maze for anxiety

Anxiety was measured using elevated plus maze according to method of Walf and Frye, (2007). The EPM apparatus used for assessing the anxiety related behavior was the same used for assessing spatial learning and memory. This neurobehavioral task was initiated by placing the each animal individually on the central platform of the maze, facing one of the open arms, and allowing it to explore the apparatus freely for 180 s. The anxiety was assessed in terms of total time spent in open arm and closed arms along with the number of entries in open arm and closed arm were recorded using computer-based video tracking system.

3.4.4 Actophotometer

The locomotor activity was measured by using actophotometer (Instruments Manufacturing Corporation, Ambala, India). This instrument consisted of infrared beams located above the floor of testing chamber and the interruption of beams by the moving animal causes beam
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break which was recorded as photobeam counts (Figure 3.4). Each animal was placed individually in plexiglass chamber (actophotometer) and the number of counts was recorded over a period of 5 min and was expressed as counts per 5 min.

Figure 3.4: Actophotometer for assessing the locomotor activity

3.4.5 Active avoidance

Active avoidance task was performed according to the method described by Kamboj and Sandhir, (2007). The apparatus consisted of two way shuttle box equipped with a stainless steel grid floor that is divided into two equal sized chambers (dark and lit compartment) with an opening in the middle having enough space for animal to pass through (Figure 3.5). Both the chambers were interconnected to each other. Acoustic and visual stimulators in the form of buzzer and light were supplied.

Figure 3.5: Active avoidance apparatus
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Each animal was kept inside shuttle box for 15 min for habituation one day before starting the task during which the animal was allowed to move freely. During learning session, the animal was placed in lit compartment where it learnt to enter the safe compartment after experiencing light and sound stimulus for 10 s each (conditioned stimulus) and anticipating an electric foot shock (Un-conditioned stimulus). A total of 10 trials were given to every animal in a day. The number of trials in which the animal responded only to un-conditioned stimulus was recorded as escape trials.

3.4.6 Passive avoidance

Passive avoidance task was performed to evaluate the learning and long-term memory in animals as described by Uzum et al., (2010). The apparatus consisted of two chambers (a dark and one illuminated chamber) with a stainless steel grid floor to deliver foot-shocks (Figure 3.6). The rat was placed in the illuminated compartment and had free access to all parts of the apparatus for 300 s for habituation (Figure 3.6).

![Figure 3.6: Passive avoidance apparatus for assessing learning and long-term memory](image-url)

One day after the last PTZ injection, the animal was placed in the illuminated chamber and allowed to explore for 30 s. After 30 s, the door between two chambers was lifted up and thereafter its latency to enter the dark compartment was recorded. The interval between placement of the rat in the light chamber and its entry into the dark chamber was measured as entrance latency. Immediately, the door was closed once animal entered the
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A dark compartment and a single electric foot shock (65 V AC, 50 Hz) for 5 s was delivered. Both the consolidation and long-term memory was assessed.

One day after learning session, the animal was placed in the illuminated chamber and the latency to re-enter the dark chamber was measured. This is known as consolidation memory. The same procedure was used for the long-term memory test which was performed three days after the learning/training session. Foot shocks were not given during the retention tests. Rats that did not enter the dark compartment during the given 5-min period were removed from the apparatus and their latency was considered to be 300 s.

3.4.7 Morris water maze

The spatial learning and memory was evaluated using Morris water maze task (Morris et al., 1982). Morris water maze apparatus consists of an open black circular pool (140 cm in diameter, 60 cm deep) which was filled with water (25 ± 1°C) to a depth of 40 cm and a platform submerged 2 cm beneath the water surface (Figure 3.7). This test relies on distal cues to navigate from start locations around the perimeter of an open swimming arena to locate a platform which is submerged in water. To track the animal and monitor (record) the swimming parameters, a camera with computerized tracking system (ANY-Maze, Stoelting, Wood Dale, IL, USA) was mounted on ceiling above the pool.

On the day before training, animals were placed in the pool with no escape platform present and allowed to swim for 90 s to habituate them to the apparatus. Six days training was given to each animal. A trial began by placing the animal gently into the maze facing towards the wall of the tank. The animal was released in the quadrant opposite to where the platform was placed and was allowed to locate the hidden platform. If the animal did not find platform within 90 s, it was gently guided/directed to the platform and was allowed to rest there for 20 s. After that, the animal was removed from the apparatus and subjected to next training. The time taken by animal to find the hidden platform (escape latency) and swimming path followed in the pool was recorded before starting the dosing (baseline) and on day 39 (final day).

Probe trial: A probe trial was carried out on next day after recoding the final readings i.e. end of dosing (40th day). This was done to assess the spatial memory in which the platform
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was removed from the pool. Each rat was placed at the same start position and the parameters were recorded that includes the time spent in the target quadrant, swim distance to the target quadrant and total number of crossings in the quadrant where the platform had been located.

Figure 3.7: Schematic diagram of Morris water maze apparatus with video tracking system.

3.4.8 Y-maze

The Y-maze apparatus was used to assess the exploratory behavior and the working memory impairment in animals. The test was performed according to the method described by Jung et al., (2008). The apparatus consists of three arms, start arm, novel arm and familiar arm with equal length (start arm: 30 cm long, 10 cm long wide and 20 cm high). All three arms were at an angle of 120° to each other, radiating from the central point (Figure 3.8). This behavioral test comprised of two trials; during first trial one of the arms of the maze was blocked (named as novel) allowing exploration of other two arms (start arm and familiar arm) for few mins. After 1 hr, second trial was given in which the animal had access to all three arms for 120 s. It is a normal tendency of an animal with novel
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recognition memory to prefer/explore the third unexplored arm (novel arm, previously blocked) whereas the animals with impaired spatial memory prefer already explored arms which is used as indicator for spatial recognition memory. The number of entries and total distance travelled in each arm was recorded using computerized tracking system (ANY-Maze, Stoelting, Wood Dale, IL, USA).

Figure 3.8: Y-maze apparatus to assess working memory

3.5 Necroscopy and Tissue homogenization

After the completion of treatment paradigm and neurobehavioral assessment, the animals were sacrificed by cervical dislocation. The hippocampus and cortex were dissected, rinsed in ice cold saline (0.9% w/v NaCl), blotted dry and stored at -80°C for further analysis. A 10% (w/v) tissue homogenate was prepared in 50 mM phosphate buffered saline (pH 7.4) using potter-Elvehjem type glass homogenizer. The homogenate was centrifuged at 1,000 g for 10 min at 4°C to remove nucleus and unbroken cells. The pellet was discarded and a portion of supernatant was again centrifuged at 12,000 g for 20 min to obtain post mitochondrial supernatant. Various biochemical assays were performed in the homogenate and post-mitochondrial supernatants.
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3.6 Biochemical Estimations

The following biochemical parameters were studied to assess the role of oxidative stress in chronic model of epilepsy and the anti-oxidant potential of curcumin.

3.6.1 Lipid peroxidation

Lipid peroxidation, was estimated according to the method of Ohkawa et al., (1979) in the hippocampus and cortex. This method is based on the formation of malondialdehyde (MDA), a byproduct of lipid peroxidation which on reaction with thiobarbituric acid (TBA) forms MDA-TBA chromophore which is measured by spectrophotometer at 532 nm.

In this method, appropriate amount of homogenate was added to Tris-HCl buffer (pH 7.4) and the contents were incubated at 37°C for 2 hr. Following incubation, ice cold 10% (w/v) trichloroacetic acid (TCA) was added to it and mixed properly and centrifuged at 2,000 g for 10 min. Supernatant obtained was mixed with 0.67% (w/v) TBA and the tubes were kept in boiling water bath for 10 min or till the pink color appeared. The absorbance was read at 532 nm using spectrophotometer and the results were expressed as nmoles MDA/mg protein using molar extinction coefficient of MDA-TBA chromophore (1.56 x 10^5 M^-1 cm^-1).

3.6.2 Superoxide dismutase (SOD EC 1.15.1.1)

Cu, Zn-superoxide dismutase activity was measured spectrophotometrically according to the method described by Kono, (1978). The activity was measured by monitoring the rate of inhibition of nitroblue tetrazolium (NBT) reduction to blue formazan mediated by superoxide anions under aerobic conditions. SOD inhibits the reduction of NBT mediated by hydroxylamine hydrochloride. Enzymatic activity of SOD is measured by the extent of inhibition.

\[
2O_2^- + 2H^+ \xrightarrow{\text{Superoxide dismutase (SOD)}} H_2O_2 + O_2
\]

In a cuvette, solution A [96 μM NBT prepared in buffer containing 50 mM sodium carbonate and 0.1 mM EDTA (pH 10.8)] and solution B [0.6% (w/v) Triton X-100 prepared in buffer containing 50 mM sodium carbonate and 0.1 mM EDTA (pH 10.8)]
were added. Reaction was initiated by adding solution C [20 mM hydroxylamine hydrochloride (pH 5.0)] along with appropriate amount of test sample. The development of blue color complex in the reaction and change in absorbance was measured at 560 nm for 3 min. The results were expressed as units of SOD/mg of protein, where one unit of enzyme is defined as the amount of enzyme inhibiting the rate of reaction by 50%.

3.6.3 Catalase (CAT, EC 1.11.1.6)

Catalase activity was estimated using the method described by Aebi, (1984). Catalase catalyzes the decomposition of hydrogen peroxide (H$_2$O$_2$) and the rate of decomposition is measure of enzyme activity.

\[
2 \text{H}_2\text{O}_2 \xrightarrow{\text{Catalase (CAT)}} 2\text{H}_2\text{O} + \text{O}_2
\]

In a cuvette, appropriate amount of test sample was added followed by the addition of 12.5 mM H$_2$O$_2$ prepared in 0.067 M sodium phosphate buffer. The decrease in absorbance was measured at 240 nm for 3 min. The results were expressed as µmoles of H$_2$O$_2$ decomposed/min/mg protein, using molar extinction coefficient of H$_2$O$_2$ ($71 \text{ M}^{-1} \text{ cm}^{-1}$).

3.6.4 Glutathione-S-transferase (GST EC 2.5.1.13)

GST activity was assayed by the procedure described by Habig et al., (1974). This method is based on the enzyme-catalyzed conjugation of GSH with the model substrate, 1-chloro,2,4-dinitrobenzene (CDNB). The extent of conjugation is used as a measure of enzyme activity. The change in absorbance was measured spectrophotometrically at 340 nm.

To 1 ml of phosphate buffer (pH 6.5), 20 mM CDNB prepared in 95% alcohol and appropriate amount of sample was added in a cuvette. The final volume of 2.9 ml was adjusted by adding double distilled water. The reaction was incubated at 37°C for 5 min and 20 mM reduced glutathione [prepared in 0.2 mM phosphate buffer (pH 6.5)] was added to the reaction. The change in absorbance was read at 340 nm for 3 min. The enzyme activity was expressed as pmoles CDNB-GSH conjugate formed/min/mg protein using a molar extinction coefficient of $9.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. 

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3.6.5 Total Thiols

Total thiol groups (TSH) were quantified according to the method of Sedlak and Lindsay, (1968) In this method 5,5-dithio-bis 2-nitrobenzoic acid (DTNB) is reduced by free thiols group (~SH) to form 1 mole of 5-thio-2-nitrobenzoic acid per mole of ~SH.

In the tube, 0.2 M Tris HCl (pH 8.2) was added along with appropriate amount of sample followed by addition of 0.01M DTNB (prepared in methanol) and the total volume of 2.5 ml was adjusted by adding methanol. This reaction was kept at room temperature (RT) for 15 min and the contents were centrifuged at 3000 rpm for 10 min. Absorbance was read at 412 nm and the results were expressed as μmole T-SH/mg protein using molar extinction coefficient of 13600 M⁻¹ cm⁻¹.

3.6.6 Glutathione (Non-protein thiols)

GSH levels were estimated in terms of non-protein thiols or low molecular weight thiols, by the method described by (Roberts and Francetic, 1993). In this method, DTNB is reduced by the free ~SH groups of glutathione to form 1 mole of 5-thio-2-nitrobenzoic acid per mole of ~SH.

0.5 ml of sample was precipitated with 0.5 ml of 4% (w/v) sulphosalicylic acid. The samples were incubated for 30 min at RT. The contents were centrifuged at 1,200 g for 10 min. Ellman’s reagent which is 0.1 mM DTNB prepared in 100 mM sodium phosphate buffer was added to 0.5 ml supernatant obtained. The absorbance was measured at 412 nm after 2 min. The results were expressed as μmoles GSH/mg protein by using molar extinction coefficient of 13600 M⁻¹ cm⁻¹.

3.6.7 Protein Thiols (P-SH)

P-SH levels were calculated from the difference between the values of total thiols and non-protein thiols (NPSH). The results were expressed as μmole/mg protein.

3.6.8 Acetylcholinesterase activity (AChE 3.1.1.7)

The activity of AChE was determined by the method of Ellman et al., (1961). Acethylocholine iodide is the ester of thiocholine and acetic acid which is used as a
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substrate in this assay. AChE enzyme hydrolyses acetylthiocholine into thiocholine and acetate. Thiocholine forms a mercaptan, which reacts with the oxidizing agent DTNB to form 5-thio-2-nitrobenzoate which has a maximum absorption at 412 nm.

\[
\text{(CH}_3\text{)}_3\text{N(CH}_2\text{-S-C-CH}_3\text{)} + \text{H}_2\text{O} \rightarrow \text{(CH}_3\text{)}_3\text{N(CH}_2\text{-SH} + \text{CH}_3\text{COOH} \quad \text{Thiocholine}
\]

An appropriate amount of sample was added in cuvette containing 0.1 M sodium phosphate buffer (pH 8.0) and Ellman’s reagent (10 mM DTNB dissolved in sodium phosphate buffer). The reaction was initiated by adding 14.9 mM acetylthiocholine iodide and the rate of change of absorbance was followed at 412 nm for 2 min. The results were expressed as nmoles substrate hydrolyzed/min/mg protein using molar extinction coefficient of 5-mercapto-2-nitrobenzoate (13.6 x 10³ M⁻¹ cm⁻¹).

3.7 Mitochondrial Studies

3.7.1 Isolation of Mitochondria and activity of mitochondrial complexes

Mitochondrial fractions were prepared according to the method described by Stahl et al., (1963). The hippocampus and cortex were weighed and homogenized separately in ice cold buffer containing 10 mM Tris HCl (pH 7.4), 0.44 M sucrose, 10 mM EDTA and 0.1% BSA and centrifuged at 2,100 g for 15 min at 4°C. The pellet was discarded and the supernatant obtained was further centrifuged at 14,000 g for 15 min at 4°C. The mitochondrial pellet obtained was washed with the same buffer and again centrifuged at 7,000 g for 15 min at 4°C. The final mitochondrial pellet was re-suspended in buffer containing 10 mM Tris HCl (pH 7.4) and 0.44 M sucrose.

Mitochondrial complexes, MTT reduction assay, mitochondrial membrane permeability along with oxidative stress parameters (protein carbonyl and ROS) were studied in isolated mitochondria.
3.7.2 NADH dehydrogenase (EC 1.6.99.3)

The activity of NADH: cytochrome c reductase was measured as described by King and Howard (1967). NADH catalyzes the reduction of electron acceptor, cytochrome c. This reaction involves catalytic oxidation of NADH to NAD$^+$ with subsequent reduction of cytochrome c on addition of mitochondrial preparation.

$$\text{NADH} + 2 \text{cytochrome } c_{\text{oxidized}} \xrightarrow{\text{NADH dehydrogenase}} \text{NAD}^+ + 2 \text{cytochrome } c_{\text{reduced}} + \text{H}^+$$

In this assay, appropriate amount of mitochondrial sample was added to reaction containing 0.2 M glycyl glycine buffer (pH 8.5), 6 mM NADH, 1 mM oxidized cytochrome c and 0.02 M NaHCO$_3$. The increase in absorbance was followed at 550 nm for 3 min. The results were expressed as nmoles NADH oxidized/min/mg protein using molar extinction coefficient of reduced cytochrome c at 550 nm (1.96 mM$^{-1}$ cm$^{-1}$).

3.7.3 Succinate dehydrogenase (EC 1.3.5.1)

The activity of succinate dehydrogenase was assayed according to the method of King et al., (1976). This assay is based on oxidation of succinate to fumarate by an artificial electron acceptor, potassium ferricyanide, catalyzed by enzyme succinate dehydrogenase.

$$\text{Succinate} + 2\text{Fe(CN)}_6^{3-} \xrightarrow{\text{Succinate dehydrogenase}} \text{Fumarate} + 2\text{Fe(CN)}_6^{4-} + 2\text{H}^+$$

In this procedure, the reaction was initiated by addition of requisite amount of mitochondrial sample to the reaction mixture containing 0.2 M sodium phosphate buffer (pH 7.8), 1% (w/v) BSA, 0.6 M sodium succinate, 0.03 M potassium ferricyanide. The change in absorbance was read at 420 nm for 3 min. The results were expressed as nmoles of succinate oxidized/min/mg protein using molar extinction coefficient of potassium ferricyanide (1000 M$^{-1}$ cm$^{-1}$).

3.7.4 Cytochrome-c oxidase (EC 1.9.3.1)

The activity of cytochrome oxidase was assayed according to the method of Sottocasa et al., (1967). This assay measures the oxidation of reduced cytochrome c by enzyme cytochrome c oxidase.
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First, the oxidized form of cytochrome c was reduced by adding few crystal of sodium borohydride and then it was neutralized to pH 7.0 by adding 0.1 N HCl. 0.3 mM of reduced cytochrome c was added to 0.075 M phosphate buffer and the reaction was initiated by addition of appropriate amount of mitochondrial fraction. The decrease in absorbance was followed at 550 nm for 3 min. The results were expressed as nmoles of cytochrome c oxidized/min/mg protein using molar extinction coefficient of cytochrome c (1.96 mM⁻¹ cm⁻¹).

3.7.5 F₁F₀ ATP synthase (EC 3.6.3.14)

Activity of mitochondrial F₁-F₀ ATP synthase was assayed by the method of Griffiths and Houghton, (1974).

\[ ATP \xrightarrow{ATP \text{ synthase}} Pi \text{ (inorganic phosphate)} + ATP \]
\[ Pi + \text{ammonium molybdate} \xrightarrow{H^+} \text{ammonium phosphomolybdate} \]

The reaction mixture containing 50 mM Tris HCl (pH 7.5), 2 mM MgCl₂, 5 mM ATP and requisite amount of mitochondrial sample were incubated at 37°C or 10 min. The reaction was stopped by addition of 10% (w/v) TCA and the contents were centrifuged at 3,000 g for 20 min. The phosphate liberated was measured on the basis of reaction of the inorganic phosphate with ammonium molybdate as described by (Fiske and Subbarow, 1927). The results were expressed as nmoles of ATP hydrolyzed/min/mg protein.

3.7.6 MTT Reduction

MTT reduction was used to study the mitochondrial function as described by Liu et al., (1997). This assay is based on the conversion of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to blue formazan by mitochondrial reductase enzymes. The blue formazan produced has maximum adsorption at 595 nm.
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Tetrazolium salt (Yellow) Formazan (Blue)

To the appropriate mitochondrial sample, 1/10th of stock MTT (5 mg/ml), was added and mixed thoroughly. This reaction was incubated for 30 min at 37°C. The contents were centrifuged to obtain formazan pellet. The solvent solution containing 0.04 N HCl in isopropanol was added to pellet and re-centrifuged at 2,000 g for 10 min. The absorbance was read at 595 nm. The results were expressed as μg of formazan formed/min/mg protein.

3.7.7 Mitochondrial swelling

Mitochondria transition pore opening leads to swelling of mitochondria. Mitochondrial swelling and contraction acts as a functional test for mitochondrial membrane integrity which was performed as described by (Tedeschi and Harris, 1958). This method is based upon the increased absorbance by mitochondria in a contracted state and decreased absorbance by swollen form due to cation influx. The swelling and contraction can be detected by the measurement of light scattering at 520 nm.

Mitochondrion swelling was assessed by adding appropriate amount of mitochondrial sample to the buffer containing 0.12 M KCl in 0.02 M Tris HCl (pH 7.4) and the absorbance was measured for 6 min at 520 nm. The mitochondrial contraction was initiated by adding 5 mM Mg\(^{2+}\)-ATP buffer to the above reaction and the change in absorbance was read at 520 nm which was normalized by protein concentration.

3.7.8 Mitochondrial reactive oxygen species (ROS) generation

ROS formed in isolated brain mitochondria were measured using the method described by Wang and Joseph, (1999). This assay measures the oxidation which is based on the ROS
mediated conversion of a non-fluorescent compound 2, 7-dichlorofluorescein-diacetate (DCFH-DA) into a highly fluorescent compound 2,7-dichlorofluorescein (DCF)

In this method, appropriate amount of mitochondrial sample was added to 10 μM of DCFH-DA dissolved in buffer (40 mM NaCl, 5 mM KCl, 1 mM MgCl2, 5.6 mM glucose, 1.5 mM CaCl2, 20 mM HEPES-Na, pH 7.4) and the contents were incubated for 1 hr at 37°C. The fluorescence of DCF produced was measured with excitation at 488 nm and emission at 530 nm using spectrofluorometer (Shimadzu, Japan). The results were expressed as pmoles DCF formed/min/mg protein.

3.7.9 Protein carbonyls

The protein carbonyl levels were determined using 2, 4-dinitrophenylhydrazine (DNPH) assay as described by Levine et al., (1990). Protein carbonylation involves the formation of carbonyl moieties (-C=O) at the amino acid side chains which are most susceptible to oxidative damage. This method is based on the formation of stable protein-hydrazone derivative (2,4 dinitrophenyl hydrazone) which is formed where DNPH selectively binds to carbonyl groups present on the proteins. The amounts of protein-hydrazones formed are measured spectrophotometrically at 370 nm.
The mitochondrial sample was dissolved in 20 mM DNPH prepared in 2 N HCl and kept at RT for 1 hr in dark. Respective negative control was prepared in which mitochondria sample was dissolved in 2 N HCl only. Following 1 hr incubation, 20% (w/v) TCA was added for protein precipitation and the mixture was centrifuged at 10,000 g for 10 min. Supernatant was discarded and the pellet obtained was washed three times with ethyl acetate: ethanol mixture (1:1) to remove excess DNPH. The final pellet was later dissolved in 6 M guanidine HCl solution at 50°C and the yellow colored complex obtained was read at 370 nm against respective negative controls. Negative controls were read at 280 nm to determine protein concentration. The results were expressed as nmoles carbonyls/ mg protein using extinction coefficient of DNPH (22 mM⁻¹ cm⁻¹).

**3.7.10 Protein Estimation**

Protein contents were estimated by the method of Lowry et al., (1951). Proteins contain peptide bonds. This test is based on the reaction of peptide bonds with Cu²⁺ and then conversion of Cu²⁺ to monovalent ion (Cu⁺) in alkaline conditions which is the first step of reaction. In second step, Cu⁺ reacts with Folin Ciocalteu Reagent (phosphomolybdic/phosphotungstic acid) and produce blue color that can be read at 650-750 nm. The blue color formed in this step depends majorly on the contents of tyrosine and tryptophan (amino acids) in the protein.

In this method, appropriate amount of test sample, BSA (standard), water (blank) was taken and the final volume (1 ml) was made up with double distilled water. Lowry’s reagent [prepared by mixing 1% (w/v) copper sulphate solution, 2% (w/v) sodium
potassium tartarate, 2% (w/v) sodium carbonate prepared in 0.1 N sodium hydroxide in ratio of 1:1:98] was added to each tube (3 ml). The contents were vortexed and incubated for 10 min at 37°C. Finally, 0.3 ml of Folin Ciocalteu reagent [prepared freshly by diluting 2 N commercially available Folin’s reagent with distilled water (1:1)] was added to each tube. The contents were again vortexed and incubated at 37°C for 30 min. The absorbance was read at 750 nm. The protein concentration was calculated as mg/ml using standard BSA.

3.8 Neuroinflammation Studies

Neuroinflammation in brain is characterized by rapid increase in activation of glia cells which releases molecules like cytokines, chemokines etc. Activation of microglia (Iba-1) and astrocyte marker (GFAP) along with different pro-inflammatory cytokines were studied using real time PCR, western blotting, ELISA and immunohistochemistry.

3.8.1 RNA extraction

Animals were sacrificed by decapitation under mild ether anesthesia; the hippocampus and cerebral cortex were dissected from brain and immediately placed in RNAlater (Ambion Inc., Austin, Texas, USA). The tissue samples were then homogenized using sterile/RNAase free plastic hand homogenizer in RNA extraction buffer (Trizol) and centrifuged at 12,000 g for 10 min. For phase separation, supernatant was collected and chloroform was added to each tube. The samples were shaken vigorously for 30 s and incubated at RT for 10 min. Upper aqueous layer was transferred to a fresh tube after centrifugation at 12,000 g and isopropanol was added to precipitate RNA. Samples were again centrifuged at 12,000 g for 5-10 min at 4°C and RNA pellet formed was washed with 75% (v/v) ethanol and air-dried for 5-10 min. The RNA pellet was re-dissolved in RNAase free water (DEPC-water). RNA samples were treated with DNase-I at 65°C for 5 min to remove any genomic DNA traces if present. The concentration and purity of the extracted RNA (OD ratio at 260/280) was measured using Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). The overall quality and integrity of RNA extracted was assessed by agarose gel electrophoresis. Agarose gel was prepared by dissolving 1 g agarose in 100 ml of Tris-acetate-EDTA buffer (89 mM Tris-HCl pH 7.8, 89 mM borate, 2 mM EDTA)
with 0.5 μg/ml ethidium bromide added to the gel. Approximately 1.5 μg of total RNA was loaded for each sample and gel was run at 100 V for 45 min. Gel was later visualize on a UV transilluminator. Three distinct bands of RNA (28S, 18S and 5S) present on gel after electrophoresis suggests a high quality of RNA.

3.8.2 cDNA preparation

The total RNA obtained was transcribed into complementary DNA (cDNA) by using cDNA synthesis Kit (Thermo Fisher Scientific, USA). In a nuclease free sterile PCR tube, 3 μg of DNase treated RNA was added with 0.5 μg/μl oligo (dT) primers followed by addition of transcription reaction mixture containing dNTP (10 mM) in reaction buffer (250 mM Tris HCl (pH 8.3), 250 mM KCl, 20 mM MgCl₂, 50 mM DTT). All the contents were mixed gently and spin down briefly. Further, this reaction was incubated for 60 min at 42°C and terminated by heating at 70°C for 5 min. cDNA prepared was stored at -80°C for further use.

3.8.3 Real time PCR reaction

Real time PCR was performed to examine mRNA expression for astrocytic (GFAP) and microglial marker (Iba1) along with cytokines (TNF-α, IL-1β, IL-6) and chemokine (MCP-1). Briefly, 2 μl of cDNA corresponding to 30 ng of total RNA and gene-specific primers at concentration of 0.25 μM (Table 3.1) were mixed with SYBR green PCR master mix (Roche Applied Science, Germany) in total volume of 20 μl. The PCR was performed on system LC480, Roche Applied Science, Germany. The real time reactions were performed in duplicates for all the target genes and β-actin was used as a housekeeping control for data normalization. Relative expression of genes was calculated using the $2^{-ΔΔCt}$ method (Edmonson et al., 2014).

3.8.4 Cycling conditions

For RT-PCR, the first step was to activate the hot-start Taq-polymerase supplied with the SYBR Green detection kit which was performed at 95°C for 5 min, followed by 40 cycles of 10 sec at 95°C, 20 sec at 58°C and 15 sec at 72°C each. During amplification, the fluorescence signal, which is proportional to the amount of double-stranded product
produced, was monitored. In this way a complete amplification profile for each of the 96 wells of a PCR plate was obtained, which was used for the analysis. At the end of the PCR run, melting curves of the amplified products were obtained, which were used to control specificity of the amplification reaction.

Crossing point (CP) was calculated, the point where the reaction’s fluorescence reaches the maximum of the second derivative of the amplification curve (acceleration of the fluorescence signal is at its maximum). The expression levels were normalized to those obtained for the housekeeping gene (β actin). All samples were run in duplicate and the average values were used for the relative quantification of mRNA expression.

3.8.5 Probe (Oligonucleotide primers)

The sequence of primers provided in Table 3.1 was designed using the Primer3 program available from the Whitehead Institute for Biomedical Research. The primer pairs are given in 5' → 3' direction.

Table 3.1: Sequence of primers used for real-time RT-PCR analysis

<table>
<thead>
<tr>
<th>Target Gene (Gene bank ID)</th>
<th>Forward primer (5'→3')</th>
<th>Reverse primer (5'→3')</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP (NM017009)</td>
<td>TCCTGGAACAGCAAAAACAAG</td>
<td>CAGCCTCAGGTTGGTTTCA T</td>
<td>224</td>
</tr>
<tr>
<td>Iba1 (NM017196)</td>
<td>GTCTTGGAAGCGAATGCTGG</td>
<td>CATTCCTCAAGATGGCAGATC</td>
<td>157</td>
</tr>
<tr>
<td>IL-1β (NM031512)</td>
<td>CACCTCTCAAGCAGAGCAGAGC</td>
<td>GGTTTCCATGGTGAAGTCAAC</td>
<td>79</td>
</tr>
<tr>
<td>IL-6 (NM012589)</td>
<td>CCTACCCCAACTTCCAATGCTC</td>
<td>TGGATGGTCTTGGTGCCTTAGCC</td>
<td>78</td>
</tr>
<tr>
<td>TNF-α (NM012675)</td>
<td>CCCAGACCCCTCACACTCAGAT</td>
<td>TTGTCCTTGAAGAGAACCTG</td>
<td>215</td>
</tr>
<tr>
<td>MCP-1 (NM031530)</td>
<td>AGCAGTTTTGGAATGGCAA C</td>
<td>AGAAGTGCTTGGAGTTGTTG</td>
<td>82</td>
</tr>
<tr>
<td>β-actin (NM031144)</td>
<td>CTCTTTGCAGCTTCCCTCGTG</td>
<td>TCACACCTTGGGTGCTAGG</td>
<td>178</td>
</tr>
</tbody>
</table>
3.8.6 Western Blot analysis

Immunoblotting was performed to analyze the protein expression of GFAP and Iba1 along with β-actin as an internal loading control according to the procedure described by Towbin et al., (1992). The hippocampus and cerebral cortex were homogenized in lysis buffer [20 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM MgCl₂, 1 mM DTT (Dithiothreitol), 1 mM PMSF (phenylmethylsulfonyl fluoride) and protease inhibitor cocktail (inhibitors with broad specificity for serine, cysteine, and acid proteases, and aminopeptidases) ] and centrifuged at 20,000 g for 20 min. The supernatant was collected and analysed for protein concentration. Approximately 30 µg of protein was loaded per lane and separated on 12% SDS-PAGE. The proteins were transferred electrophoretically to PVDF membrane in a transfer buffer (25 mM Tris-HCl, 192 mM glycine and 20% (v/v) methanol, pH 8.3). The membranes were blocked by incubating with 5% non-fat dry milk in Tris-buffered saline containing Tween (TBST: 25mM Tris, pH 8.0, 150 mM NaCl and 0.5% Tween 20) overnight at 4°C. The membranes were then probed with primary antibodies for GFAP, Iba-1 and β actin diluted at 1:1000, 1:600 and 1:2000 respectively in 2% non-fat dry milk/TBST. After washing with TBST, membranes were incubated with the respective horse radish peroxidase (HRP) conjugated secondary antibodies diluted in 2% non-fat dry milk/TBST. The membranes were washed three times with TBST and the proteins were detected with 3,3 di-aminobenzidine tetrahydrochloride (DAB) and H₂O₂ as substrates. The reaction was stopped by washing the membrane briefly with water. Densitometric scanning and quantification of intensities (scanned blots) were carried out using AlphaEase software (Alpha Innotech Corp., San Leandro, CA, USA).

3.8.7 Quantification of cytokines and chemokines using ELISA

The protein expression of cytokines and chemokine in the hippocampus and cerebral cortex regions of brain was studied using ELISA kit from BD biosciences, San Jose, CA, USA (OptEIA Set with specific antibody) for TNF-α (Tumor necrosis factor-alpha), IL-1β (Interleukin 1- β), IL-6, MCP-1 (monocyte chemotactic protein-1) kit was purchased from Ray Biotech Inc., USA. Enzyme-linked Immunosorbent Assays (ELISAs) technique involves the measurement of antigen or antibody concentration in any liquid state sample.
This is based on the detection of an analytes (antigen/protein) using specific antibodies coupled to an easily assay enzymes.

ELISA plates were coated with specific antibody, sealed and incubated overnight at 4°C. Next day, standard, sample and control were added in duplicate to the appropriate coated 96-well plate and incubated for 2 hrs at RT. After the antigen was immobilized, detection antibody was added forming a complex with the antigen. Between each step, the plates were washed with the washing buffer. Enzyme reagent (streptavidin-HRP) was added after the last wash and plates were incubated further for 30 min at RT followed by addition of substrate solution (stabilized chromogen) which starts the colour reaction. Stop solution was added to stop the reaction and the absorbance was read at 450 nm. Wavelength correction was done by subtracting absorbance at 570 nm from absorbance 450 nm. The concentration of cytokines and chemokine was determined from the regression line for their respective standards with different ranges from 7.8-10,000 pg/ml and expressed per mg protein.

3.8.8 Immunohistochemistry

Immunohistochemical analysis for GFAP and Iba-1 was done in hippocampus and cerebral cortex region. The animals were anesthetized with ether and perfused via ascending aorta with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% (w/v) paraformaldehyde in 0.1 M PBS (pH 7.4). The brain was removed and post fixed in the same fixative followed by immersion in 30% (w/v) sucrose for cryoprotection. The tissue was cut in 30 µm thicknesses using a cryostat at -20°C. Sections were air dried at 37°C for an hour and washed in PBS and later treated with 1% triton X-100 in PBS for 30 min. Non-specific proteins were blocked with normal goat serum in a humid chamber. Thereafter, rabbit polyclonal anti-Iba-1 (1:800) and rabbit polyclonal anti-GFAP (1:2000) antibodies were used to stain microglia and astrocytes respectively. Next day, the sections were incubated with 1:100 biotinylated anti-rabbit secondary antibodies for 1 hr at RT. Tissue sections were rinsed with PBS and further incubated with 1:200 tertiary complex which is streptavidin biotinylated HRP (cat. RPN 1051, Amersham, UK) for 2 hr at RT. The signals were visualized with freshly prepared diaminobenzidine (DAB) substrate. Slides were washed in running tap water, followed by distilled water and air dried for 40-50 min. The
sections were dehydrated, cleared in xylene and mounted in DPX and were visualised and photographed using a light microscope (Leica-DFC 295, Wetzlar, Germany). The immunoreactive astrocytes and microglia were quantitated by Ostu’s clustering based image thresholding method using MATLAB (version R2010b, Mathworks, Inc., Natick, MA US) as described by Kozlowski et. al. (Kozlowski and Weimer, 2012).

3.9 Blood Brain Barrier studies

Blood brain barrier (BBB) permeability was assessed in terms of uptake of two different dyes sodium fluorescein and Evans blue. These dyes differ in their molecular weight (MW). Sodium fluorescein (MW 376 Da) is one of the smallest and Evans blue (EB, MW 961 Da) is one of the largest molecular weight intravital dye used as tracers in BBB permeability studies (Kozler and Pokorny, 2003).

3.9.1 Sodium fluorescein (NaFl) extravasation

The animals were injected intravenously with 2 % (w/v) NaFl at a dose of 5 ml/kg, 5 min before the administration of PTZ challenge dose. After 1 hr, the animals were perfused with saline under light ether anesthesia until the perfusion fluid comes colorless. Colorless perfusion fluid (free of blood) is an indicator for good perfusion. To quantify the NaFl extravasation, the brain was removed and the cortex and hippocampus were separated and weighed on pre-weighed aluminum foil. The brain regions were homogenized in PBS (pH 7.4) and 60% TCA was added to the homogenate. The contents were incubated for 30 min at RT followed by centrifugation at 14,000 g for 10 min. Fluorescence intensity of NaFl in the supernatant was measured at excitation 440 nm and emission 535 nm using spectrofluorometer (Shimadzu RF-5301PC, Japan). The results were expressed as ng dye/ing brain tissue using NaFl as standard.

3.9.2 Evans Blue (EB) extravasation

The BBB integrity was assessed using Evans blue dye which is known to bind the albumin after i.v injection and is commonly used as a tracer for serum albumin. The Evans blue 2% (w/v) was injected intravenously (penile vein) at a dose of 4 ml/kg. After 1 hr, the rats were perfused transcardially with saline until the fluid from the right atrium become colorless.
and the cortex and hippocampus regions were separated after decapitation. The cortex and hippocampus region were weighed and homogenized separately in N,N-dimethylformamide (DMF) followed by incubation at 37°C for 72 hr (dark). The volume was made up 1 ml using DMF and centrifuged at 14,000 rpm for 15 min. The supernatant obtained was spectrofluorometrically measured at excitation 620 nm and emission 680 nm (Weissman and Stewart, 1988). The results were expressed as ng/mg brain tissue using Evans blue as standard.

3.9.3 Measurement of brain water content

Brain edema was examined by measuring the water content in different brain regions as described in the method by (Mansfield et al., 1996). After decapitation, brain was rapidly removed and different brain regions including cortex, hippocampus and rest of the brain were separated. Immediately, the separated brain regions from each animal were placed separately on the pre-weighed filter paper to obtain the wet weight of tissues. The samples were then placed in incubator maintained at 60°C for 72 hr. After 72 hr, the samples were re-weighted on filter paper using same weighing balance to obtain the dry weight. Edema was calculated using formula:

\[
\text{water contents (\%)} = \frac{\text{Wet weight} - \text{dry weight}}{\text{Wet weight}} \times 100
\]

3.9.4 Gelatin Zymography for MMP-9 and MMP-2

Gelatin zymography was used to determine the activity of MMP-9 and MMP-2, which was performed in brain homogenates as described in the method of Koistinaho et al., (2005). Proteins were separated by electrophoresis in a 10 % SDS-PAGE gel containing 0.1% gelatin. Equal concentration of protein was used for all the tissue samples. Pre-stained molecular weight standards (Bio-Rad) were also run with the samples. After the gels were completely run, they were washed twice with 2.5% Triton X-100 (20 min each) to remove SDS and incubated at 37 °C with developing buffer (50 mM Tris–HCl pH 7.5, 10 mM CaCl₂, 0.02% NaN₃) for 24 hr. Enzymatic bands were visualized after staining for 1 hr with Coomassie blue R-250 for 20-30 min, and de-stained with a solution containing methanol/acetic acid/water at ratio of 50:10:40 (v/v/v). The gels were photographed and
the intensity of bands was quantified using Alpha ease FC software.

3.9.5 Real time PCR (MMP-9 and MMP-2)

RNA was extracted by the method described above and real time PCR was performed to examine the mRNA expression for MMP-9 and MMP-2 using SYBR Green dye on system LC480, Roche Applied Science, Germany. Briefly, 2 μl of cDNA corresponding to 30 ng of total RNA and gene-specific primers (Table 3.2) were mixed with SYBR green PCR master mix (Roche Applied Science, Germany) in total volume of 20 μl. The real time reactions were performed in duplicates for all the target genes and β-actin was used as a housekeeping control for data normalization. Relative expression of genes was calculated using the 2^ΔΔCt method (Edmonson et al., 2014).

Table 3.2: Sequence of primers used for real-time RT-PCR analysis of MMP-9 and MMP-2

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Forward primer (5’→3’)</th>
<th>Reverse primer (5’→3’)</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>CGGTATTATTTGGCGGACAGT</td>
<td>GCCTCATAACACAGGTCAATCTT</td>
<td>107</td>
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<td>(NM031054.2)</td>
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<tr>
<td>MMP-9</td>
<td>TCGACTCCAGTACAGCAATCC</td>
<td>CGGAACTTCACAATACCGAC</td>
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<tr>
<td>(NM031055.1)</td>
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<td></td>
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</tr>
</tbody>
</table>

3.10 Electrophoretic mobility shift assay (EMSA)

The transcription factors help regulate gene expression by binding to DNA and may play an important role in the response to injury induced by seizures and in activation of cytokines or chemokines. The transcription factors NF-kB was assessed using electrophoretic mobility shift assay (EMSA) which is based on relative differences in electrophoretic mobility between protein-DNA complexes and un-complexed DNA under non-denaturing conditions.

To detect the NF-κB DNA-binding activity, nuclear protein was extracted from hippocampus and cortex region by the method described by Prusty et al., (2005). The NF-kB consensus sequence 5’-AGT TGA GGG GAC TTT CCC AGGGC-3’ used was synthesized from Applied Biosystems DNA synthesizer using phosphoramidite chemistry.
These oligos were annealed and labeled with $[\gamma^{32}P]$ ATP by T4 polynucleotide kinase and gel purified onto a 15% polyacrylamide gel. Briefly, a binding reaction of 10 µg nuclear extract with $[\gamma^{32}P]$ labeled oligo probe was performed in a 25 µl reaction volume containing 50% glycerol, 60 mM HEPES (pH 7.9), 20 mM Tris-HCl (pH 7.9), 300 mM KCl, 5 mM EDTA, 5 mM DTT, 100 µg/ml of bovine serum albumin, 2.5 µg of poly (dI-dC) for 30 min at RT. The DNA–protein complexes were resolved on 4.5% nondenaturing polyacrylamide gel (29:1 cross-linking ratio), dried and exposed overnight to KODAK X-Omat films (Kodak India, India).

3.11 Assessment of neuronal injury

Fluoro-Jade B and TUNEL staining was performed to access the neuronal cell death. It has been reported that staining the tissues with Fluoro-Jade B and TUNEL, respectively, shows an overlap thereby suggested that Fluoro-Jade B could be a useful marker of apoptotic cell death (Zuch et al., 2000).

3.11.1 Fluoro jade B

Fluoro-jade B is a novel fluorescent marker used for identification of cell degeneration, preferentially brain neurons. This method was performed as described by Kundrotiene et al., (2004). Brain sections were incubated in 100% (v/v) ethanol for 3 min followed by 1 min incubation in 70% (v/v) ethanol and washing with distilled water. Sections were treated with potassium permanganate (0.06%) for 15 min to protect the tissues from photo bleaching and to avoid background staining and later washed with water for 1 min. Thereafter, the sections were stained with 0.001% (w/v) Fluoro-Jade prepared in 0.09% (v/v) acetic acid for 20-30 min. At last, the sections were washed with water and allowed to dry at RT (dark). Sections were examined using Leica DM2500 microscope (Leica microsystems, Wetzlar, Germany) attached with a Leica DFC295 CCD camera.

3.11.2 Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) Assay

Cell death/apoptosis was detected using in situ nick end labeling by terminal deoxynucleotidyl transferase enzyme (TdT) which was performed using TACS XL blue label apoptosis detection kit (Trevigen Inc., Gaithersburg, MD, USA.). Briefly, the tissue
samples were de-paraffinized before starting the labeling reaction and later rehydrated by transferring the slides through graded ethanol series of 100% (v/v) to 95% (v/v) to 75% (v/v) and final wash with PBS (15 min). The sections were incubated with proteinase K for making the cell permeabilized followed by washing with water. The endogenous peroxidase activity was inactivated by covering the sections with quenching solution which was 2% (v/v) hydrogen peroxide. Further, the sections were labeled with labeling reaction mixture containing terminal deoxynucleotidyl transferase and nucleotide mix of brominated dNTPs for 1 hr at 37°C in a humid chamber and the reaction was stopped by adding stop buffer. The samples were incubated with anti-BrdU (bromodeoxyuridine) antibody for 1 hr followed by incubation with peroxidase-streptavidin conjugate solution and finally labeled with TACS blue for 5 min. Samples were washed with deionized water and counterstained with nuclear fast red and visualized using bright field microscope.

3.12 Histopathological Studies

In order to evaluate and correlate biochemical and functional changes in the brain histopathological studies were carried out by performing haemotoxylin and eosin staining (H&E) and cresyl violet staining. Briefly, rat was deep anesthetized and perfused transcardially with saline followed by 0.4% (w/v) paraformaldehyde prepared in phosphate buffer saline. Brains were removed and hippocampus and cortex sections were separated, processed and embedded in paraffin blocks. Coronal sections were prepared and mounted on poly-L-lysine coated slides and stained with following staining techniques.

3.12.1 Haemotoxylin and Eosin staining

The brain sections were stained with routine H&E stain. Paraffin fixed sections were de-waxed at 57°C in incubator followed by xylene solution and downgraded (rehydration) through various grades of alcohol to water (absolute alcohol-5 min, 90% (v/v) alcohol-2 min, 70% (v/v) alcohol-2 min, water-1 min). The tissues sections were stained with haematoxylin for 20 min and then washed with running water for 10 min. Thereafter, the slides were treated with acid water and ammonia till blue nuclei became visible in clear cytoplasm followed by dehydration. Finally, the sections were stained with alcoholic eosin for 30 s followed by dehydration steps: 90% (v/v) alcohol (single dip), absolute alcohol (5-
7 min) until excess eosin is removed. Slides were viewed under microscope and cleared using xylene solution (2 changes, 2 min each).

3.12.2 Cresyl violet Staining
The brain sections were stained with 0.1% (w/v) cresyl violet using method described by Ogita et al., (2003). Paraffin sections were de-waxed at 57°C and incubated in xylene solution for 10 min (twice), followed by rehydration in absolute alcohol (10 min). The slides were immersed once in 90 % (v/v) ethanol, once in 70 % (v/v) ethanol followed by immersion in 50 % (v/v) ethanol. Then, the slides were dipped in 30% ethanol (10 min), rinsed two times in distilled water and finally immersed in cresyl violet staining solution for 5 min. After this, the slides were rinsed in water and air dried. The slides were subjected to two changes of n-butanol followed by cleaning in xylene solution (5 min). Slides were mounted in DPX and dried overnight. Images were acquired using Leica DM2500 microscope (Leica microsystems, Wetzlar, Germany) equipped with a Leica DFC295 CCD camera.

3.12.3 Transmission electron microscopy
Transmission electron microscopy was done as described by Gao et al., (2013). The hippocampus and cerebral cortex were removed after decapitation under mild anesthesia (ether) and fixed in 3% (w/v) glutaraldehyde (diluted in 0.2 M Sorensen's buffer) for 24 hr at 4°C. Brain sections were diced into small cubes of 1 mm size and postfixed in osmium tetroxide, dehydrated in a graded ethanol series, cleared in propylene oxide at RT and finally embedded in EPON mixture containing Taab/812. Thick sections of 0.5 μm size were cut and toluidine blue staining was done to confirm the presence of neurons. 60 nm ultra-thin sections were cut, mounted on nickel grids and were double contrast stained using uranyl acetate and lead citrate. Sections were examined and photographed on a Hitachi H-7500 transmission electron microscope (Hitachi, Tokyo, Japan).

3.13 Statistical analysis
All values were expressed as mean ± standard error of mean (SEM) with seven animals per group. The statistical significance was analyzed by using one-way analysis of variance (ANOVA) followed by Newman-Keuls test using SPSS software. Values with p<0.05 were considered as statistically significant.