Materials & Methods
4.1. Materials:

4.1.1. Animals

In the present study, male Wistar rats of two age groups: young (4 months) and old (18 months) were used. Animals were obtained from the central animal house facility of the Jawaharlal Nehru University, New Delhi, as per the experimental protocols approved by Committee for the Purpose of Control and Supervision on Experimental Animals (CPCSEA) and Institutional animal ethical committee (IAEC) of Jawaharlal Nehru University, New Delhi, India. The animals were housed in pairs in standard laboratory cages 8”x12”x5” made of polypropylene with stainless steel coverings and maintained at 26±4°C, under light condition of 12h light/ 12h dark cycle. Each animal was provided with *ad libitum* access to food and water. Each rat was checked for health status by observing various criteria such as tail sores, posture hunch, grooming, nose red rim, red eye rims, tumors and teeth etc (Markowaska et al., 1990; Sharma et al., 1993).

4.1.2. Chemicals

All the chemicals used were of analytical grade and obtained from Sigma Aldrich Co, USA or from standard Indian companies like BDH, SRL, Merck and Qualigen. The drugs L-deprenyl and curcumin was purchased from Sigma-aldrich chemical company, USA.

4.2. Methods

4.2.1. Experimental setup:

Experimentation was carried out in two parts. First part constituted investigation of alterations associated with FeCl₃-induced epileptogenesis. Groups of randomly selected rats of 4 and 18 months of age were designated as 1) Untreated controls- maintained on normal rat feed (obtained from Hindustan Lever Limited). 2) Saline injected controls- injected with 5μL saline intracortically for five minutes and 3) Epileptic rats- injected with 5μL FeCl₃ intracortically for five minutes. Electrophysiological, biochemical, microscopic and behavioral studies were performed on every animal of each group. Single group constitute minimum six animals (n=6).
All the parameters were studied in the cortex and hippocampus regions of experimental rats and compared with age matched controls. Progression of epileptogenesis was monitored till the FeCl₃ injected old rats.

The second part of the experimental work was designed as shown in Fig-1, to evaluate the affect of dietary curcumin and L-deprenyl treatment on epileptic rats. Anticonvulsive or anti-epileptic potentials of these compounds were determined in both young and old rats. Curcumin was fed in diet as a dietary supplement at a concentration of 500ppm and 1500ppm. In long-term treatment, rats of both age-groups were allowed to feed curcumin supplemented diet three months before the FeCl₃ injection and continued thereafter, for 22 weeks in young and 16 weeks in old rats. For short-term treatment rats of both age groups
initially maintained on normal diet, were switched over to curcumin supplemented diet for five weeks after FeCl$_3$ injection. Similar treatment plan was followed for L-deprenyl. In both long and short term treated animals electrophysiological and behavioral recordings were done regularly for 22 weeks in young and 16 weeks in old rats in order to monitor the development and progression of epileptic seizures.

4.2.2. Brain regions studied
1) Cerebral cortex.
2) Hippocampus.

4.3. Electrophysiological experiments.

a) Equipments: The equipment used to carry out electrophysiological recordings was as follows:

1) Rat Stereotaxic apparatus (INCO, India)

2) Polygraph: (Embla A10 instrument attached with Somnologica Analysis software).

Synchronized video recording was performed along with electrophysiological recordings. Digitized values were obtained to analyze the typical transient seizures with the help of MATLAB analysis software

3) Accessories

Screw and wire electrodes: For recording the cortical (ECoG) and depth electroencephalographic activity (EEG), screw and insulated tungsten wire electrodes were obtained from PlasticsOne, Roanoke, Virginia, USA. Screw and wire electrode were manually fabricated as shown in fig-2 for ECoG and depth EEG recordings. During surgery, free ends of these electrodes were affixed through insulated wire to a fifteen pin adaptor and fixed on the scalp of the rat skull with the help of dental cement for multi-channel animal recording.

4) Brain atlas: Stereotaxic coordinates for electrode implantation were according to the rat brain atlas of Paxinos and Watson (1982).
Figure 8. Showing recording setup for multi-animal recording (A) Embla A10 polygraph. (B) Computer attachment for recording and analysis of electrophysiological signal. (C) Operated rat attached to polygraph.
a tranquilizer such as Xylazine. Level of anesthesia was observed by using the tail pinch response and breathing pattern. If at any point of time during the surgery the anesthetic seems to be wearing off, another booster dose (~40% of the initial dose) was administered.

B) Surgery

After mild anesthetization, animal was made to rest on the stereotaxic platform and head of the animal was fixed with the help of ear bars. During the surgery, petroleum (Neosporin) jelly was applied over the eyes, and a midline incision of 2 cm was made along the scalp. Burr holes of 0.5 mm diameter was drilled on the surface of the skull marked stereotaxically for placement of electrodes and intracortical injection (Fig-1). Insulated (except at the tip) flexible insulated wires were connected bilaterally to dorsal neck muscles and muscles near external epicantus of eyes to record bilateral electromyogram (EMG) and electrooculogram (EOG), respectively. Each electrode was connected through wire to the individual pin of the 15 pin adaptor. Later 15 pin was affixed on the surface of the skull with dental acrylic cement to make a robust platform.

FeCl₃ injection

Coordinate for FeCl₃ injection was antero-posterior = -1.0mm; lateral = 1.0mm and ventral (depth) =1.5mm. The depth of electrode is expressed relative to the duramater. 5 μL of 100 mM FeCl₃ or physiological saline (pH=2) (vehicle) was injected through the burr hole in the somatosensori region of the cortex with the help of injector cannula. After injection burr hole was sealed with bone wax.

Electrode implantation

Electrodes were placed in the burr holes drilled on the scalp at coordinate points determined following the rat brain atlas of Paxinos and Watson (1982) in the following region of the brain.

Cortex

Coordinates for cortical electrodes: 2mm posterior and anterior to bregma and 2mm lateral surrounding somatosensori region (Fig-1). Four stainless steel electrodes were placed epidurally in four different burr holes to record ECoG. One screw electrode was placed upon the frontal sinus to serve as animal-ground.
Hippocampus
Stereotaxic coordinate for placement of CA1 electrodes: AP = 2.8 mm, Lateral 2.5mm, ventral= 2.71 mm. Exact location was verified, following the histological procedures.

Fig-10. Representing the positioning of epidural screw and ground electrodes on the skull of Wistar rat for recording field potential in the form of Electrocouticogram (ECoG). G: Animal ground electrode; I: Ipsilateral side; C: Contralateral side; SI: Site of injection for FeCl3/ Saline pH=2. Bipolar electrodes were placed in the CA1 field to record field potential in the form of depth EEG. Additional Electromyogram and Electrooculogram electrodes were placed to record EMG and EOG.

C) Recovery
Operated rats were provided with optimal post-operative care and habituation before video-EEG recording. Nebasulf was applied around the wounds to prevent infection. Rats were monitored continuously; in case of low water consumption or dehydration a 1cc saline or 10%sucrose was injected subcutaneously. Each rat was checked for health status by observing various criteria such as tail sores, posture hunch, grooming, nose red rims, red eye rims, tumors, teeth etc (Sharma et al., 1993; Markowaska et al., 1990). Recording was started after complete recovery from the surgery and enough habituation (five days) in the recording chamber.

D) Recordings
EcoG and depth EEG were recorded from the ipsilateral and contralateral sides of the cortex and CA1 field of hippocampus. Bipolar ECoG recordings were performed from the epidural screw electrodes placed in the parietal cortex on ipsilateral and contralateral sides of injection site (SI). Similarly, bipolar depth EEG recordings were performed with the
help of insulated tungsten wire electrode placed in the CA1 field of ipsi- and contralateral hippocampus. ECoG and EEG records were obtained minimum from six animals from each control as well as experimental groups.

4.3.2. Digital Electro-behavioral recording setup and analysis

After recovery rats were placed for continuous synchronized video-EEG recording with the help of Embla A10 and Somnologica Studio software. The signals were sampled at a frequency of 99 Hz and filtered with a low-pass filter cut off at 45 Hz and high pass filter cut-off at 1 Hz with the help of Embla A10 instrument and recorded through Somnologica software. Differential EEG recording was done in order to minimize EEG artifacts. In ECoG the non-ictal discharges were distinguished from ictal discharges based on waveform morphology and frequency and the associated behavioral alterations respectively as described by Kharlamov et al., (2003). Qualitative and quantitative ECoG analysis was performed off-line, paying special attention to transient ictal discharges and associated behavioral alterations. Somnologica automated analysis was not sufficiently sensitive to discriminate arrival of seizures. Therefore, seizures were later verified manually by routing signals through FFT and CWT. Each animal from every group was subjected for 8 hours video-EEG recordings in one week and a total recording was done minimum for 22 weeks in young and 16 weeks in old. In old epileptic rats generalized grade IV seizures started appearing in 12th week. We used Racine behavioral score of kindling in its modified form described by (Racine et al., 1972; D’ Ambrosio et al., 2004) to categorized different classes of epileptogenesis in this study (Table-1). Recordings obtained from different set of experiments were analyzed randomly on the basis of broad classification of seizures described in table 1. In addition, several typical transient seizure discharges were indentified to analyze for the spectral composition by employing Fast Fourier transform (FFT) and continuous wavelet transform (CWT) algorithms. After recording brain from each animal was taken out and fixed in formal dehyde. Paraffin serial sections of 10µm were cut and stained with cresyl violet stain to verify the site of electrode implantation in hippocampal CA1 region of the brain (Fig-7).
4.3.3. Spectral analysis:

FFT was performed to analyze the dominant frequencies of transient discharges appearing in the EEG traces with the help of Somnologica analyzing software. Signals were transformed to a complex exponential function (or a sinusoidal function) to get resultant signal appeared in the frequency domain. FFT was used traditionally to analyze EEG signal, as infinite basis functions in Fourier analysis. It is able to extract frequency information specifically from periodic, non-transient signals. Since, FFT analysis is unable to capture the transient features in a given signal and the time frequency information, any local behavior of a signal cannot be easily traced with Fourier transformation. Therefore, continuous wavelet transform (CWT) was performed to resolve frequency in relation to time with the help of MATLAB analysis software.

CWT is a more suitable and powerful tool for analyzing transient signals as both frequencies (scales) and time information can be obtained. Long time intervals (corresponding to smaller values of scaling parameter “a”) are used for more precise low-frequency information and shorter intervals (corresponding to larger values of a) for the
time locality of high-frequency information. Hence, if the wavelet function (eq-2), has a finite duration, then the frequency information obtained from the wavelet transform is localized in time. Therefore, for transient signals such as EEG, the wavelet analysis is superior to Fourier transform.

If \( f(t) \) is a square integral function of time, \( t \), then the CWT of \( f(t) \) is defined as (Chui, 1992) eq-1.

\[
W_{a,b} = \int_{-\infty}^{+\infty} f(t) \frac{1}{\sqrt{|a|}} \psi^* \left( \frac{t - b}{a} \right) dt
\]

(1)

\[
\psi_{a,b}(t) = \frac{1}{\sqrt{|a|}} \psi \left( \frac{t - b}{a} \right)
\]

(2)

Eq. (1) can be expressed as

\[
W_{a,b} = \int_{-\infty}^{+\infty} f(t)\psi_{a,b}^*(t) dt
\]

(3)

where \( a, b \in \mathbb{R} \), \( a \neq 0 \), \( \mathbb{R} \) is the set of real numbers, the star symbol '*' denotes the complex conjugation, and the wavelet function is defined as The factor \( 1 = \sqrt{|a|} \) is used to normalize the energy so that it stays at the same level for different values of \( a \) and \( b \). The wavelet function \( \psi_{a,b}(t) \) becomes narrower when \( a \) is increased and displaced in time when \( b \) is varied. Therefore, "\( a \)" is called the scaling parameter which captures the local frequency content and \( b \) is called the translation parameter which localizes the wavelet basis function at time \( t=b \) and its neighborhood.

In the present study, coefficients for CWT was generated by using Daubechies order 4 wavelet (Daubechies, 1988). Daubechies family of wavelets is known for its orthogonality property and efficient filter implementation. Daubechies order 4- wavelet was reported to be the most appropriate for analysis of epileptic EEG data (Adeli et al., 2003).
4.4. Biochemical experiments

4.4.1. Brain regions

Biochemical assays were made on the following regions:

i) Cerebral cortex: assays were performed separately in the ipsilateral and contralateral side of the cortex

ii) Hippocampus: Ipsilateral and contralateral hippocampus of each rat was pooled during homogenization to obtain optimum amount of tissue sample for biochemical assays.

4.4.2. Homogenate Preparation

After electrophysiological recordings, each rat was sacrificed by cervical dislocation and brain was removed immediately for biochemical analysis. Each brain was washed with normal saline to remove blood smear and cooled in a deep freezer. Ipsilateral and contralateral cortical hemisphere and hippocampi were rapidly dissected out on ice plate, according to the stereotaxic atlas by Paxinos and Watson (1982). Specimens dissected out from Ipsi and contralateral hemisphere were homogenized separately in 50mM Tris buffer (pH=7.4) containing protease arrest kit (Genetech) with Potter-Elevehijam type homogenizer fitted with teflon plunger. Assays were done separately minimum in six animals of each group. The homogenate was diluted 1:10 (with Tris, pH=7.4 buffer) and centrifuged at 6000 rpm for 10 minutes, in refrigerated centrifuge (Sorvall RCS or RC5C). The resulting pellet (P1), consisting of nuclear and cellular material, was discarded. The supernatant (S1) was further centrifuged at 14,000 rpm for 25 min. to separate synaptosomes and mitochondria (P2) from microsomes and cytosol (S2). Na-K ATPase and membrane fluidity was performed in the crude synaptosomal fraction, while lipid peroxidation, protein oxidation and cytosolic protein kinase C activity was performed in the cytosolic fraction (S2).

4.4.3. Estimation of Lipid Peroxidation (TBARS Content)

Lipid peroxidation was estimated by measuring the TBARS content as per the protocol of Ohkawa et al., 1979 with minor modifications.
**Principle**
The test material was heated at low pH with TBA and the resulting pink chromogen was measured by absorbance at 532 nm or by fluorescence at 553 nm (Gutteridge and Helliwell, 1990). The chromogen formed by reaction of one molecule of malondialdehyde (MDA) with two molecules of TBA. Several other aldehydes formed in peroxidizing lipid systems also give different chromogens with TBA.

**Procedure**
To 250 µl of tissue homogenate (S2 fraction), 100 µl of 4% sodium dodecyl sulfate (SDS) was added to which 750 µl of 2M HCl in 1% CH₃COOH and 0.8% TBA were added in a glass centrifuge tube in the same sequence. Mixtures were incubated at 95°C in water bath for 1 hour. After incubation, solutions were cooled at room temperature and centrifuged at 2000 rpm for 20 minutes. The pink colour developed was recorded at 532 nm using UV-260A spectrophotometer. The amount of TBA-RS formed was calculated as TBARS content per mg protein. Tetra-methoxy propane (TMP) was used as standard.

4.4.4. Estimation of Protein oxidation (Carbonyl content)

**Principle:**
Protein oxidation is most often indexed by the presence of carbonyls. Protein carbonyls arise from direct free radical attack on vulnerable amino acid side chains or the protein backbone or from the products of glycation, glyoxidation and lipid peroxidation reaction of proteins. Carbonyl groups are composed of stable C=O organic radicals which react with 2, 4-dinitrophenyl hydrazine to form a 2, 4-dinitrophenyl hydrazone. Spectrophotometric determination of protein bound hydrazone was performed at 375 nm.

**Procedure:**
Protein carbonyl content, as an index of protein oxidation was measured according to the modified method of Dubey et al., (1996) and Liu et al., (2003). For each homogenate sample containing 2 mg of soluble proteins were divided into two equal volumes. Four volumes of 10mM DNPH in 2 M HCl were added to one of the sample pair, and four volumes of 2M HCl alone were added to the other one (for reagent blank assay). Samples were then incubated for 1h at room temperature in the dark with continuous stirring and were precipitated with an equal volume of 20% trichloro acetic acid (TCA). After 10 min.
on ice samples were centrifuged at 3000X g for 5 min and supernatants were discarded. Protein pellet were washed in 10% TCA once and in ethanol/ethyl acetate (1:1) three times to remove free DNPH and additional lipid contaminants. Finally protein precipitates were dissolved in 6M guanidine HCl solution. The difference in absorbance between the DNPH-treated and HCl-treated samples were determined by spectrophotometry at 375 nm, and the amount of carbonyl contents was calculated by using molar extinction coefficient (ε) of 22,000-1 cm⁻¹ [ C in nmol/ml = A₃₇₅ (Δ X 10⁶ / ε) ]. Values were expressed as nmoles carbonyl per mg of soluble extracted protein.

4.4.5. Estimation of Na-K ATPase

Na-K ATPase assay was performed as described by Akagawa and Tsukada (1979) with some modification. Activity of ouabain sensitive Na-K ATPase activity was measured as inorganic phosphate (iP) released per mg protein per hour.

Principle:
Na-K ATPase estimation was done by spectrophotometric method. Activity is defined as ouabain sensitive hydrolysis of ATP in the presence of Na⁺, K⁺ and Mg²⁺. Na-K ATPase activity was determined spectrophotometrically by measuring iP liberated during the reaction.

Na-K ATPase enzyme

ATP + H₂O → ADP + Pi

Procedure:
Na-K ATPase activity was measured in the crude synaptosomal fraction (P2) according to the method of Akagawa and Tsukada (1979). The fraction P2 was prepared according to the method of de Robertis et al., (1963) and Gray and Whittaker (1962). An aliquot containing 50 µg protein was incubated with a reaction mixture at 37°C for 30 min. The reaction mixture contained 100mM NaCl, 20mM KCl, 5mM MgCl₂, 3mM ATP and 50mM Tris pH 7.4. Reaction was stopped with addition of 1 ml chilled 10% TCA solution. The tubes were centrifuged at 1000g and liberated inorganic phosphate was spectrophotometrically estimated following method by Fiske and subbarow. (1925). The reaction mixture comprised 1 ml of supernatant, 8.1 ml distilled water, 0.5 ml of acid ammonium molybdate (0.5% ammonium molybdate in 5N H₂SO₄). After incubation at room temperature for 10 minutes, 0.4 ml of reducing agent (0.5g amino napthol sulfonic
acid, 15% sodium bisulfite, 20% sodium sulfite) was added and the absorbance of the
colour developed was read at 600nm in a shimadzu UV-160A spectrophotometer. Ouabain
(1 mM) was used as a specific blocker of Na–K ATPase activity. The ouabain sensitive
Na–K ATPase activity was estimated and expressed as n moles of inorganic phosphate
released per mg protein per hour.

4.4.6. Estimation of Ca$^{2+}$ dependent Protein kinase C (PKC).

**Principle:**
Cytosolic PKC activity was determined by mixing the gamma P$^{32}$ labelled proteins with
cytosolic fraction and incubating mixture containing CaCl$_2$ and HEPES (pH=6.8). In this
condition Ca$^{2+}$ dependent PKC proteins utilizes radio-labeled ATP to phosphorylate their
substrates. Phosphorylation intensity was estimated by using β-counters.

**Procedure:**
PKC assay was performed as described by Hetherington et al., (1982) with some minor
modifications. The assay was performed blindly. Cortical and hippocampal tissues were
homogenized separately in 40 volumes of 1 mM sodium bicarbonate (pH 7.5). The
homogenate was centrifuged at 600xg for 10 min at 4°C. The homogenate was suspended
in incubation medium (100 mM HEPES, 120 mM NaCl, 1.2 mM MgSO$_4$, 2.5 mM KCl, 15
mM NaHCO$_3$, 10 mM Glucose, 1 mM EDTA). Protein kinase activity was assayed in a total
volume of 0.5 mL of incubation medium [50 mM Heps (pH 7), 10 mM MgCl$_2$, 0.5 mM
CaCl$_2$, and 0.2 mM EGTA (free calcium level of 0.1 mM)]. After addition of 100 mg
protein, the reaction was initiated by addition of P$^{32}$ labeled ATP (specific activity, 3000
Ci/mmol ATP) Incubation was carried out at 25°C. Samples of 50 ml were taken out at
appropriate intervals (30–60 sec) and pipetted onto 3 mm filter discs which had been
pretreated with 10% trichloroacetic acid (TCA), 20 mM Sodium pyrophosphate, and 10
mM EDTA. These filter discs were dropped into 500 ml of the TCA mixture and left
overnight at 0°C. Filters were washed once in 5% TCA, heated to 90°C for 15 min in 10%
TCA. Filters were then washed in 5% TCA and extracted in hot ethanol/ether (3:1 v/v)
before drying. Radioactivity was measured by a Beckman-β counter.
4.4.7. Measurement of fluorescence anisotropy

**Principle:**
Membrane fluidity was measured by observing the change in fluorescence anisotropy \( r \), which is inversely related to fluidity of the membrane. DPH probe was used to observe the change in fluorescence anisotropy under different treatment conditions.

**Procedure:**

DPH anisotropy was assessed by employing the method described by Lebel and Schatz (1990) and Muller et al. (1995) with some minor modifications. The crude synaptosomes isolated from every brain tissue was diluted in 50 mM Tris-HCl (pH 7.4), to a final protein concentration of \(~50\, \mu g/ml\). 1 ml of crude homogenate was mixed with an equal volume of 2 mM 1, 6-diphenyl-1, 3, 5-hexatriene (DPH). The DPH diluted from a stock solution of 2 mM in tetrahydrofuran in 50 mM Tris-HCl (pH 7.4). The mixture was incubated at 37°C for 30 min in a shaking water bath. At stable anisotropy fluorescence intensity was recorded using excitation wavelength of 365 nm and emission wavelength of 428 nm for DPH on VARIAN spectrofluorimeter. The DPH fluorescence anisotropy was calculated by using the standard formula:

\[
r = \frac{(I_{VV} - I_{VH})}{(I_{VV} + 2I_{VH})}
\]

Where, \( I_{VV} \) and \( I_{VH} \) represent the intensities of light when polarizes were in parallel and perpendicular orientation. The value was multiplied by the correction factor (G) (Lakowicz 1983; Sanganahalli et al., 2000) and measurements were done at room temperature (25°C).

4.4.8. Estimation of protein

Protein estimation in the cytosolic fraction & synaptosomal fraction was performed following the protocol of Bradford et al., 1976 with some modifications using bovine serum albumin (BSA) as a standard. This is a simple, rapid, inexpensive and sensitive assay, works by the action of comassie brilliant blue G-250 dye (CBBG).
**Principle:**
Protein reacts with Bradford reagent to give a dark blue colored complex. CBBG specifically binds to proteins at arginine, tryptophan, histidine and phenylalanine residues in their anionic form, which has absorbance maximum at 595 nm (blue). Thus, absorbance was measured spectrophotometrically at 595 nm to detect CBBG complex formed with protein. The detection limit of Bradford is 1-20 μg (micro assay) and 20-200 μg (macro assay) so this assay requires small amount of sample for protein estimation.

**Procedure:**
Different concentrations (1, 2, 3 to 10 μg) of bovine serum albumin (BSA) were taken to generate standard curve before starting protein estimation of fresh sample. For protein estimation, 3 μl of homogenate + 47 μl of d H2O + 950 μl of Bradford reagent were taken in each tube. Quantitations of protein were performed at 595 nm using Shimadzu UV-260A spectrophotometer.

**4.5. Histological experiments**

**4.5.1. Regions and area studied**
Transmission electron Microscopy (TEM) and simple light microscopy were used to investigate the cytomorphological impairments consequent to epileptogenesis and curcumin or L-deprenyl treatment in the cortical cells and hippocampal pyramidal cells of CA1 field. Since electrophysiological recordings were performed on cortex and CA1 field, it was rational to study these regions at microscopic level.

**4.5.2. Tissue Processing: (Perfusion and Block preparation)**
Both experimental and control group, rats were anaesthetized with Ketamine (50 mg/ kg i.p.). Each rat was transcardially perfused with physiological saline and then fixed with a fixative containing 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer. Perfusion was performed by infusing the saline into the left ventricle of animal, as the left ventricle channels blood to systemic circulation through aorta. Simultaneously, right auricle was cut to wash out blood and the perfusate from the system. For rats, 200 ml of saline (approx 4 times blood volume) perfusion for 6-8 minutes washes out all the blood.
Following this, 200-300 ml of fixative (phosphate buffer containing 4% para formaldehyde and 2.5% glutaraldehyde) was perfused for 15 mins. After perfusion cranium of each rat was opened and brain was removed and fixed in 10% formalin. After washing, dehydration and paraffin embedding, tissue was cut into 5-7 µm thick sections using rotary microtome for light microscopic studies.

4.5.3. Light microscopy

Light microscopy was performed to verify the positioning of wire electrodes in the CA1 field of hippocampus as well as counting the number of cells in the cortex and CA1 field of hippocampus. The cresyl violet staining was performed according to the method of Disbrey and Rack, (1970). The working solution of cresyl violet was made by mixing 9.5 ml of cresyl violet (1% aqueous solution of stain), 50 ml of dH2O and 0.5ml of 10% acetic acid. The paraffin sections (5 microns) were deparaffinised in xylene and then hydrated through descending grades absolute and different gradation of alcohol. Finally sections were dipped in water and then transferred to cresyl violet stain for 10 min at 60°C. The stained sections were differentiated and dehydrated through ascending grades of alcohol, cleared in xylene and mounted with DPX to make permanent preparations. The slides were observed under Motic microscope attached with an image analyzing software Motic Image plus 2.1.

4.5.4. Electron microscopy

Electron microscopy was performed to investigate the ultra-structural alterations associated with epileptogenesis and curcumin or L-deprenyl treatment. Fei-Philips Morgagni 268D, (100 kV TEM) of maximum magnification = x 2,80,000, was used to observe the sections. Digital image analysis system was used to capture the images after focusing the sub-cellular structures. Pictures were viewed and analyzed for details with the help of Soft imaging software (SIS-viewer).

Principle:
The basic principle behind TEM is that an electron beam of wave length 0.005 nm is used to illuminate the specimen. Electron beam is generated by tungsten filament, which is then focused to the specimen by condenser. Unlike light microscope, doughnut-shaped electromagnets called magnetic lenses were used to focus the beam. The column containing the lenses and the specimen must be under high vacuum to obtain a clear image because
electrons would be deflected by collision with air molecules. The specimen scatter electron passing through it and the beam is focused by magnetic lenses to form an enlarged, visible image of the specimen on a fluorescent screen. Only extremely thin slices of tissue (20-100 μm thick) can be viewed under TEM.

**Procedure:**

The ipsilateral cortex and CA1 field of hippocampus were dissected out of the brain and 1-2 cm pieces were chopped off and fixed in Kanovsky's fixative (4% paraformaldehyde + 2.5% glutaraldehyde in PBS) for 18 hours at 4 °C. Tissues were then washed in PBS and post-fixed in 1% OsO4 for 2 hours at 4°C. For dehydration tissue was washed in PBS and then dehydrated in 30% acetone (2x15 min.), 50% acetone (2x15 min), 90% acetone (2x15 min.), dry acetone (15 min.). Finally tissues were cleared in toluene (2x30 min) at room temperature.

Infiltration was carried out using resins and the component araldite+ hardener (10 ml) + Accelerator (0.4 ml). Tissues were put in decreasing grades of toluene and embedding medium until they were treated with only embedding medium for filtration at 50 °C. Block of the tissues was prepared by embedding in bean capsules with embedding fluid. Polymerization was carried out at 60°C for 12 hours and 60°C for 24 hours.

Ultrathin sections were cut on ultra-microtome using glass and diamond knives. First semi-thin sections were taken on slides stained with methylene blue and observed under light microscope to select the area to be sectioned for ultrathin sections. Next, ultrathin (60-90 nm) sections were cut, stretched using chloroform vapours and lifted on copper grids.

Sections were stained with uranyl acetate and lead citrate. One drop of each stain was taken on a piece of parafilm floating on water in a petri-dish. Each grid was placed on the drop such that the slides containing the section faced down on the stain. Cortical and hippocampal sections were stained in uranyl acetate for 10-15 min., washed in d H2O and stained in lead acetate for 5-6 min. Grids were washed, dried and stored in covered Petri dishes for TEM.

**4.5.5. Micrometric estimations**

Cell counting was performed on cresyl violet stained sections using the optical dissector method described by Tondon et al., (1999); West et al., (1991). At least ten sections
...
screened for their swimming abilities, by recording latencies to acquire platform. Platform was painted white and kept exposed 1.5 cm above the water surface in order to make easily visible to the animal. Animals were habituated to the experimental conditions prior to experimentation by placing them on the water tank for 60 sec. without platform (minimum for 4 days) animals exhibiting significantly lower swimming speed were discarded for learning and memory test. After 4 days of testing, rats were trained to exit the bath onto platform by using the visual cues. Each rat was placed inside the water tank facing the tank wall, at one of the four randomly selected entry points once in every block of four trials. Minimum eight trials per day were performed, and on each trial the latency to reach platform was measured. In case animal fails to find the platform within 60 seconds, it was guided to reach the platform and allowed to remain on the platform for 20 seconds. Each rat was tested for five consecutive trials per day, with an inter-trial interval of 60 sec. Each rat was exposed to the task for four consecutive days (minimum of 20 trials). Location of the platform was fixed during the acquisition period. Morris water maze training was recorded using a web camera mounted at about 1 mt height on the top of the tank (Ozdemir et al. 2005). The data were analyzed by recording latency period for reaching the platform both offline and online. This method is useful to assess the rats’ visuospatial learning abilities. MWM tests were performed between 13:00 h-16:00 h in order to minimize circadian light/day rhythm related variations.

4.6.3. Open field Test

The open field is a very popular animal model of anxiety-like behavior (Prut and Belzung, 2003). The procedure consists of subjecting an animal to an unknown environment from which escape is prevented by surrounding walls. In such a situation, rodents spontaneously prefer the periphery of the apparatus to activity in the central parts of the open field. Open field was performed by modifying the previously described method by Li et al., 2005. In
the present study, the test was performed in a square open field with white-painted wall and floor (diameter 106 cm). Exploratory behavior of rats in the open field was observed in a square arena (70x70x106 cm) with a floor divide into 49 identical squares of 10 cm length. At the beginning of the test, the rat was placed in the center of the open field. The animals were placed individually in the center of the open field. Behavior was observed for 3 minutes. Before each trial, the field was cleaned thoroughly with 0.1% acetic acid solution. The locomotor activity (horizontal) defined as number of squares crossed and rearing frequency (vertical) defined as number of times the animals stood on their hind legs (Sanchez et al., 1998; Colomina et al., 1999) were evaluated. Furthermore, defecation index was also counted by counting the number of faecal boles. The defecation index (number of feces) is an indicator of potential anxiety. (Sanchez et al., 1998). The number of squares entered with the forepaws by the rat within a period of 3 min was recorded. Rearing were recorded manually by the experimenter as described by Brandt et al., (2007).

4.7. Correlation studies

Pearson's correlation test was performed between different parameters with the help of SYSTAT statistical software. Scatter Plot matrix and correlation matrix was plotted to investigate the relationship between different parameters. Correlations were drawn between different parameters studied in epileptic rats of young and old age groups. A correlation value is a measure of relationship between two variables. The Pearson product moment correlation coefficient 'r' is a dimensionless index that ranges from -1.0 to +1.0 and reflects the extent of linear relationship between two data sets. In usage, an r-value of 0.8 and above is considered a high coefficient, and r around 0.5 is considered moderate, and an r of 0.3 and below is considered a low coefficient. Zero value represents no relationship between two parameters.
\[ r = \frac{n(\Sigma XY) - (\Sigma X)(\Sigma Y)}{\sqrt{[n \Sigma X^2 - (\Sigma X)^2][n \Sigma Y^2 - (\Sigma Y)^2]}} \]

Correlation coefficient tells us about two things. a) Magnitude of correlation between two parameters. b) Direction of correlation. Positive sign shows that the two variables are positively related while a negative sign represents inverse relationship. Correlation was performed between electrophysiological, biochemical, behavioral and histological parameters in order to investigate how these parameters are related in epilepsy and aging.

4.8. Statistical tools for data analysis:

Data are presented as mean ± SD (Standard deviation). Ninety-five percent confidence intervals of various means were also determined for calculating significance by using Graph Pad prism statistical package. One way analysis of variance (ANOVA) was used to analyze the data. Bonferroni Posthoc Test was performed for multiple comparison tests for significance between different parameters.