SYNOPSIS

Epilepsy is a second most common neurological disorder, characterized by recurrent seizures of cerebral origin. It occurs due to imbalance between excitatory (glutamate) and inhibitory (GABA) neurotransmitters, which leads to increased influx of sodium (Na^+) ions in neurons, thus lowers the seizure threshold resulting in recurring fits. According to current WHO – ILAE (International League Against Epilepsy) reports, epilepsy afflicts 50 million people worldwide including 5.5 millions in India. Its prevalence rate is 6 per 1000 individuals and the condition is most common in children.

Epilepsy can be classified in to two major types: when a focal area of the brain is involved, the condition is classified as "Partial seizures" (e.g. Simple and Complex partial) and when the entire brain areas are involved simultaneously, the condition is classified as "Generalized seizures" (e.g. *Petit mal* and *Grand mal*, Atonic and Status epilepticus). The commonest type is *Grand mal*, generally referred as the "epileptic fit". Approximately 60% of all epilepsies are "idiopathic" or "cryptogenic", whereas in "symptomatic" cases, it could be due to brain injury, metabolic disturbances, infections, cerebrovascular diseases, brain tumors etc.

The antiepileptic drugs (**AEDs**) used in the treatment of epilepsy are structurally dissimilar; but functionally these fall in two groups: GABA enhancers and glutamate inhibitors. The conventional AEDs include Carbamazepine, Phenytoin, Phenobarbital, Valproate, Benzodiazepines etc. Although they have a wide range of side effects; these drugs are ineffective in about 30% of cases. The newer generation AEDs include Lamotrigine, Gabapentin, Vigabatrin, Topiramate, Clobazam and Felbamate have improved efficacy and tolerability. Besides being used as antiepileptics these drugs have

emerged as potential agents for the treatment of other neuropsychological disorders. However, in spite of their wide use, sufficient information regarding their long-term efficacy, toxicity etc. is not well documented.

Since cerebral work directly in human subjects is impracticable, several animal models of epilepsy that includes chemically induced or genetic strains are been employed to study the above mentioned aspects.

However, what leads to specific form of epilepsy is not well understood. Nevertheless, an extensive survey of the studies carried out so far in humans as well as in animals reveals some metabolic and functional abnormalities. In spite of this, only few investigations have been carried out to evaluate the basic biochemical defects underlying epilepsy at subcellular levels. The effects of AEDs on GABA, glutamate and amine neurotransmitters and receptor binding activities have been reported, although, in depth biochemical investigations to evaluate their action and/or effects at subcellular levels are lacking.

The present studies have been focused to elucidate biochemical mechanism(s) underlying epilepsy. The studies were carried out to decipher the possible defects at subcellular membrane systems – mitochondria and lysosomes – and to check whether these defects are corrected by the AEDs viz. Carbamazepine (CBZ), Lamotrigine (LTG) and Clobazam (CLB). Studies reported in the present thesis have been carried out in brain tissue of picrotoxin (PTX) induced epileptic model of rat. Parallel studies were also carried out on liver – major metabolic and detoxification organ – that served as internal control.

Chapter 1 of the thesis embodies **"Introduction"** which gives a general overview of the literature on epilepsy; classification, epidemiology, pathophysiology and diagnosis of the epilepsy. A general overview of the recent advances in epilepsy is also included. A large number of conventional and new generation AEDs is used for treatment and management of epilepsy. The pharmacological properties, reported mode of action, toxicity / adverse reactions and biochemical effects etc. with special emphasis on the AEDs in focus – CBZ, LTG and CLB - are described in detail in "Introduction" chapter. Since the studies were carried out on mitochondria and lysosomes, a brief account along with relevant reports on lysosomes; mitochondrial energy conversation mechanisms and membrane structure-function relationships is also included.

Mitochondrial oxidative phosphorylation provides the majority of the ATP required for neuronal metabolism, which is utilized to maintain the neuronal plasma membrane potential. Mitochondria can also modulate synaptic transmission at the neuromuscular junction. In addition to its energy providing function, mitochondria are an important intracellular Ca^{2+} sequestering system in neurons. Since, mitochondrial function is a key determinant of both excitability and viability of neurons, it was of interest to assess the status of mitochondrial oxidative energy metabolism in the chronic condition of generalized (tonic-clonic) seizures. These results are summarized in **Chapter 2** of the thesis.

It was observed that, after PTX-induced convulsions, state 3 and state 4 respiration rates were decreased (27-41%) in glutamate as the substrate. The ADP-phosphorylation rate decreased by 44%. When pyruvate + malate was used as the substrate pair, state 3

respiration rate decreased by 16% whereas 1.3 folds increase was observed for state 4 respiration rate. ADP/O ratio decreased by 33% therefore corresponding decrease by 44% in ADP-phosphorylation rate was seen. However, the extent of uncoupling of ATP synthesis was higher when succinate was used as the substrate. Here also, state 3 and state 4 respiration rates were decreased marginally (13-14%) with drastic reduction in ADP-phosphorylation rate by 65%. An opposite trend was observed when ascorbate + TMPD were used as the electron donor, with 1.4 to 1.5 folds increase in ADP/O ratio and ADP-phosphorylation rate.

Glutamate dehydrogenase (GDH) activity increased by 4 folds after PTX-induced convulsions. The malate dehydrogenase (MDH) activity (mitochondrial and cytosolic both) and succinate dehydrogenase (SDH) activity were decreased by 21-80%, with maximum effect being seen for SDH. Cytochrome b content was decreased significantly after PTX treatment. While the ATPase activity increased in basal (-/-) and DNP-stimulated conditions by 13-30%; whereas Mg-stimulated activity was decreased, therefore the total activity (DNP + Mg stimulated) reflected the composite effect. These results are clear indicative of mitochondrial respiratory chain dysfunction in brain in the chronic epileptic condition.

Since the major metabolic site for PTX is liver, where it's converted in to pircotoxinin and picrotin of which picrotoxinin being an active component. In view of the above, liver mitochondrial functions were assessed, which showed decreased state 3 and state 4 respiration rates by 13 to 57% for all the substrates except when ascorbate + TMPD was used as the substrate (1.3-1.6 folds increase). The uncoupling of mitochondria with

drastic decrease in ADP-phosphorylation rate was observed for glutamate, pyruvate + malate and succinate as the substrates. Differential alterations were seen for GDH, MDH and SDH activities and cytochrome contents. Basal, Mg and DNP stimulated ATPase activity was elevated after PTX treatment.

Since mitochondrial ATPase plays an important role in ATP synthesis, the effect of PTX-induced convulsions and AED treatment on brain F_oF_1ATPase was checked and the results are summarized in **Chapter 3** of the thesis.

PTX-induced seizures caused significant alterations in ATPase activity in the epileptic rat brain. When the epileptic animals were treated with AEDs (CBZ, LTG and CLB); all the three AEDs decreased basal and DNP-stimulated activity (21-57%), which was found to be elevated in epileptic condition. CBZ and CLB treatment to epileptic animals increased the Mg-stimulated and total ATPase activity by 1.5 to 2.7 folds with compared to controls. However, LTG treatment restores the Mg-stimulated and total activity in epileptic animals.

AEDs treatment to control animals revealed decrease in Mg-stimulated and total activity by 41-46% in CBZ treatment. LTG elevated the basal and DNP-stimulated activities by 1.2 folds, whereas the total activity was elevated by 1.3 fold in the CLB treatment group. Thus, differential effects of AED treatment to control animals were noteworthy, however treatment with AEDs to the epileptic animals reflects the restoration of ATPase activity to some extent.

In the view of the changes in ATPase activities, further studies were conducted to examine the effect of PTX-induced convulsions and AED treatment on substrate and temperature kinetic properties of the mitochondrial ATPase. The substrate kinetics analysis had shown that brain mitochondrial ATPase was resolved in two kinetic components with different Km and Vmax (Vm) values. In epileptic condition, Km and Vm of component I increased by 1.4 folds. Whereas, the Km of component II was increased by 1.2 folds. Treatment of epileptic animals with AEDs showed 1.7 to 1.9 times increase in Km and Vm of component I with all the three AEDs under study, as compared to controls. Increase in Km and Vm of component II was observed with the change of substrate saturation curves after CBZ treatment to the epileptic animals.

AEDs treatment to control animals in general resulted in differential decrease in Km and Vm for component II.

Studies on temperature – dependence of ATPase revealed that the Arrhenius plots in the control group were biphasic with two energies of activation E1 and E2 and a phase transition temperature (Tt). PTX-induced seizures resulted into 1.2 fold increase in both E1 and E2 while Tt was decreased by 4 °C. CBZ treatment to epileptic animals resulted into the restoration of E1 and E2 and thus Tt back to the controls. LTG treatment decreased E1 and E2 and thereby composite decrease in Tt was observed. Treatment with CLB decreased E1 by 10% with 5 °C decrease in Tt in the epileptic animals.

On the other hand, treatment with AEDs to the control animals revealed 1.2 to 1.5 times increase in E1 with maximum extent was seen for CBZ treatment. Tt was decreased by

3.3 °C after CBZ treatment while 4 to 6 °C elevation was observed in LTG and CLB treated groups.

Thus, altered substrate and temperature kinetics following PTX-induced seizures suggests that the system is trying to compensate for the decreased respiratory activity by changing the kinetic properties of ATPase. However, these alterations under the epileptic condition were not corrected by the treatment with AEDs; in-fact these AEDs exert their own effect on the system, as evident from the AED treated control animals.

In the next series of experiments kinetic properties of liver mitochondrial ATPase were monitored. These results are described in **Chapter 4** of the thesis.

Thus basal, Mg and DNP stimulated ATPase activities were elevated by 1.12 to 1.9 folds with decrease in total activity by 33% after PTX treatment. CBZ treatment to the epileptic animals resulted in 1.5 fold increase in total ATPase activity, while the basal and DNP-stimulated activities were restored back to normal. 1.5-fold increase in the total ATPase activity was observed in treatment with LTG to the epileptic animals. CLB treatment decreased DNP-stimulated and total ATPase activity by 14-16% with 1.7 to 1.9 folds elevation in basal and Mg-stimulated activities in the epileptic animals.

CBZ, LTG and CLB treatment to the control animals resulted into 1.3 to 1.5 fold increase in the Mg-stimulated activity. LTG treatment showed 1.3-fold increase in DNP-stimulated ATPase activity.

The substrate kinetic analysis of liver mitochondrial ATPase resolved in three-component system with different Km and Vm values. PTX exposure resulted into increased Km and Vm for component II with the loss of component III. CBZ treatment to the epileptic animals resulted in increased Km and Vm of component I and II. Similar trend was observed for LTG treatment to the epileptic animals. CLB treatment in general showed increased Vm for component I and II by 1.2 to 1.8 folds in the epileptic animals. Component III was not restored in all the AED treatment groups.

AED treatment to the control animals resulted in increased Km (3.5-5.4 folds) and Vm (1.8 to 2.2 folds) with the loss of component III.

In the temperature kinetic analysis, marginal decrease in E2 in liver was observed in PTX-treatment group. When, the epileptic animals were treated with AEDs, a generalized decrease in E1 and E2 was seen for all the three AEDs. Tt was decreased by 5 °C after CBZ treatment to the epileptic animals. Similar trend of generalized decrease (10-35% decrease) was observed for all the three AEDs when these are given to the control animals. CBZ treatment elevated Tt by 5.1 °C.

Taken together, chronic PTX treatment resulted in altered substrate kinetic properties of the liver mitochondrial ATPase. Temperature kinetics revealed only marginal change. However, AED treatment in general to the epileptic animals showed tissue specific differential alterations.

Since mitochondrial respiratory chain including F_oF_1ATPase are membrane bound moieties, any alteration in lipid / phospholipid (PL) environment can result into functional impairment of the system as a whole. Hence, the studies were extended to examine the effect of PTX-induced seizures on the lipid composition of mitochondria. These results on brain mitochondrial membrane are described in **Chapter 5** of the thesis.

PTX-induced convulsions resulted in increased lysophosphatidic acid (Lyso), sphingomyelin (SPM) and phosphotidylcholine (PC) components; while phosphotidylserine (PS), phosphotidylinositol (PI), phosphotidylethanolamine (PE) and diphosphotidylglycerol (DPG) were reduced by 17 to 78%; with maximum reduction was observed for DPG. Total phospholipid (TPL) and cholesterol (CHL) contents decreased significantly without affecting the TPL/CHL molar ratio. Brain mitochondrial membrane was somewhat more fluidized under the epileptic condition. CBZ treatment to the epileptic animals resulted into 1.2 to 1.9 folds increase in Lyso, SPM, PS and PI components; while DPG and PC were decreased significantly as compared to controls. Drastic decrease (43 to 51%) in the TPL and CHL contents was seen with composite decrease in the TPL/CHL ratio. While LTG treatment to the epileptic animals imparts restoration of Lyso, SPM, PC, PI and PS to the normality with slight increase in PE while DPG was decreased by 55%. When the epileptic animals were treated with CLB, decreased PS, PI and PC was observed with 1.2 to 1.5 fold elevation in Lyso and DPG. Both, LTG and CLB treatment to the epileptic animals decreased the TPL and CHL contents by 18-27% with increased mitochondrial membrane rigidity.

CBZ treatment to control animals resulted into increased Lyso, SPM and PS with decreased PI, PC and DPG components. TPL content was decreased by 46% with the lowering of TPL/CHL molar ratio. Whereas, LTG treatment caused increase in SPM, PS and PE with decrease in PC and DPG percentage composition. CHL content was decreased by 16%. Mitochondrial membrane became more fluidized in CBZ and LTG treated control group. Decrease in PI and DPG with increase in SPM component was noted in CLB treatment to the control animals. Here, TPL content was decreased slightly with increased membrane rigidity. Hence, AEDs differentially alters the lipid composition.

Thus, massive alterations in the distribution of the individual phospholipids of the brain mitochondrial membrane as observed in the epileptic condition was not normalized by the AED treatment, except for LTG treatment, which restores the majority of PLs to some extent.

In the next set of experiments, effects of PTX and AED treatment on liver mitochondrial lipid composition was evaluated. These results are described in **Chapter 6** of the thesis.

PTX treatment resulted in marginal changes in the percentage composition of individual PLs. CHL content was increased by 1.2 folds with 14% decrease in TPL/CHL ratio. Membrane fluidization was prominent in PTX treatment. CBZ treatment to the epileptic animals resulted into 1.4 to 4 folds elevation in Lyso, SPM, PS and PI with decreased PC and DPG components. TPL content was further decreased by 50% with increased CHL content and thereby decreased TPL/CHL ratio. LTG treatment caused increased Lyso,

SPM and PI by 1.3 to 1.7 folds as compared to controls with marginal effects on PS, PC, DPG and PE components. Lyso, SPM and DPG were elevated by 1.3 folds in CLB treated epileptic animals. TPL content decreased with the lowering of TPL/CHL ratio.

CBZ treatment to the control animals resulted into increased PS, PI and DPG with decreased PC and PE components. Similar increase in PS and PI was also observed with decreased DPG in the LTG treatment. Opposite trend was seen for PS, PI and DPG after CLB treatment. General increase in Lyso and SPM was noted by 1.5 to 4 folds after AEDs treatment to the control animals. All the three AEDs caused decrease in TPL content by 12 to 26%, thereby lowering of TPL/CHL ratio.

Therefore, in general, AEDs can exert alterations in the liver mitochondrial lipid composition in control animals. However, despite of marginal change in PTX treatment group, the AED treatment to the epileptic animals differentially affected the PL profile, which is clearly pinpointing the sole cause of AED treatment in liver mitochondria.

Sudden burst of electrical activity in epilepsy results into neurodegeneration because of excitotoxicity. Such hypoxic neuronal death results into the necrotic process, which may involve the role of lysosomes as a cause or consequence. Hence, studies on the lysosomal functions were carried out in PTX-induced convulsions and in AED treatment. These results are summarized in **Chapter 7** of the thesis.

In the brain, PTX-induced seizures resulted in increased free RNAse activity by 1.3 folds, whereas free DNAse activity was decreased. Total activity for acid phosphatase (β-

glycerophosphatase, BGPase), ribonuclease (RNAse) and deoxyribonuclease (DNAse) was decreased by 35 to 41%. Cathepsin D activity was completely abolished in the epileptic condition. The ratio of Total activity / Free activity (T/F ratio), which is an indicator for lysosomal membrane integrity, was lower for all the enzymes with maximum extent seen for RNAse. When the epileptic animals were treated with AEDs, CBZ treatment caused 1.2 folds elevation in free BGPase activity with 23% decrease in the total activity. Free as well as total RNAse activities were decreased by 38 to 60% with compared to controls. Cathepsin D activity was not restored by CBZ treatment. While DNAse activity was further abolished. LTG treatment restored the cathepsin D activity in the epileptic animals, with loss of DNAse and RNAse activities; which was tending to decrease in the epileptic condition. The T/F ratio was improved for catehpsin D activity in LTG treatment to the epileptic animals. CLB treatment caused increased free BGPase and RNAse activities, whereas cathepsin D and DNAse activities were lost. T/F ratio in general was reduced after CLB treatment to the epileptic animals.

AED treatment to control animals resulted into increased free cathepsin D activity in CBZ and CLB treatment groups; whereas LTG treatment showed 2.5 folds elevation in free RNAse activity. The total activity, in general, decreased with the AED treatment. Therefore, generalized decrease in T/F ratios was observed in AED treatment.

Thus, in brain, lysosomal dysfunction was evident in the epileptic condition, which were not corrected by the AED treatment. In fact, AEDs adversely affected the lysosomal functions.

In the liver, increased free and total nucleases activities were observed in PTX treatment. Cathepsin D activity remained undetected. The T/F ratio was low for all the enzymes. However, CBZ treatment to the epileptic animals caused significant increase in free DNAse and BGPase activity, with decreased total activity for RNAse and BGPase. Cathepsin D activity was not restored in the epileptic animals treated with CBZ. Decreased total activity by 31-62% was seen for BGPase, DNAse and RNAse in LTG treatment to the epileptic animals. Cathepsin D activity was restored with 1.3 folds increase in the total activity and improved T/F ratio. CLB treatment showed increased free activity for BGPase, DNAse and RNAse with increased total activity. In general, treatment with all the AEDs decreased the T/F ratio in the epileptic animals.

CBZ, LTG and CLB treatment to the control animals in general, resulted into increased free activity of DNAse in liver. The total RNAse activity was increased with decreased free BGPase and cathepsin D activities after CBZ treatment. LTG treatment resulted into increased free cathepsin D and decreased total BGPase and cathepsin D activities. In CLB treatment, free cathepsin D and RNAse activities were elevated. Whereas, total cathepsin D, RNAse and DNAse activities were increased by 1.8 to 3 folds. T/F ratios in general were lower with all the three AED treatment to the control animals.

Thus, increased liver lysosomal membrane fragility was noted upon prolonged PTX treatment. AEDs adversely affected the lysosomal function and also failed to correct the lysosomal dysfunctions in the epileptic animals, which might be responsible for possible precipitation of the toxic / adverse effects seen in AED treatment.

In conclusion, the results of the present studies thus suggest that functions of subcellular organelles are been adversely affected in the epileptic condition and that AEDs failed to correct these biochemical defects by exerting their own toxic effects. While application of the AEDs are not only essential for the treatment but also for the management of epilepsy. Thus, membrane-stabilizing compounds should be developed to treat and prevent the adverse condition in epilepsy apart from controlling the seizures.

One part from the studies presented in the thesis dealt with the tyrosine estimation in the assay of protease. The method employed for the tyrosine estimation was first described by Folin and Ciocalteu (1927) which was subsequently reviewed by Anson (1937), Spies (1957), Barret and Heath (1977) and Turk *et al.*(1984) for its use in the estimation of tyrosine for proteases assay. However, these methods were less sensitive and not so economic in terms of the use of Folin-Ciocalteu (FC) reagent. Hence, attempts were made to develop a method, which is more sensitive and economic, and at the same time, it can be applicable in tyrosine determination in enzyme assays and in biological tissues. These experiments are described in the supplementary chapter (**Chapter 8**) of the thesis. In the present method, varying concentration of FC was standardized and the most suitable one was employed for the assay. It was found that, in 1.5 ml of assay system, 0.1 ml of suitably diluted FC with final concentration of 0.2 N served as a best reagent for the color development, thus reducing the excess use of FC in the method. A stable blue color with absorption maxima at 750 nm was developed. The time course of color development was determined. Unlike the conventional procedure described by Anson, the total volume of the assay system was reduced, with 2.5 times improvement of the molar extinction co-

efficient, which imparts the sensitivity to the present procedure. After standardizing the optimum conditions, the applicability of the method for enzyme assays e.g. Cathepsin D and for the determination of tissue tyrosine pool was checked. The results obtained by following conventional and those obtained using our improved procedure were in close agreement.

In conclusion, a modified micro-method for tyrosine estimation has been developed which is more sensitive, economic and easily applicable for tyrosine estimation in routine biochemistry.