Characterization of miR-3120 in the experimental model of epilepsy and its response to dietary curcumin in rat brain

Thesis submitted to Jawaharlal Nehru University for the award of the degree of

DOCTOR OF PHILOSOPHY

Submitted by

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JULY, 2017

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DECLARATION

I hereby declare that the research work entitled **"Characterization of miR-3120 in the experimental model of epilepsy and its response to dietary curcumin in rat brain"** presented in this thesis embody the results of original work carried out by me under the supervision of **Prof. Deepak Sharma**, School of Life Sciences, Jawaharlal Nehru University, New Delhi. This work has not been submitted in part or full for any degree or diploma of this or any other University.

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(Candidate)

Date:
July 20, 2017

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CERTIFICATE

This is to certify that the research work embodied in this thesis entitled as **"Characterization of miR-3120 in the experimental model of epilepsy and its response to dietary curcumin in rat brain"** submitted for the award of degree of **Doctor of Philosophy** has been carried out by **Mr. Prince Kumar** under the supervision of **Prof. Deepak Sharma**, School of Life Sciences, Jawaharlal Nehru University, New Delhi, India.

The research work is original and has not been submitted so far, in part or full for the award of any other degree or diploma of any other university.

Prince Kumar
(Candidate)

Prof. Deepak Sharma
(Supervisor)

July 20, 2017

for 21/7/17

Prof. Shyamal K. Goswami
(Dean)

If roses grow in heaven,
Lord please pick a bunch for me,
Place them in my Mother's arms
And tell her they are from me.

Dolores M. Gracia

Dedicated in the memory of my MOTHER

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We cannot solve our problems with the same thinking we used when we created them.

--Albert Einstein.

Pursuing a Ph.D. is a venture through a long journey to reach a destination of glee. I commenced my voyage in 2011, as I can view the journey's end; I take a while to look back. My journey is filled with ups and downs where I can feel the support of copious warm hands in the form of kind help and guidance. This warmth feeling keeps me aware that completing this task took a village and a result of the incomparable contribution of people in a direct or indirect manner, which succoured to reach this accomplishment. Though, it may not be enough to express my gratitude in words to all those people who helped me. Yet, I would like to give my many and many thanks.

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Saving the last for best, I convey my deepest gratitude to my family. Their hard work, believe and encouragement vented in this denouement. This journey would be impossible without their sacrifices; love and support are treasured to me. I also feel immensely pleased to thank my sister Ila for being strong and supportive in our adversities.

Above all I bow my head before Almighty God with whose grace and beatitude, I moved through this venture. Thank you God for all your benediction and shower of blessings on me and being with all the time.

Prince Kumar

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LIST OF ABBREVIATIONS

Abbreviations	Full forms
5-mC	5-methylcytosine
AEDs	Antiepileptic drugs
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APS	Ammonium persulfate
BDNF	Brain derived neurotropic factor
BSA	Bovine serum albumin
CaMK II	Calmodulin-dependent protein kinase II
CBZ	Carbamazepine
CoX-2	Cyclooxygenase-2
DAB	3,3′-Diaminobenzidine
DNMT1	DNA methyltransferase 1
ECoG	Electrocorticogram
EDTA	Ethylenediamine tetracetic acid
$FeCl_2$	Ferrous chloride
$FeCl_3$	Ferric chloride
GABA	Gama-aminobutyric acid
GABRD	GABA-A receptor δ subunit
i.p.	Intraperitoneal
IHC	Immunohistochemistry
iNOS	Inducible nitric oxide synthase
KA	Kainic acid
KEGG	Kyoto encyclopedia genes and genome
miRNAs	Micro-RNAs
mTOR	Mammalian target of Rapamycin
MUA	Multiple unit action potentials
MWM	Morris water maze
NF-κB	Nuclear factor kappa B
NMDA	N-methyl-D-aspartate
nt	Nucleotide
^oC	Degree centigrade
PIKK	Phosphatidylinositol 3-kinase-related kinase
pri-miRNA	Primary miRNA
PTE	Post-traumatic epilepsy
PTEN	Phosphatase and tensin homolog (protien)
Pten	Phosphatase and tensin homolog (gene/RNA)
PTMs	Post translational modifications
RT	Reverse transcription
SAM	*S*-adenosylmethionine
SDS	Sodium dodecyl sulfate
SE	Status epilepticus
SEM	Standard error of mean
STRADalpha	STE20-related adaptor protein α
TBI	Traumatic brain injury

TEMED	Tetramethylenediamine
TLE	Temporal lobe epilepsy
TNF	Tumor necrosis factor
TRAIL	TNF receptor apoptosis-inducing ligand
mg	Milligram
µl	Microlitre
µg	Microgram
µM	Micromolar
nM	Nanomolar

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INTRODUCTION

1. Introduction

MicroRNAs (miRNAs) are endogenous non-coding small RNAs. They regulate gene expression by inhibiting/degrading protein coding mRNAs. However, miRNAs can also upregulate translation (Cao et al., 2006; Kosik, 2006). miRNAs have been found to participate in the molecular mechanisms of many pathophysiological processes including those of neurological disorders. miRNA-214 targets Huntington's gene (Sinha et al., 2011). It is also involved in NMDA receptor-related memory processes (Wang et al., 2014). miRNA-214 has been reported to modulate NMDA receptor-mediated neurobehavioral dysfunction (Wang et al., 2014). Growing body of evidence from human and animal studies have demonstrated numerous miRNAs to be altered, such as let-7, miR-9, miR-146, miR-214 independently (Dogini et al., 2013). In addition to this, expression of some miRNAs was also altered during Traumatic brain injury (TBI) in the cortex of the rat brain (Lei et al., 2009). miR-214 belongs to the family of miR-199 cluster, which is known to be involved in the progression of epilepsy (Aranda et al., 2015; Baumgarten et al., 2013). Moreover, miR-214 has been reported to be dysregulated during Temporal lobe epilepsy (TLE) (Dogini et al., 2013). miR-3120 is another member of miR-199 cluster family having high sequence similarity with miR-214. It is co-expressive with miR-214; thus, both miRNAs together are called as mirror miRNAs (Scott et al., 2012). miRNA-3120 is a brain-specific miRNA which is involved in uncoating of vesicles (Scott et al., 2012) and is also involved in learning.

Changes in the expression of miRNAs occur in epilepsies as miRNAs target a variety of pathways i.e. inflammation, apoptosis, dendritic growth, and spine dynamics, neurites growth, Ca^{2+}- calmodulin-dependent protein kinase-II and thus NMDA receptors. miRNAs are thus seem to be involved in the pathogenesis of epilepsies. For example, miRNA-146a was shown to be upregulated in human temporal lobe epilepsy (TLE), miR-219 was found to decrease in kainic acid model of epilepsy and in the CSF of epilepsy patients, and silencing of miR-219 was found to induce seizures (Dogini et al., 2015). Several miRNAs were found altered in a lithium-pilocarpine model of status epilepticus. miRNA-214 was found to be down-regulated in mesial temporal lobe epilepsy patients (Li et al., 2014). Brain-specific miR-219 and 134 were found to be significantly upregulated with seizures (Li et al.,

2014), miRNAs have even been considered as biomarkers of epilepsy as different miRNAs may be involved in different epilepsies (Li et al., 2014). "Epilepsy is a brain disorder characterized predominantly by recurrent and unpredictable interruption of normal brain function, called epileptic seizures" (Fisher et al., 2005). It is the second most common neurological disorder in the world and it can occur in individuals of all ages; however, the rate of epilepsy is higher among children and in the older population (Hb et al., 1999; Long et al., 2000). This neurobiological disorder affects cognitive, psychological, and social consequences in humans. Further, mortality of epilepsy patients is three times higher than that of the unaffected population. Sudden unexpected death in epilepsy is one of its causes where frequency of deaths in mild epilepsy is 1 in 2500, and in severe epilepsy it is 1 in 100 (Shorvon, 2009). Epilepsy may be sporadic or genetic in origin, thus, categorized on the basis of symptoms. It is characterized as "Symptomatic" when the cause is known, "Cryptogenic" when the cause is unknown, and "Idiopathic" when genetic causes are present (Shorvon, 2009).

Post-traumatic epilepsy (PTE) is one of the most common symptomatic epilepsies in which seizures arise because of traumatic brain injury (TBI). It occurs more frequently in young adults and military personnel because they are more likely to get injured (Annegers et al., 1998; Annegers and Coan, 2000; Salazar et al., 1985). Pathophysiology of PTE may vary in relation to the type of injury, i.e. closed head injury causes edema, axonal damage or ischemic conditions leading to discharge of amino acids or lipids (Evans, 2006), while non-penetrating head injury causes focal contusion and intercranial hemorrhage (Agrawal et al., 2006). These injuries further cause epileptogenesis where alteration in levels of neurotransmitters such as glutamate, gama-aminobutyric acid (GABA), dopamine occurs (Werner and Coveñas, 2015). This may be due to the alteration in the channel proteins e.g Ca^{2+} and Na^{+} channels, GABA receptors and glutamate receptors in the neurons (Demchenko et al., 2017; Mishra et al., 2013; Peng et al., 2004; Zamponi et al., 2010) which further leads to hyperexcitation of neurons followed by neurotoxicity, neurodegeneration or neuronal cell death (Ono and Galanopoulou, 2012). The neurodegeneration mostly occurs by activation of apoptotic pathway in which various pro-apoptotic proteins are involved and regulate through specific signaling cascades such as PTEN and caspase-9 and DNA damage signaling (Schwarzenbach et al., 2012; Zhao et al., 2004; Zhu et al., 2006). There are enumerable reports of possible mechanisms of epilepsies;

however, it is warranted to understand the exact mechanisms of epileptogenesis in various types of epilepsies including PTE.

Iron-induced experimental epilepsy in rodents models the human clinical post-traumatic epilepsy (Willmore et al., 1978). This experimental model has often been used to investigate the mechanism of epileptogenesis and pharmacology of epilepsy (Willmore, 1990). In iron- induced epileptogenesis, glutamatergic mechanisms have been implicated. For example glutamate transporters are down-regulated, glutamate receptors are upregulated and extracellular glutamate levels increase in iron-induced epileptogenesis (Ueda et al., 2001). In the present study, this model was adopted to further investigate the possible involvement of miRNAs in the pathogenesis of epilepsy as miRNAs may show differential responses in different epilepsies/models (Li et al., 2014), Glutamatergic mechanisms are involved in iron-induced epilepsy (Mishra et al., 2013; Ueda and Willmore, 2000) and miRNAs are involved in the regulation of glutamatergic mechanisms (Harraz et al., 2012; Kawashima et al., 2010; Morel et al., 2013). In the present study, we explored the correlation between miRNA-214, miRNA-3120 and development of epileptiform activity in iron-induced epileptogenesis by investigating their expression profiles in epileptogenic tissue.

The second aim of the present study was to determine whether curcumin's anti-epileptic effect (Jyoti et al., 2009) involves curcumin's action on miRNAs. Curcumin is diferuloylmethane. It is obtained from the rhizome of the plant *Curcuma longa* and is a common agent used as a spice in Indian food. Curcumin has been termed as a cureamine and experimentally it has been found to have multiple pharmacological and therapeutic properties of possible clinical importance (Satoskar et al., 1986; Strimpakos and Sharma, 2008). Neurologically it has been found to be a neuroprotective agent (Cole et al., 2007), and exerts antiepileptic action in experimental epilepsy (Noor et al., 2012). It significantly attenuates electrographic and behavioral seizures and their biochemical measures in iron-induced experimental epilepsy (Jyoti et al., 2009). Curcumin has been shown to alter expression of several miRNAs i.e. miR-103, 140, 146a, 148a, 199a, 21, 22, 204, 98, 7 in a variety of experimental conditions (Sun et al., 2008). In the current study we, therefore investigated whether the antiepileptic action of curcumin is mediated by its action on the expression of miRNA-214, miR-3120 and Pten gene in iron-induced experimental

epilepsy. Curcumin appears to be an inhibitor of mTOR signalling (Meng et al., 2013) and therefore curcumin's and pten gene's influence on Pten gene is of interest.

In the current study, the experiments were performed to determine the response of miR-214 and miR-3120 in epilepsy. Thereafter, the antiepileptic effect of dietary curcumin was assessed on the brain of $FeCl_3$ injected rats. The study is divided into the following parts: 1) electrophysiology, 2) behavioral testing, 3) molecular biology experiments (semi-quantitative PCR and western blotting), 4) immunohistochemistry experiments, and 5) bioinformatic analysis.

Electrophysiological recording was performed for the validation of seizures after $FeCl_3$ injection into the somatosensory cortical region of the brain of awake and conscious rats in both ipsilateral and contralateral sites. We also validated the effect of dietary curcumin on the development and progression of seizures, both in young as well as in aged rats.

Morris water maze (MWM) test was performed to verify the behavioral changes due to PTE. The test suggested memory deficit due to $FeCl_3$ injection and development of seizure. Similarly, validation of counter effect of dietary curcumin was also assessed using this behavioral test.

In molecular biology studies, microRNA assay technique was used to quantify the expression of both miRNA-214 and miRNA-3120. Reverse transcription (RT)-semi quantitative PCR was performed for the amplification of miRNAs, and gel quantification method was used to check their expression during PTE epileptic condition in the cortex and hippocampus of the rat brain. Further, we quantified the expression of both miRNAs after dietary supplementation of curcumin in PTE rat models.

In-silico analysis was performed for sequence analysis and target prediction of miRNAs, using different databases i.e. MicroRNA target prediction and function study database (miRdB), mirbase, Kyoto Encyclopedia Genes and Genome (KEGG). The pathways to which these miRNAs are related were searched and their relationship with epilepsy was assessed.

As depicted from bioinformatics analysis CACNA1A and GABRD are target proteins of miRNA-3120, immunohistochemistry (IHC) was used to monitor the

changes in the expression of these target proteins in epileptic as well as curcumin-fed animals. Further, western blotting was performed to validate the IHC studies. Quantification of the expression pattern of both the channel proteins was also done. In addition to that, as a target of both miRNAs, PTEN mRNA expression was also quantified.

REVIEW OF LITERATURE

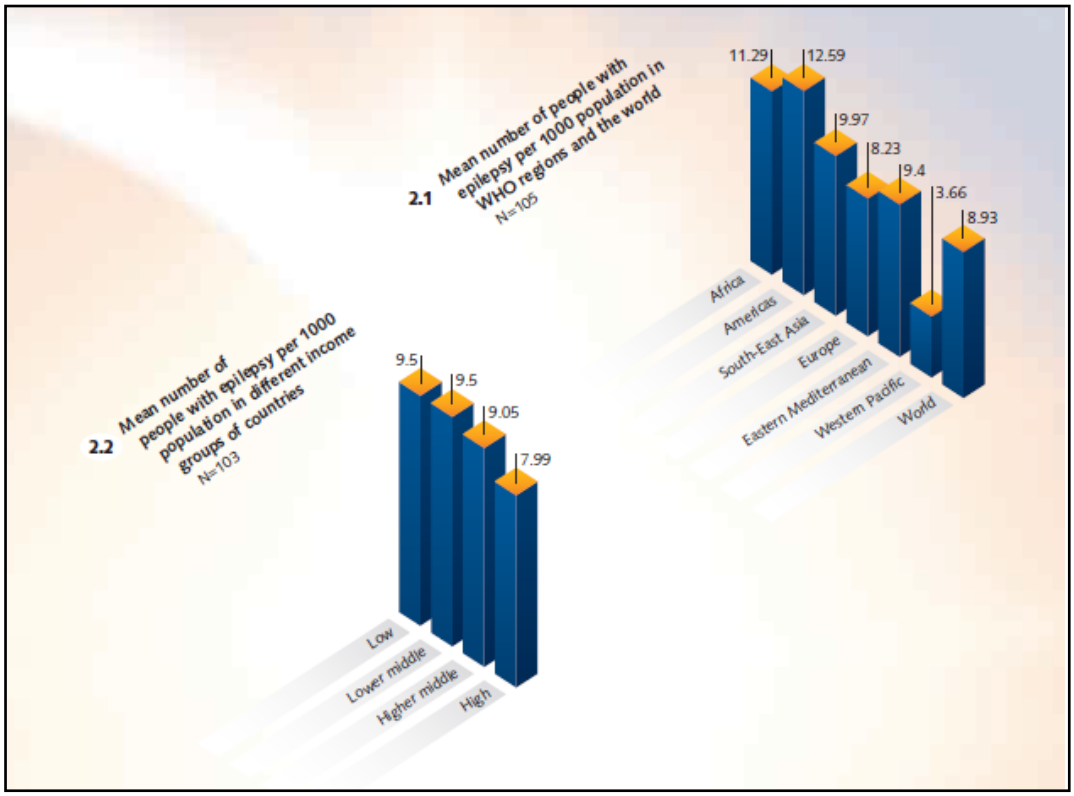
2. Review of literature

2.1. Epidemiology of epilepsy

Epilepsy is one of primeval known neurological diseases in all age of individuals. It is defined by WHO recurrence of unprovoked seizures. It has been estimated that approximately sixty million population worldwide have a diagnosis of epilepsy (Fisher et al., 2005; Thurman et al., 2011). However, most of the population affected by this condition is not evenly distributed all around the globe (Figure.2.1). The increased occurrence of incidence of epilepsy may be co-related to different factors e.g. exposure to eurocysticercosis, limited access to health care, socioeconomic status etc. It has been estimated that 2.4 million population of world are diagnosed with epilepsy annually. In high income countries the prevalence of new cases are 30 to 50 in 100000 people of the common population. However, in middle and low-income countries, this number can increase up to two times (Fisher et al., 2014; Global Campaign against Epilepsy, 2005). The recent reports also demonstrate the occurrence of epilepsy in middle-income or developing country is about 80% (Global Campaign against Epilepsy, 2005).

It is one of the disorders that have a very high risk to become endemic in nature such as neurocysticerosis or malaria due to higher incidents of road accidents, birth-related injuries and medical infrastructure variations including negligence of preventive health programmes and accessible care.

(a)



(b)

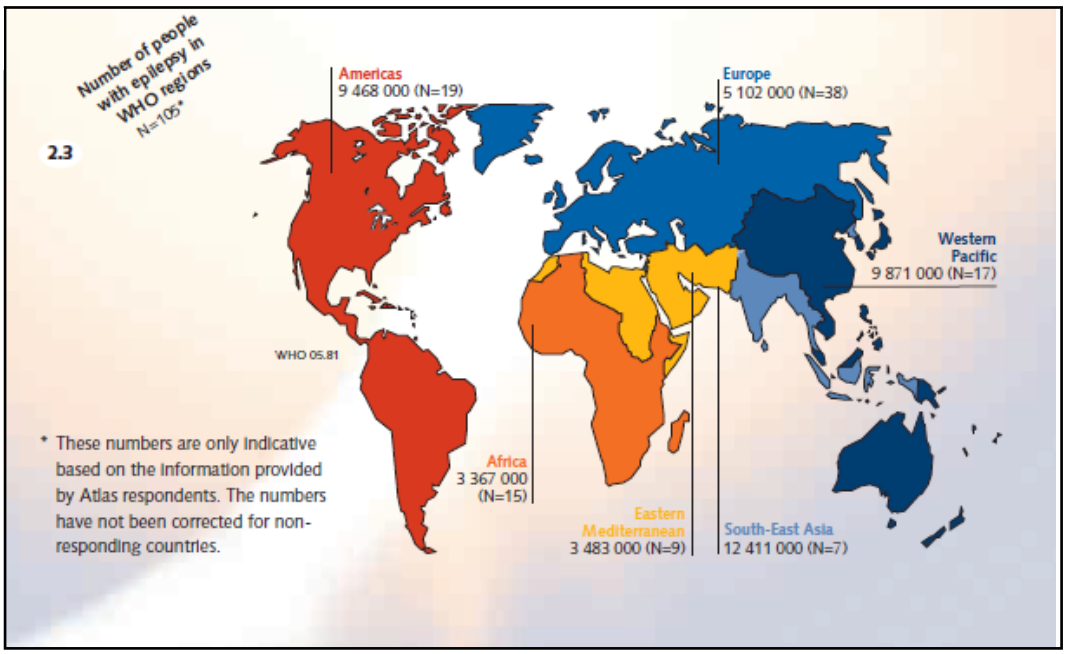


Figure.2.1. Epidemiological distribution of epilepsy around the globe, (a) comparison between income groups and prevalence of epilepsy shows lower income group have higher occurrence of epilepsy, (b) shows the number of occurrence of epilepsy around the world (Global Campaign against Epilepsy, 2005).

There is lack of sufficient data of mortality rate in Asian people. However, the accessible present data reported high rate of occurrence of epilepsy in developing countries of this region e.g. An extremely high mortality rate was found in Laos in which about 90.9 deaths are reported per 1000 person per year. This survey was done in a mountainous zone where a few people had availability of antiepileptic drugs (AED) (Tran et al., 2006). The data from a door-to-door survey of a management project and followed-up reports of patients was small; however, the results from the report clearly indicates the possible condition of mortality rate, Since, Laos in considered as least developed in comparison to all the countries of this region, approximately all the patients were either untreated or inadequately treated in this country (Tran et al., 2006). Interestingly, in china, distribution of mortality was found as 3·9 per 2455 people with epilepsy during the appraisal of epilepsy management at the primary health-care level in rural area (Ding et al., 2006), however, a thorough study is needed to understand low prevalence in rural areas. Similarly, higher mortality is reported in developing countries of other regions of the world also for example 28.9 among 1000 person per year in a rural area of Cameroon, and 31.6 among 1000 person per-years in rural areas of central Ethiopia (Kamgno et al., 2003). Similarly, rate of mortality due to epilepsy is low in most of the developed countries in Asia. In Japan, long study of 18.9 yrs on childhood epilepsy in the general population showed mortality rate of 45 per 1000 (Wakamoto et al., 2000). The study in adult Taiwan patients demonstrated mortality rate of 9 per 1000 person (Chen et al., 2005). Epilepsy patients in Taiwan reported to have 3.5 times higher risk of death than the normal population with mortality ratio of 3.47, 95% (Mac et al., 2007; Neligan et al., 2012).

In India, the affected population is distributed unevenly in different regions. Various studies of many different regions of India showed that about of 3-10 individuals per 1000 which are affected (Fisher et al., 2014). However, in rural areas the occurrence of epilepsy cases were higher due to lack of good health care resources (Amudhan et al., 2015 Santhosh et al., 2014). Survey reports from various states such as Chandigarh, Haryana, Uttarakhand, Kashmir, West Bengal and Tamil Nadu shows higher rate of prevalence in rural areas as compared to urban population (Das et al., 2008, 2006; Gourie-Devi et al., 2004; Santhosh et al., 2014). However, some studies

have also shown higher rate of occurrence in urban population as well (Amudhan et al., 2015; Gourie-Devi et al., 2004; Pandian et al., 2006).

2.2.Etiology of epilepsy

Etiology of epilepsy is an important factor to determining the clinical condition of this disorder, thus, the modern classification of epilepsy is based on etiology. It is divided into four main categories 1) Idiopathic epilepsies 2) Symptomatic epilepsies 3) Provoked seizures 4) Cryptogenic epilepsies (DeLorenzo et al., 2005; Engel, 2001). However, there are some cases which are difficult to categorize in any of these classes of epilepsies. There are many factors involved for causing this dilemma such as multifactorial cause of epilepsy, the knowledge of mechanisms of epileptogenesis rather than knowing the cause of epilepsy only and degree of investigation by using advanced and thorough techniques (DeLorenzo et al., 2005).

The mechanism of epileptogenesis is associated with oxidative stress that can autonomously cause the progression of disease after neuronal injury. Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) are involved in many neurodegenerative disorders (Beal, 1998). While, the actual mechanism of involvement of ROS/RNS in the epilepsies in not fully known; however, numerous reports showing that seizure-induced neuronal death is implicated with ROS-induced oxidation of cellular macromolecules due to repeated seizures (Liang et al., 2000; Waldbaum and Patel, 2010), and depletion of antioxidant defense system (mimetics, vitamin C, melatonin) which functions as prevention of the epileptogenesis and its pathology (Drion et al., 2016; Gupta et al., 2000; MacGregor et al., 1996; Tan et al., 1998). Although, population of all age groups have prevalence of epilepsy, the occurrence of epilepsy is higher in elderly (Hauser and Stiene-Martin, 1991). Similarly, a physical injury (trauma) to the brain causes a cascade of excitation in the neurons which later results in neurotoxicity and seizures (Agrawal et al., 2006).

Information is received by neurons from many different sources. Information between neurons is generally transmitted by synapse. The transfer of information begins with the discharge of neurotransmitters from presynaptic cleft. The information transfer come to its end by transformation of the chemical signal of neurotransmitter into an electrochemical one via opening of ionotropic receptors

which causes flux of ions and further activation of secondary messengers via metabotropic receptors.

2.3. Involvement of neurotransmitters in epilepsy

Neurotransmitter imbalance is an important contributor of epileptiogenesis. There are many reports showing elevated levels of excitatory amino-acids such as glutamate and aspartate, after brain injury in epileptogenic rats (Hillered and Persson, 1999; Nilsson et al., 1994; Wenzel et al., 2000) as well as humans (Carlson et al., 1992; Ronne-Engström et al., 1992). During epilepsy, a network of neurotransmitter mediated signaling takes place in between neurons and supporting cells. Figure.2.2 shows that involvement of various neurotransmitters such as glutamate, glutamine, GABA and gliotransmitters in epileptoform activity. In the neurons, action potential is regulated by Voltage-gated Na^+ and K^+ channels of presynaptic neurons which leads to the release of exocytotic synaptic neurotransmitter glutamate (Bergles and Jahr, 1997). Glutamate activates α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) and N-methyl-D-aspartate (NMDA) receptors of the postsynaptic membrane further leads to influx of Na^+ and Ca^{2+} to generate of excitatory synaptic potentials. If excitation potential is stronger than optimum, this synaptic excitation leads to epileptiform discharges (Tanaka et al., 1997), thus excessive glutamate is taken up into reactive astrocytes by the EAAT1 (GLAST) and EAAT2 (GLT-1) transporters (Bergles and Jahr, 1997), and further this glutamate is converted to glutamine by glutamine synthetase enzyme this glutamine is a substrate which is converted to GABA in inhibitory GABAergic neurons (Benedetti et al., 2011). Loss of glutamine synthetase in reactive astrocytes causes a decrease in GABA production thus hyperexcitation of neurons (Eid et al., 2012). In addition, K^+ is released from neurons by voltage-gated K^+ channels enters in the astrocytes through inwardly rectifying K^+ channels and is distributed into blood capillaries. Ca^{2+} waves activate the release of gliotransmitters that can influence neuronal excitability. This leads to movement of adenosine into astrocytes by the equilibrative nucleoside transporters ENT1 and ENT2, and concentrative nucleoside transporter CNT2. Excessive adenosine kinase in reactive astrocytes causes the elevation of removal of adenosine, hence enhances the hyperexcitability (Boison, 2012; Devinsky et al., 2013).

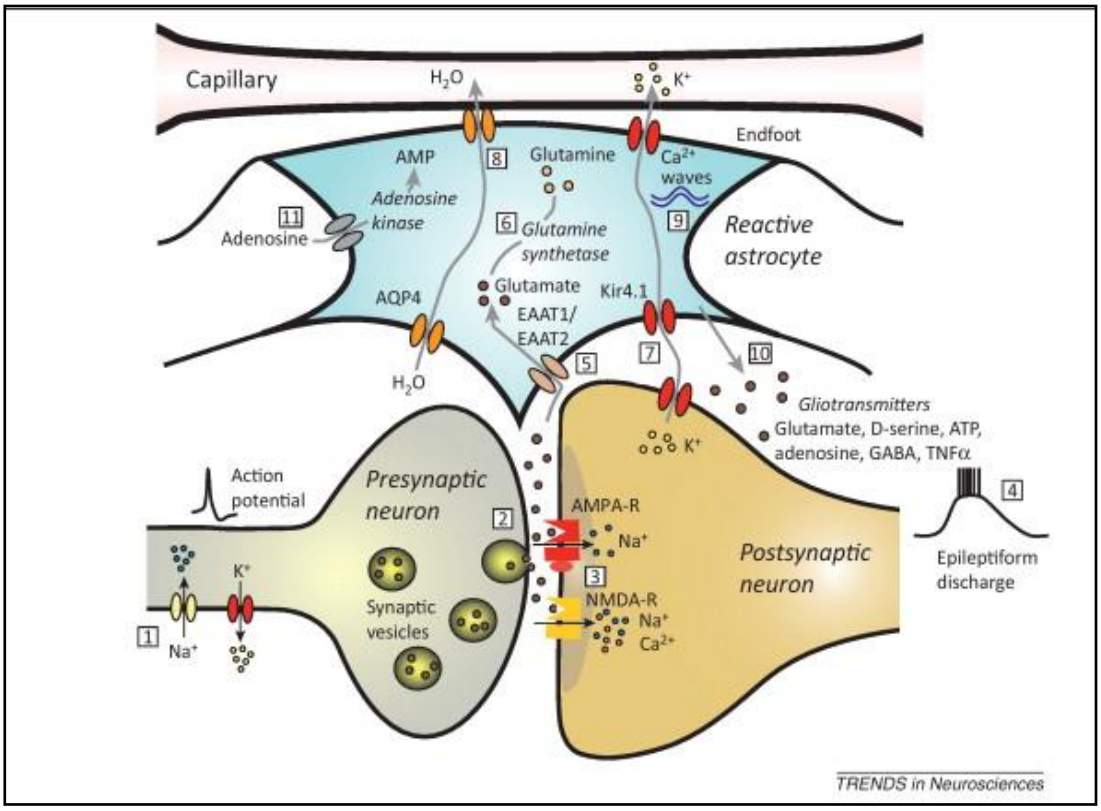


Figure.2.2. Schematic model demonstrate selected interactions between astrocytes and excitatory neurons (Devinsky et al., 2013).

2.4. Role of channel proteins and receptors

Channel proteins and receptors are membreane proteins present on the surface of neurons and glial cells. Hence, channel proteins and receptors are main regulators of neurotransmitters, any alteration in expression or activation of these proteins can cause hyperexcitation or hypoexcitation of neurons. There are different channel proteins and diverse receptor for binding, and release of neurotransmitters and ions such as voltage gated Ca^{2+} channel, voltage gated Na^+ channel and K^+ channel, similary, glutatate receptor and GABA receptor. Thus, several paroxysmal neurological disorder is caused by mutation in ion channels and receptors gene. . In brain, potassium and sodium channel genetic mutation have been reported to be associated in two rare, human epilepsy phenotypes. There are several reports on involvement of K^+ channels, Ca^{2+} and Na^+ channels in epilepsy and alteration in these membrane proteins have been observed in different epileptic models (Devinsky et al., 2013). Similarly, numerous reports have demonstrated involvement of GABA receptor in epilepsy is reported also (Belelli and Lambert, 2005; Benedetti et al., 2011; Peng et al., 2004).

2.5. Post-traumatic epilepsy

PTE is the symptomatic development and progression of seizures due to secondary damage to the brain because of head injury. Thus, prevention of PTE is important to reduce the extent of functional morbidity associated with TBI. It is important to understand the difference between provoked and unprovoked seizure after the TBI. The occurrence of seizures within 24 hrs of TBI are called immediate seizures; however, seizures occurring between 4 hrs to first 7 days after TBI are called as early post-traumatic seizures and comes in the class of provoked seizures (Beghi, 2003). The absence of any precipitating factor in the seizure leaded to its name as unprovoked seizure; however, acute systemic, toxic and metabolic insult causes occurrence of provoked seizures. Reports showed that the best way to determining the difference between early and late epilepsy is the focal hemorrhagic brain damage (D'Alessandro et al., 1982).

There are different types of head injuries that further leads to post-traumatic epileptogenesis. In closed-head injury in which there is no penetration in the skull where on the other hand there is open head injury in which an object penetrates into the skull (Adams et al., 1997), such as, in case of a gunshot or shrapnel wound; leading to temporary unconsciousness, resulting from violent jerks or collision of the head, resulting jarring or shaking of brain in the skull. This can cause a contusion of cerebral tissues and further can cause functional impairment. Although, these kind of open head penetrating injuries are uncommon, army persons are very prone to these injuries especially in the course of battle or war like situations; similarly, children and contact sports athletes e.g boxers are closer to the danger of concussion. There are numerous strategies to segregate different head injuries are been in use, however, a well defined classification for head injury severity is yet to be developed.

2.6. Mechanism of post-traumatic epileptogenesis

In PTE a latent period of variable duration is present which can deviate from weeks to years starting from brain injury to the occurrence of first unprovoked seizure. This can lead to an array of changes in the brain such as cell death, changes in excitatory and inhibitory neurotransmitters, axonal sprouting and network reorganization which further causes hyper-excitation of neurons and spontaneous seizure generation (Chang and Lowenstein, 2003). Many different theories have been

proposed on post-traumatic epileptogenesis after head injury (Figure.2.3). The theory of inhibitory function of brain due to excitatory activity of neurons resulting from free radical formation by blood in parenchyma of the brain is most accepted. Bulk displacement of brain tissue which is caused by mechanical trauma of the injury produces secondary responses including change in cerebral blood flow, alteration in intracranial pressure and altered vascular permeability (Willmore, 1990).

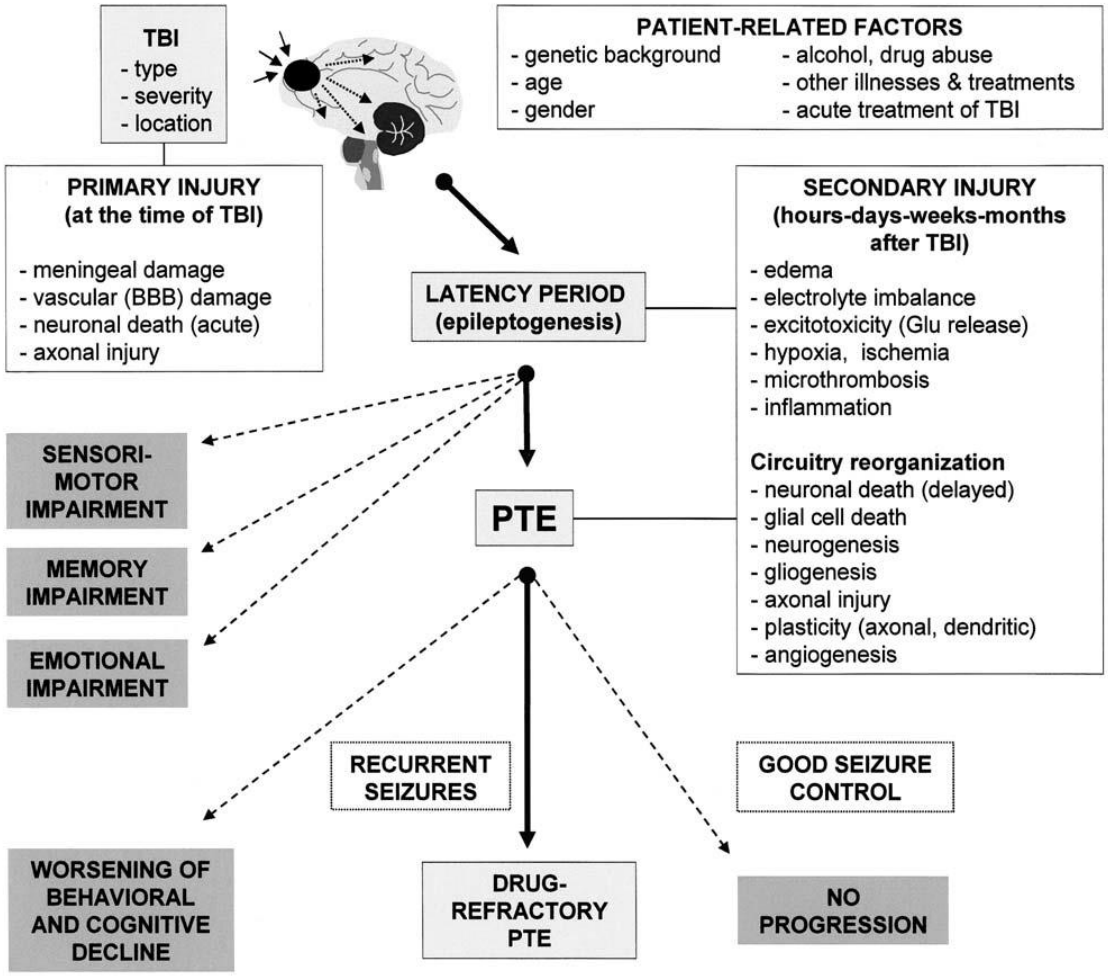


Figure.2.3. Representative diagram of contributing factors in the process of post-traumatic epileptogenesis after TBI.

There are numerous models of PTE, which resemble the human post-traumatic condition, e.g cortical undercut model, lateral fluid-percussion TBI etc. (Schmidt and Rogawski, 2002; Stables et al., 2002; White, 2003). These models are used for studying the role of pathophysiological substrates of epileptogenesis and to perform the preclinical screening of antiepileptic drugs (AEDs).

2.7. Signaling pathways in epilepsy

Epilepsy is a complex disease and progression of epileptogenesis associated with various signaling pathways. All these signaling pathways either function in an interconnected manner or independently in the process of epileptogenesis; inflammatory pathway, Apoptotic pathways (intrinsic and extrinsic), PI3-AKT mediated mTOR pathways are common pathways associated with epilepsy (Henshall, 2007); however, the involvement of various signaling pathways in different types of epilepsies and different stages of epileptogenesis is still yet to be understood.

2.7.1. Inflammatory pathway

Inflammatory pathway of neuronal excitability involves cytokines and its inflammatory cascades which work on cellular and neuronal network levels; IL-1 TLRs, TGF-β and COX-2 are mainly involved in these pathways (Kulkarni and Dhir, 2009; Riazi et al., 2010). Studies also show that the expression of proinflammatory cytokines (IL-1β, TNF and IL-6) occur initially in activated microglia and astrocytes; and expression of cytokine receptor is up-regulated in microglia, astrocytes and neurons (Vezzani et al., 2013, 2011). Induction of cyclooxygenase-2 (CoX-2) is further followed and so, prostaglandins, and components of the complement system in microglia is up-regulate in astrocytes and neurons, chemokines and their receptors are generated chiefly in neurons and in activated astrocytes also. Figure.2.4. shows the signal cascade of involvement of inflammatory pathway in epileptogenesis and its interconnecting links with other pathways also. Accumulating body of evidence suggest the involvement of inflammation in several neurological disorders including epilepsy. Rogers et al.,(1994) showed the presence of autoantibodies to glutamate receptors in Rasmussan's encephalitis, Allan et al. (2005) showed the involvement of IL-1 in the neuronal injury.

Epilepsy an important neurological disorder affecting human population has been reported to activate the inflammatory pathway. Recently numerous studies have been demonstrated altered expression of various inflammatory cytokines of inflammatory cascade in different animal models. In connection to this a study conducted by Minami et al. (1991) reported increased mRNA expression of IL-1, IL-6 genes and TNFα. Similarly, an another study showed that protein expression of IL-1β, IL-6, TNFα and LIF was up-regulated in the Kainic acid (KA)-induced seizures rat

model (Lehtimäki et al., 2003). Moreover, studies have also shown activation of complement system along with inflammation in epilepsy. Aronica et al. (2007) have suggested the involvement of the complement cascade during epileptogenesis in TLE in human and rats. Study reported unregulated expression of C1q, C3 and C4 in CA3 region of hippocampus in 1 week and 3–4 months after SE.

In addition, brain inflammation is an essential characteristics of hyperexcitable pathological brain tissue in drug resistant epilepsies (Vezzani et al., 2011). Reports also suggested that brain inflammation contributes in seizure threshold in seizure vulnerable brain regions to contribute in seizure recurrence (Dubé et al., 2005; Kulkarni and Dhir, 2010; Riazi et al., 2010; Vezzani et al., 2011).

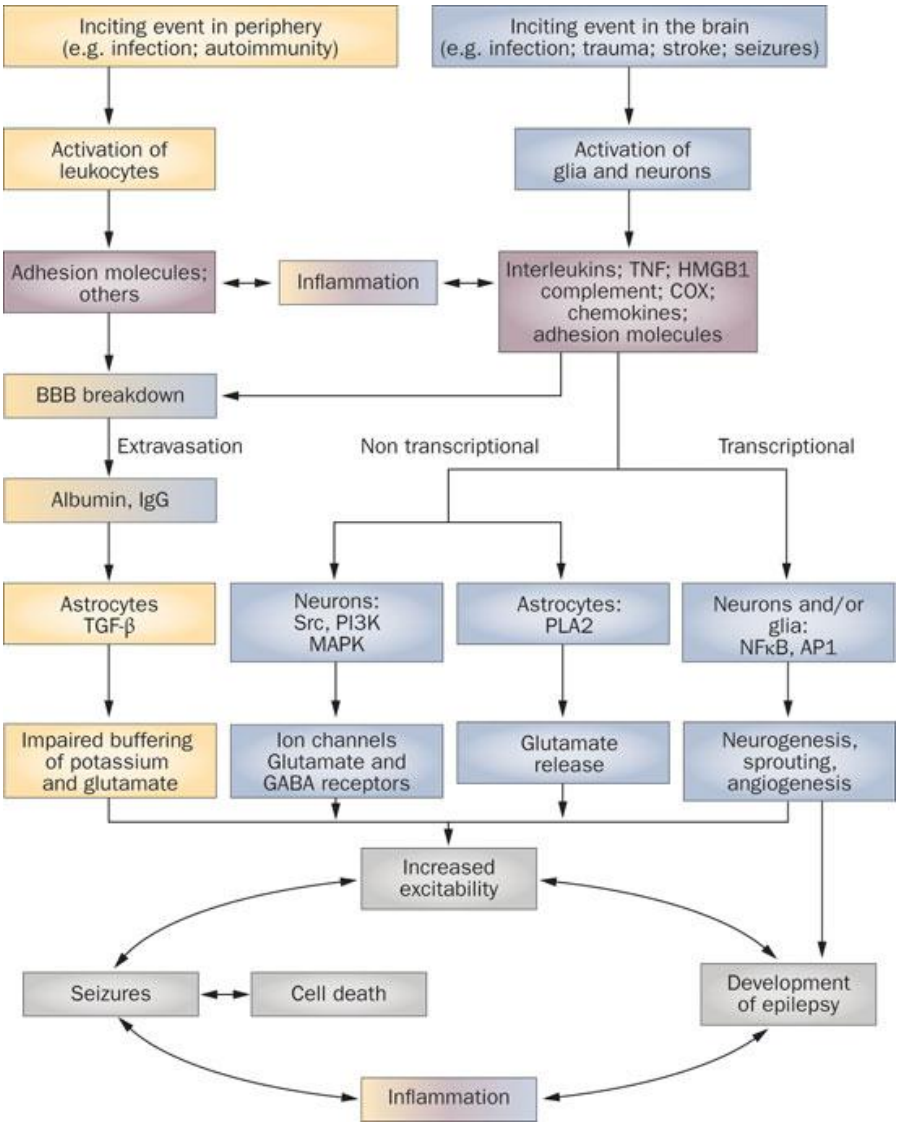


Figure.2.4. Schematic representation depicts the inflammatory pathway involved in epileptogenesis (Vezzani et al., 2011).

2.7.2. Apoptotic pathways

"Apoptosis is a morphologically distinct form of cell death characterized by cytoplasmic condensation, preservation and packaging of intracellular organelles, DNA fragmentation, dispersal and phagocytosis of the cell as apoptotic bodies" (Henshall, 2007). In epilepsy, hyperexcitation of neurons mold themselves to neuronal cell death pathways such as necrosis or apoptosis, and apoptosis is a prominent pathway in epileptogensis. There are two ways of triggering apoptotic pathways, one is intrinsic pathway and other is extrinsic pathway (Henshall, 2007). Extrinsic pathway is initiated by activation of cell surface receptors, belongs to tumor necrosis factor (TNF) family (Shinoda et al., 2003; Yamamoto et al., 2006). TNF superfamily receptor including TNFR1, Fas (CD95), DR4 (TRAIL receptor-1) and DR5 (TRAIL receptor-2), are activated by binding of ligand molecule to one or more receptors on the cell surface such as TNFα, Fas ligand and TNF receptor apoptosis-inducing ligand (TRAIL). After activation, oligomerization of receptor occurs which originate promulgation of cell death signals into the cell and activating signaling cascade starts by FADD and initiator caspases e.g. caspse 8 or caspase 10) present in the intracellular side of the plasma membrane ultimately causing activation mitochondrial disintegration and DNA damage (Henshall, 2007). Whereas, in intrinsic pathway, apoptosis is activated by intracellular molecules such as increased level of intracellular $Ca^{2+,}$ proapoptotic Bcl-2 protein activation or due to presence of ROS (White et al., 2005). All these events initiates the release of cytochrome c from mitochondrial inter-membrane space, and trigger mitochondrial-originated intrinsic pathway (Henshall and Simon, 2005). Further, cytochorme c binds with APAF-1 with the assist of dATP and engage the initiator caspase (caspase-9) (Henshall, 2007; Henshall and Simon, 2005) and formation of functional apoptosome takes place. Similarly, raised Ca^{2+} level or misfolding of proteins may also trigger the formation of apoptosome and apoptosis (White et al., 2005).

In case of DNA damage, the tumor suppressor phosphatase PTEN can promote apoptosis of mitotic cells by inhibiting activation of the cell survival kinase Akt. PTEN is essential for normal embryonic development, PTEN expression is associated with neuronal differentiation, and deletion of PTEN in the mouse brain results in seizures (Zhu et al., 2006), ataxia, and other abnormalities (Zhu et al.,

2006). However, the possible roles of PTEN in regulating neuronal survival are not known.

There are several reports of involvement of intrinsic pathway in epilepsy for example, apaf1/ cytochorme-c complex and activation caspase 9 causing neuronal cell death (Henshall et al., 2001); involvement of Bim in TLE (Shinoda et al., 2004). Similarly of extrinsic pathway of epilepsy is also reported to be involved in epilepsy, such as Shinoda et al.,(2003) showed TNF receptorm1 triggers the apoptotic signals during seizures; Yamamoto et al.,(2006) showed endoplasmic stress causing apoptosis in TLE model.

2.7.3. mTOR pathway

mTOR is a serine/threonine kinase and belongs to the phosphatidylinositol 3-kinase-related kinase (PIKK) family (Figure.2.5). It exists in two multi-protein complexes mTORC1 and mTORC2 (Meng et al., 2013), the opposite effects on a in-between modulator of AKT and mTOR generally results in activation or inhibition of mTOR through upstream pathways, and it is formed by tumor supperssor protein tuberous sclerosis 1 and 2 (TSC1 and 2). Recent findings also demonstrated that phosphatase and tensin homolog (PTEN) and STE20-related adaptor protein α (STRADalpha), other upstream regulator downregulate mTOR pathway. These studies also demonstrated that mutation in these upstream genes causes up-regulation of the mTOR pathway which further can cause cellular alterations such as abnormal differentiation, growth and proliferation and high comorbidity of epilepsy (Berdichevsky et al., 2013).

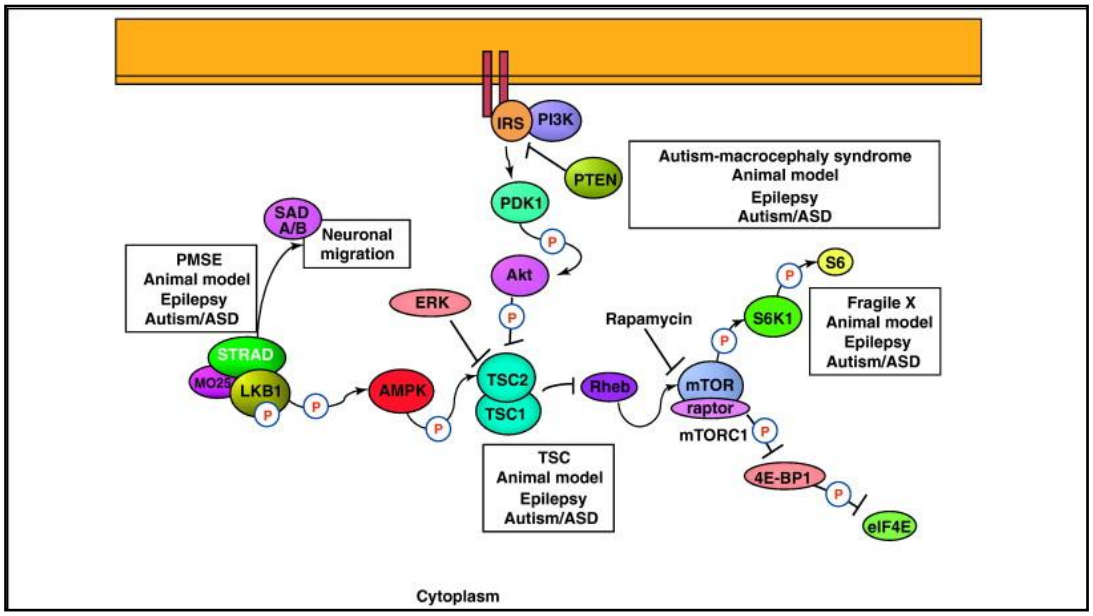


Figure.2.5. Schematic diagram represents the involvement of mTOR pathway in different neurological disorders including epilepsy; (Crino, 2011)

mTOR may be involved in epileptogeneis associated axonal sprouting and neurogenesis based on the role of mTOR in neuronal development and plasticity. The use of this mTOR signaling in healthy and safe clinical strategies will be focus of future research as animal model studies suggested that mutation in mTOR pathways show epileptic seizures (Meng et al., 2013). In addition, mTOR pathway also regulates synaptic plasticity, neurogenesis, neuronal death, apoptosis, inflammatory response and cell growth morphology which include antiepileptogenic mechanism (Figure.2.6). In contrast, the antiseizure mechanism due to mTOR pathway includes decreased excitability of cortical neurons, and regulation of synthesis of ion channels, neurotransmitter receptors and proteins which functions in neuronal signaling pathways.

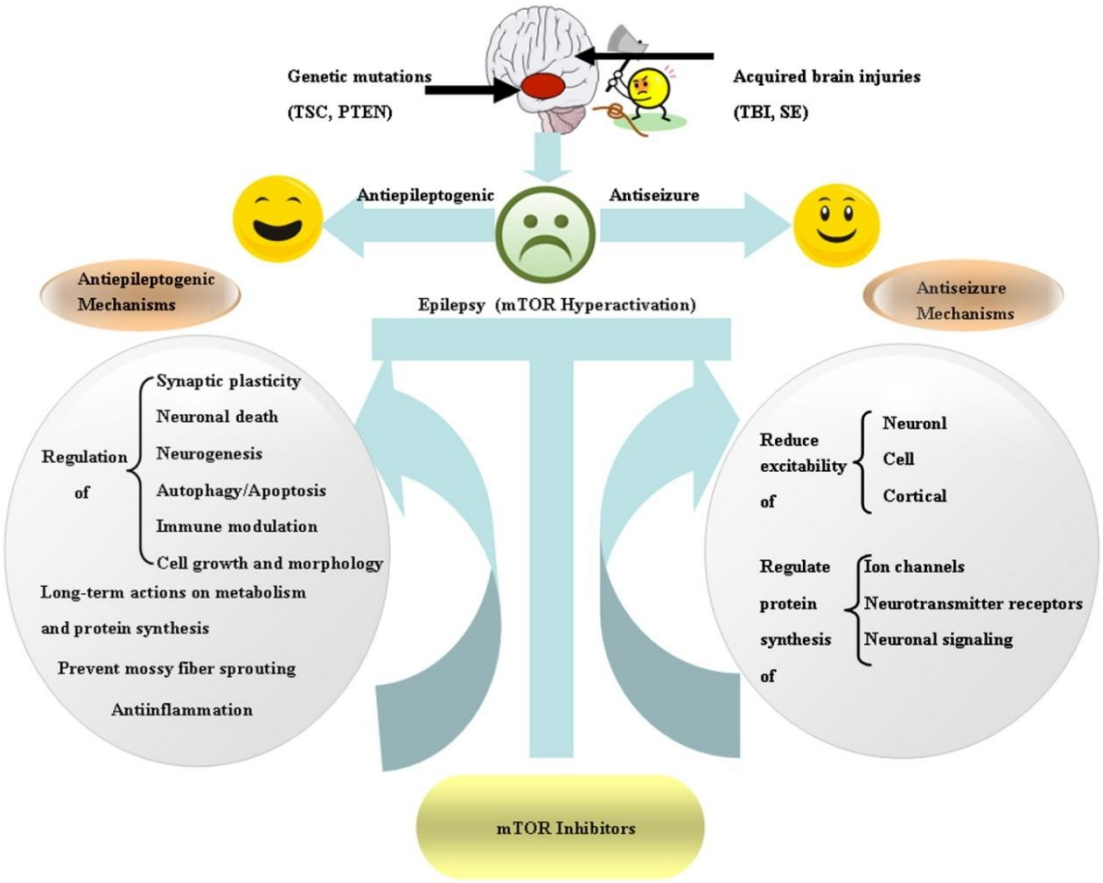


Figure.2.6. Effect of activation of mTOR in epileptogenesis ; left balloon shows the antiepileptogenic mechanism, right balloon shows the antiseizure mechanism both concur by hyperactivation of mTOR pathway (Meng et al., 2013).

2.8. Epigenetics in epilepsy

"Epigenetics is defined by the changes to the physical structure that supports genes" (Gräff et al., 2012). These changes include heritable changes which do not include any changes to the DNA code,. As the nondividing neurons cannot inherit epigenetic changes, "neuroepigenetics" term was proposed to encompass and understand epigenetic processes of the brain (Sweatt, 2013). The epigenome involve around the diverse structural modifications to DNA and histones that thereby, either support or oppose transcription. Interestingly, miRNAs regulation of gene expression which majorly includes obstruction of translation is also a part of epigenetic mechanism.

2.8.1. DNA methylation

Methylation of DNA functions as silencing agent of transcriptional activity is considered to influence the most long lasting epigenetic changes (Dulac, 2010). It

involves covalent addition of a methyl moiety from *S*-adenosylmethionine (SAM), the cell's primary methyl donor binds with cytosine base to form 5-methylcytosine (5-mC). In somatic cells, 5-mC is mainly restricted to palindromic CpG dinucleotides islands which are typically methylated in a uniform manner. There are numerous studies on different epilepsy models suggest the essential role of DNA methylation, such as Miller-Delaney et al. (2012) demonstrated the hypomethylation of 275 genes and hypermethylation of 13 gene in KA-induced status epilepticus(SE) mice model. In other report decreased brain derived neurotropic factor (BDNF) methylation in KA mice model, increased reelin methylation in TLE (Kobow and Blümcke, 2012); similarly, Ryley Parrish et al. (2013) showed increase Grinn2b methylation. In post traumatic epilepsy, increased DNMT1 methylation has been reported (Lundberg et al., 2009).

2.8.2. Histone modification

Histones are core components of the nucleosome, and belong to a family of conserved basic proteins. In nucleosome, DNA is wrapped around octameric complex of histones, and consists of two subunits of core histones H2A, H2B, H3, and H4 (Gräff et al., 2012). The amino terminal of histones consist different sites that are susceptible to post translational modifications (PTMs) that influence the affinity for DNA and other binding proteins. PTMs of histones include acetylation, methylation, phosphorylation, and ubiquitination. Though the process of histone modification is yet to be fully understood, its involvement in epilepsy is being studied in different experimental models for example, decreased H4 acetylation at GluR2/Gria2, and increased H4 acetylation at BDNF in pilocarpin induced rat model (Huang et al., 2012; Sng et al., 2006), showed increase H4 acetylation and H3 phosphoraylation in KA induced SE rat model. In post-traumatic epilepsy, reduced H3 acetylation, increased H4 acetyalation and showed to HDACi involvement in learning/memory enhancement (Gräff et al., 2012; Henshall and Kobow, 2015).

2.8.3. miRNAs and epilepsy

MicroRNAs (miRNAs) are 20-22 nucleotide long endogenous non-coding small RNAs. They regulate gene expression by inhibiting/degrading protein coding mRNAs. However, they have also reported to protein synthesis translation regulation (Cao et al., 2006; Kosik, 2006). Biogenesis of miRNAs take place inside the nucleus

and form primary miRNA (pri-miRNA) which are exported to cytoplasm through exportin, further processed by dicer to synthesize a mature miRNAs; mature miRNA binds with mRNA target to block translation process of gene expression (Cannell et al., 2008) (Figure.2.7).

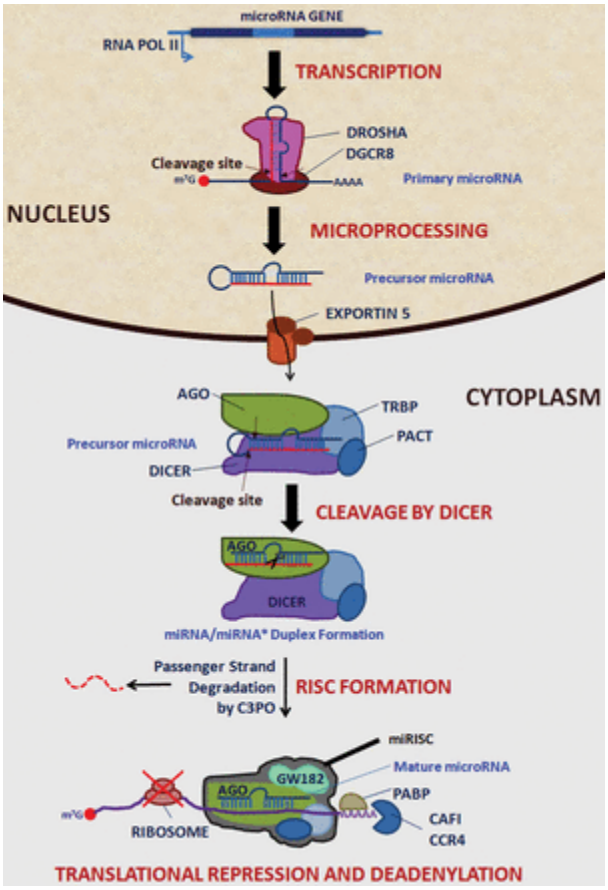


Figure.2.7. Schematic diagram represents the biogenesis mechanism of miRNAs where two major processing pathways of miRNAs takes place from nuclear to cytoplasmic transport (Gupta et al., 2012)

The enzymatic machinery as well as sequences remains conserved during biogenesis and maturation of miRNAs across animals and plants. There is simultaneous regulation of hundreds or even thousands mammalian mRNA targets take place simultaneously; that miRNA functions as master regulator of gene expression. Further, there are reports s that showed that deletion of dicer, a miRNA processing enzyme in Ca^{2+}/ calmodulin-dependent protein kinase II (CaMkII)-positive neurons caused improved learning and memory in mice model (Wang et al., 2012); however, dicer expression is crucial for long-term memory formation (Ashraf et al.,

2006; Lugli et al., 2005). miRNA processing machinery is observed to be present in dendritic spines and in postsynaptic densities including specialized cell granules called as processing bodies (P-bodies) of fully differentiated neurons (Cannell et al., 2008). There may be a variable pattern of miRNAs in different brain regions, and these differences might be in relation to involvement of miRNAs with synaptically localized mRNAs as these mRNAs are targeted by many different miRNAs (Pichardo-Casas et al., 2012). There are numerous reports showing involvement of different miRNAs to regulate molecular mechanisms of many physiological processes in brain, like cortical development (Figure.2.8), and neuronal migration associated with various neurological disorders. During cortical development, first step is cellular proliferation and differentiation takes place (Bushati and Cohen, 2007; Gupta et al., 2012; Im and Kenny, 2012); animal studies have suggested involvement of miR-9, miR-124, miR-137, miR-184 and let-7 are shown to be involved in the process of cell proliferation in the cortex (Bushati and Cohen, 2007). Similarly, miR-137 and miR-125 are reported to be involved in premature differentiation and migration of neural stem cells (Im and Kenny, 2012).

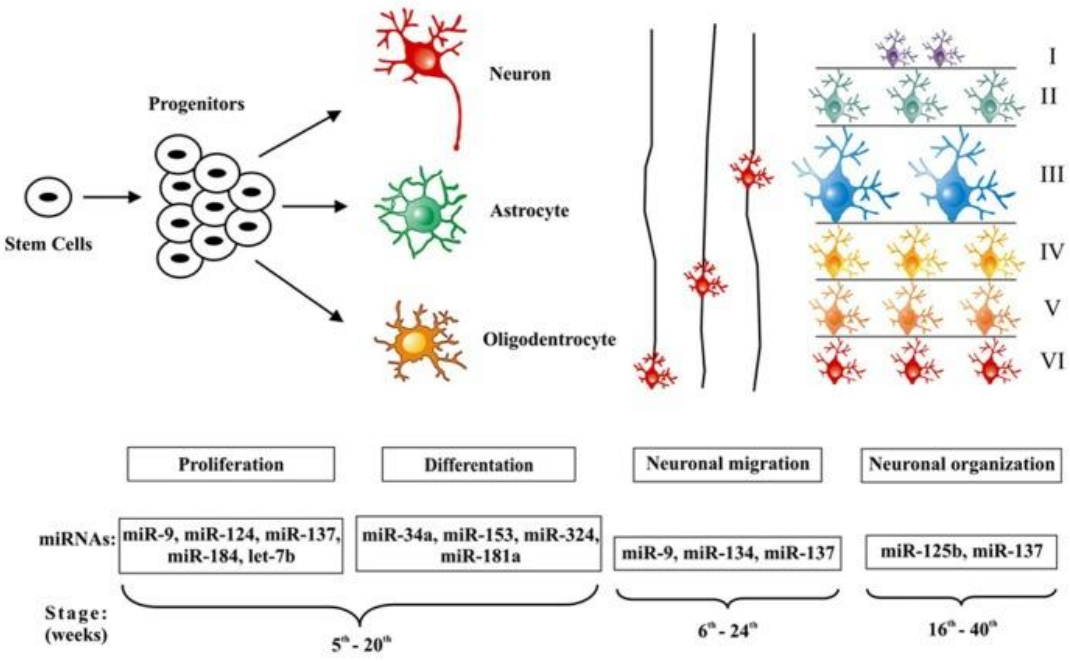


Figure.2.8. Schematic diagram showing miRNAs involve in the regulation of cerebral cortex development (Dogini et al., 2013).

Expression of various miRNAs was checked using different epilepsy models. Studies demonstrated that miRNAs were up-regulated while others were down-regulated (Jimenez-Mateos and Henshall, 2013) miR-21, miR-132, miR-7, miR-9, miR-122, miR-155, 3 prime region of miR-362 and 5 prime region miR-450 were shown to down-regulating in status epilepticus. Similarly, miR-30a/b, miR-138, miR-187, miR-324 and miR-330 have shown to be down-regulating in TLE; whereas, miR-134, miR-146a, miR-132, miR-9, miR-99a, miR-27a, miR-203 and miR-135a are reported to be up-regulated (Jimenez-Mateos and Henshall, 2013) (Figure.2.9).

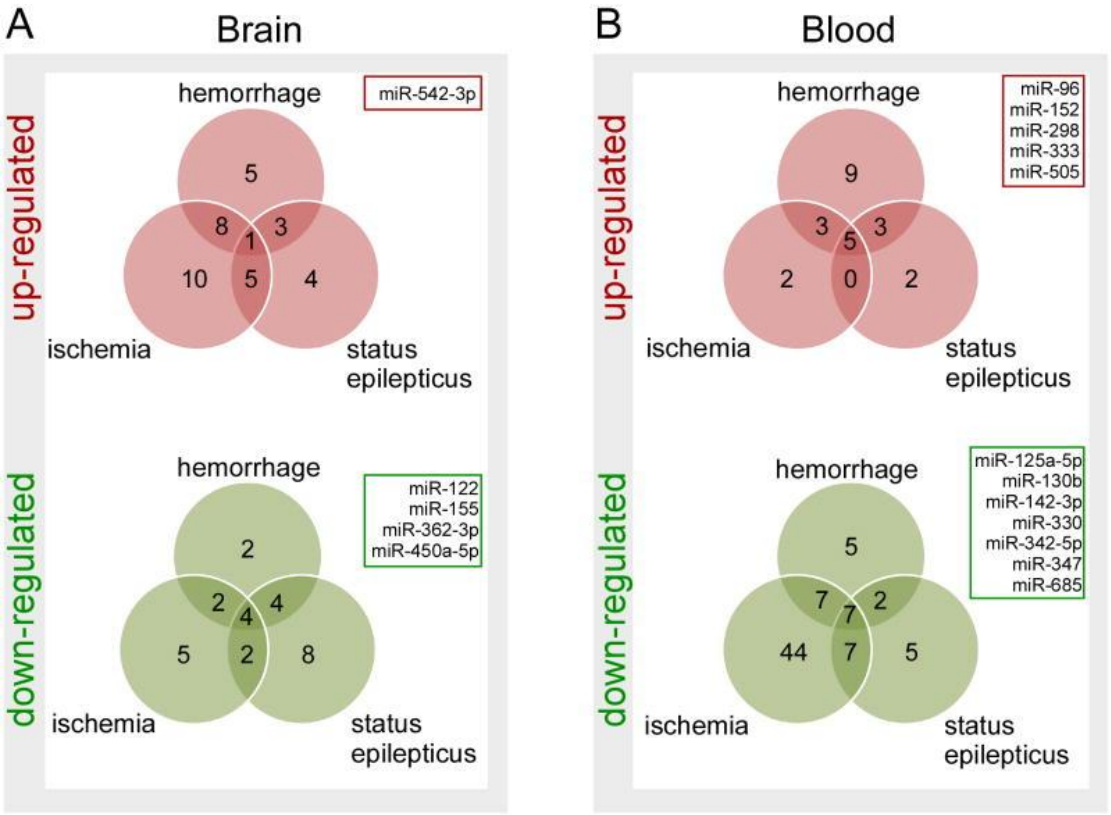


Figure.2.9. Schematic venn diagrams of different set of miRNAs either up-regulating or down-regulating, and involve in hemorrhage, status epilepticus and ischemia (A) brain (B) blood (Liu et al., 2010)

In PTE, miR-19 and miR-21 has been reported to be up-regulated in a time dependent manner during epileptogenesis in the cortex of experimental rats (Liu et al., 2010). In hippocampus of rats, miR-50 was down-regulated, whereas, miR-35 was up-regulated after TBI (Dogini et al., 2013). In human, miR-33, miR-99, miR-16,

miR-92a and miR-765 were reported to be altered (Jimenez-Mateos and Henshall, 2013).

2.9. miR-3120 and miR-214

miR-3120 and miR-214 are first fully processed and functional mirror miRNAs of mammals which belong to miR-199 cluster. Growing body of research in recent years investigated that miR-3120 present in intronic region of dynamin-3 (DNM-3) gene (Aranda et al., 2015; Scott et al., 2012) and miR-214 present in antisense strand of same intronic region of DNM-3 and produced by antisense transcription ((Scott et al., 2012, p. 3120)). These are co-transcribed miRNAs, and are often coordinately express with their host gene DNM-3 in neurons (Aranda et al., 2015; Scott et al., 2012). Though, there are chances of existence of other similar mirror miRNAs in the mammals, this is the first characterized example in mammals indicating the rare nature of both miRNAs to sustain beneficial effect and still retained by natural selection (Scott et al., 2012). miR-3120 have already been reported to be altered in renal cancer as well as bladder cancer tissues (Blondeau et al., 2015). In neurons, it is already evident that miR-3120 targets the clathrin-uncoating enzyme Hsc70, a chaperone and its co-chaperon auxilin suggesting its role in vesicular trafficking in neurons (Scott et al., 2012) (Figure.2.10).

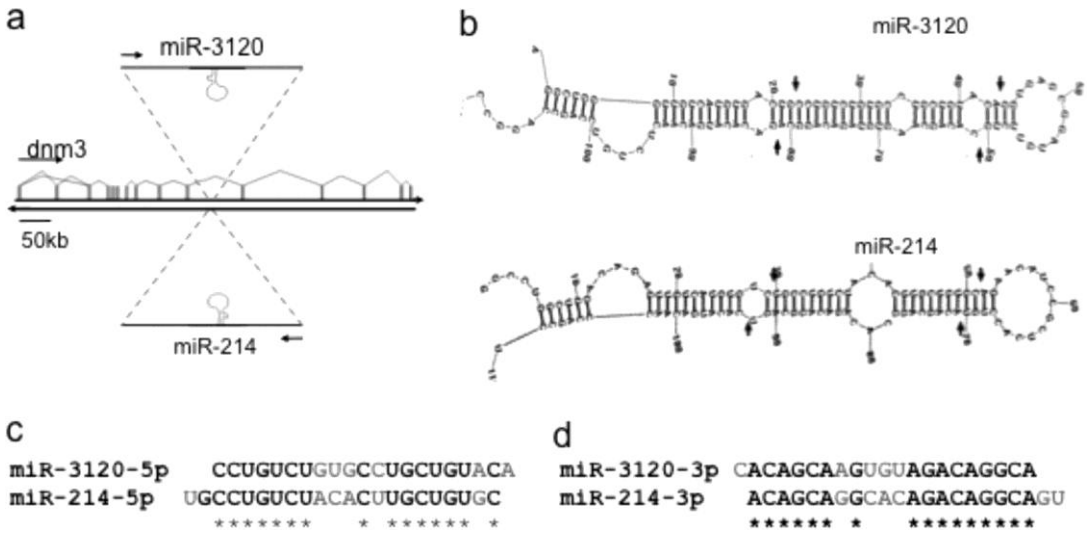


Figure.2.10. Schematic diagram showing sequences and structures of miR-3120 and miR-214 in the dynamin-3 (dnm3) (Scott et al., 2012).

For miR-214, increasing body of evidences suggested the role of miR-214 in bone formation by targeting ATF-4 gene, and in many different kinds of cancers. In brain, Chen et al., (2010) have shown miR-214 are distinctively expressed during cortical development, neuroblastoma differentiation, embryonic differentiation and control neurite outgrowth. Other studies showed that RNA-214 targets Huntington's gene (Sinha et al., 2011). Several studies evinced the role of miR-214 in apoptosis where it is targeting several genes which are involved in the apoptosis signaling pathway e.g. PTEN, Bax and caspases (Schwarzenbach et al., 2012; Zhao et al., 2015). Gao et. al., (2017) found that, miR-214 helps in triggering apoptosis in stroke. It has been reported to be involved in NMDA receptor-related memory processes, where it modulates NMDA receptor-mediated neurobehavioral dysfunction (Wang et al., 2014). In cause of epilepsy, numerous finding showed the alteration in miR-214 expression. However, the results are different in different experimental models (Gorter et al., 2014; Kan et al., 2012) miR-214 was down-regulated in TLE mice model (Kan et al., 2012), whereas, up-regulated in the hippocampus of SE rat model (Gorter et al., 2014).

2.10. Pharmacological interventions

The prevention of epilepsy is prime motive without causing any side effects of the AEDs. There are several drugs in the market most of the early drugs were based on the mechanism of blocking the AMPA receptors to inhibit AMPA-induced Ca^{2+} calcium influx and neuronal cell death. The newer generation of AEDs are more diverse in their function as phenytoin, carbamazepine, lacosamide, lamotrigine and esilcarbazepine acetate work on the voltage gated sodium channels, valproate elevate the GABA turnover, retigabin blocks potassium channels to inhibit the mechanism of epileptogenesis which blocks signaling pathways and function of neurotransmitters (Schmidt and Rogawski, 2002).

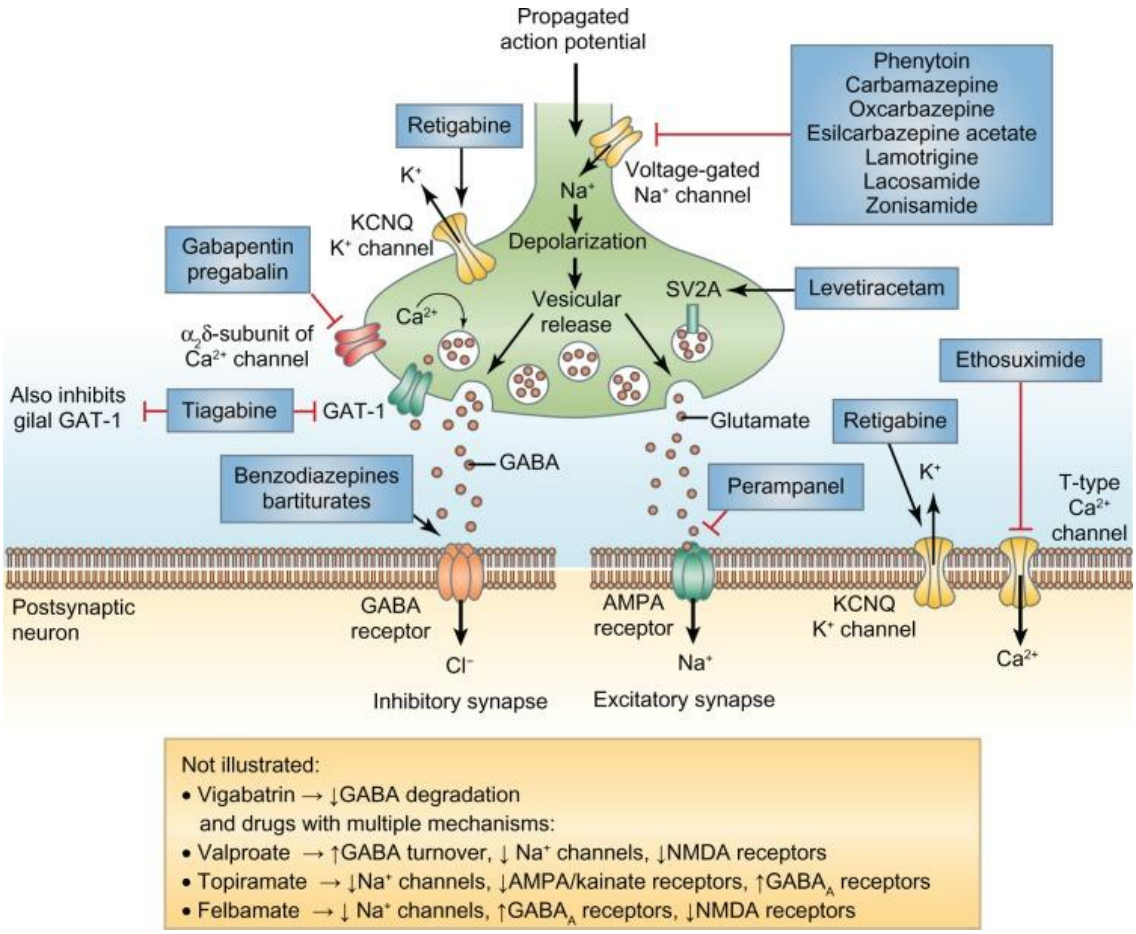


Figure.2.11. Mechanism of antiepileptic drugs, involve in blocking of channel proteins or activation of receptors; by allosteric or competitive inhibition of targeted proteins (Schmidt and Rogawski, 2002).

In PTE, the latent period between brain insult and seizure development provide opportunity for the treatment with the possible AEDs. Administration of anticonvulsants is frequently done soon after brain injury in an attempt to inhibit seizure activity, and the process termed as anticonvulsant prophylaxis. Though the prophylactic use of anticonvulsants is common, there are no available AEDs in the market which demonstrate effective protection against development of late seizures. Moreover, it has been shown that use of AEDs for prophylaxis of PTE adversely affect the brain, and can even enhance the adversity in the presence of brain injury (Eddy et al., 2011). Negative effects of AEDs have been observed as impaired cognition and behavior in the patients with head injury. Temkin et al., (2003) reported the effect of AEDs on different PTE experimental models to demonstrate the ineffectiveness of most of AEDs (Table.2.)

Table.2.1 Effect of AEDs in different PTE models (Temkin. 2003)

Drug	Model	Effect
Carbamazepine	Amygdala-kindled rats	Ineffective
	Amygdala-kindled cats	Weakly attenuated
	Pentylenetetrazol-induced kindeling in rats	Ineffective
Diazepam	Amygdala-kindled rats	Attenuated
	Pentylenetetrazol-induced kindling in rats	Attenuated
Ethosuximide	Pentylenetetrazol-induced kindling in rats	Attenuated
Felbamate	Amygdale-kindled rats	Weakly attenuated
Lamotrigine	Homocysteine thiolactone administration	Ineffective
	Amygdala-kindled rats	Ineffective
Levetiracetam	Amygdala-kindled rats	Attenuated
	Corneally kindled rats	Protected
Phenobarbital	Pentylenetertrazol-induced kindling in rats	Attenuated
	Amygdala-kindled rats	Attenuated
	Amygdala-kindled cats	Attenuated
	Hippocampus injection of penicillin cats	Attenuated
	Hippocampus injection of penicillin in rats	Attenuated
	Alumina-gel injection in monkeys	Ineffective
Phenytoin	Amygdala-kindled rats	Raised seizure threshold but ineffective in

		preventing epileptogenesis.
	Amygdala-kindled cats	Ineffective
	Homocystein thioacetone administration	Attenuated
	Flurothyl seizure in mice	Attenuated
	Kindling induced by cortical penicillin in rats	Attenuated
	Alumina-gel injection in monkeys	Mixed
Tiagabine	Amygdala-kindled rats	Attenuated
Topiramate	Amygdala-kindled rats	Ineffective
Valproate	Amygdala-kindled rats	Markedly attenuated
	Pentylenetetrazole-induced kindling in rats	Attenuated
	Flurothyl seizures in mice	Attenuated, retarded reorganization
	Rat brain slice	Mixed
Vigabatrin	Amygdala-kindled mice	Attenuated
	Amygdala-kindled rats	Ineffective
	Corneally kindled rats	Attenuated

Based on results from animal studies, the antiepileptogenic effect has been considered definite for some AEDs, like diazepam, levetiracetam, Phenobarbital, tiagabine, valproate. The antiepileptic effects are also probable for other compounds like phenytoin, topiramate, vigabatrin and absent for carbamazepine (CBZ), felbamate, gabapentin oxcarbazpine (Beghi, 2003).

Clinical and experimental trials of AEDs also showed the potential of side effects such as anorexia, dissiness, ataxia, fatigue, double vision and sleep disturbance (Kr et al., 2014) Same report showed the teratogenic effect of AEDs, hence, various herbal drugs are considered as an alternative of AEDs (Kr et al., 2014). Keeping the need to develop of efficient treatment of epileptogenesis a variety of natural anti-oxidants have been tested as anticonvulsant. Accumulating body of reports suggested that these naturally occurring anti-oxidants can attenuate seizures in $FeCl_3$-induced epilepsy and can be very useful alternative medications for prevention as well as attenuation of the occurrence of epileptic seizures without negligible side effects (Mori et al., 1998).

There is no concrete evidence regarding the effect of AEDs on the expression of genes involved in activation of epileptogenic pathways. As the expressions of these genes are regulated by many other factors there is a need of drugs which can alter the expression of these genes and ameliorate the epileptogenesis.

2.11. Curcumin

Curcumin is yellow coloured flavoring compound extracted from rhizome of *Curcuma longa* commonly known as turmeric plant. Turmeric contains primarily curcumin along with other compounds combined called as curcuminoids (Srinivasan, 1952). The IUPAC name of curcumin ($C_{21}H_{20}O_6$) is diferuloylmethane. Curcumin II (demethoxycurcumin), curcumin III (bisdemethoxycurcumin), and cyclocurcumin are the curcuminoids present in turmeric (Aggarwal et al., 2003).

2.12. Biotransformation of curcumin

Curcumin have been reported to have low bioavailablity and a rapid biotransformation in humans as well as in animals. The biotransformation of curcumin is a four step process in which dihydrocurcumin converts to tetrahydrocurcumin then to subsequently convert to monoglucuronide conjugates.

Intraperitoneal (i.p.) administration of curcumin (0.1g/kg) to mice showed its distribution in different body parts, about 2.25 mg/ml of curcumin was observed in the plasma after 15 min. The levels of curcumin were 177, 26. 26.9 and 7.5 µg/g tissue weight in the intestine, spleen, liver, and kidney respectively after one hour of administration (Jen-Kun Lin et al., 2000). In human, concentration of curcumin in serum was observed at highest at 1-2 hours after oral intake of curcumin and dwindled within 12 hours. The average peak concentration of curcumin in the serum after taking 4000 mg, 6000 mg, and 8000 mg of curcumin were 0.51 ± 0.11 µM, 0.63 ± 0.06 µM and 1.77 ± 1.87 µM respectively. Curcumin has also crosses the blood-brain barrier and move to brain by blood flow. In the human brain, levels of curcumin was about 0.1 µM; it resembles the concentration mark required to inhibit central nervous system AP-1 mediated transcription *in-vivo* (Luo et al., 1999), and also with the suppression of inducible nitric-oxide synthase (Chan et al., 1998) as well as for anti-oxidative activities. Moreover, due to lipophylic in chemical nature and with a small size it can cross the blood brain barrier (Kelloff et al., 1996).

2.13. Antioxidative nature of curcumin

Innumerable investigations on anti-oxidative potential of curcumin demonstrated the *in-vitro* protection against H_2O_2 –induced oxidative stress in renal cell line (Cohly et al., 1998), induction of hemeoxygenase in endothelial cells (Motterlini et al., 2000), suppressive effect against trichloroethane-indiced oxidative stress (Watanabe and Fukui, 2000) and inhibition of oxidative damage of cellular DNA (Jaruga et al., 1998). The electrophilic nature due to β-unsaturated carbonyle group as well as the equilibrated keto-enol form of curcumin provide it an antioxidant nature and help it to react with neucleophile such as glutathione, thus , curcumin have the potential to inhibit lipid peroxidation, neutralize reactive oxygen and nitric-oxide base free radicals (Chan et al., 1998). Additionally, the primary metabolite of curcumin tetrahydrocurcumin (THC) along with also act as an antioxidant with β-diketone moiety, by breaking the C-C bond at the active methylene carbon between the two carbonyl groups (Pan et al., 1999). As a potential free radical scavenger during apoptosis curcumin increases the level of glutathione (Jaruga et al., 1998), ability of curcumin inhibit nuclear factor kappa B (NF-κB)-mediated transcription of inflammation cytokines (Xu et al., 1997), inducible nitric oxide synthase (iNOS)

(Chan et al., 1998), and Cox-2 (Plummer et al., 1999). These all investigations indicate its anti-oxidant potential and anti-inlamatory capabilities (Figure.2.12).

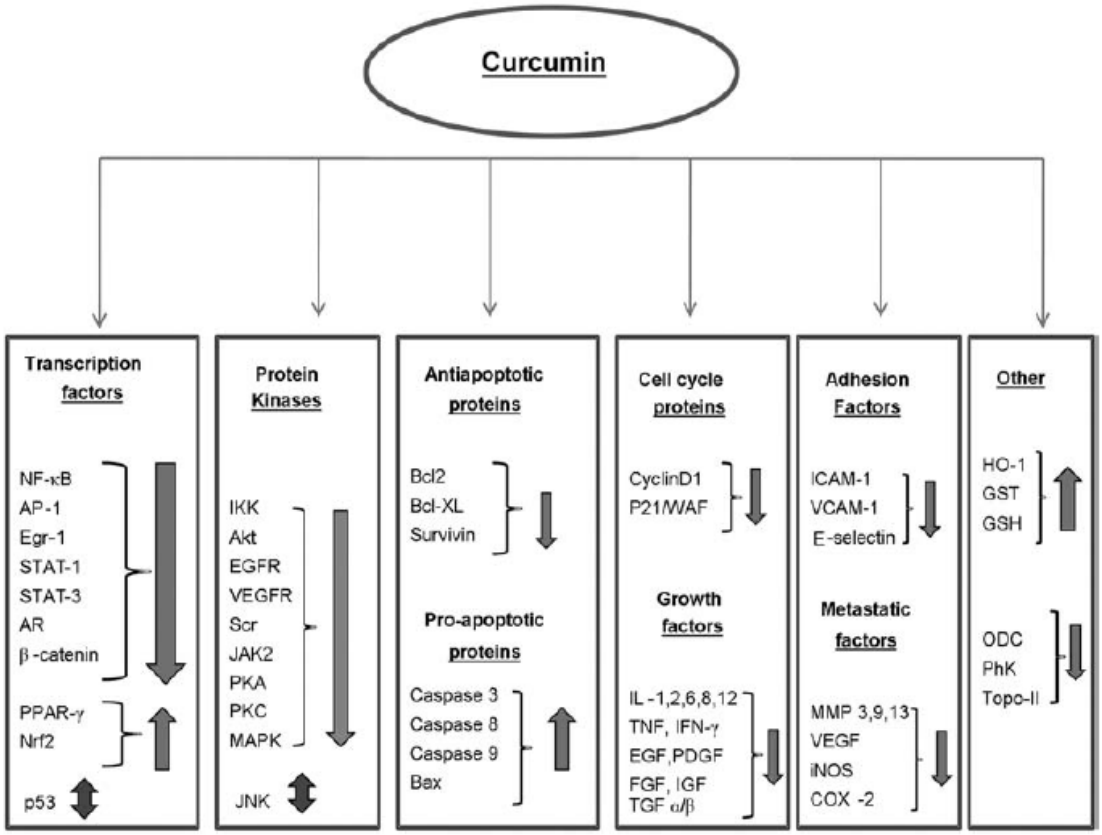


Figure.2.12. Diagram showing regulation of diverse signaling pathways by targeting different signaling proteins: a representation of therapeutic applications of curcumin.

2.14. Therapeutic use of curcumin

Curcumin has history of well tolerated long term use in human with almost no side effect (Kelloff et al., 1996). Srimal and Dhawan (1973) showed no mortality of any experimental mice fed with curcumin at a dose of 2000mg/kg of body weight. Another study by Lim et al.,(2001) did not notice any adverse effect of curcumin which was fed at a dose of 5000 ppm. However, 13 to 1 year study plans week study of high dose from 1000 to 50000 ppm curcumin fed to rats, showed minimal toxicity with no mortality of animals which included mice and rats (National toxicology program, 1993). Due to favorable toxicity profile and antioxidant characteristic, curcumin is extensively studied natural occurring compound. Figure.2.13. shows the broad spectrum of therapeutic applications of curcumin in various diseases and disorders including liver diseases, lungs diseases, skeletal of bone abnormalities, heart diseases, endocrine disorder, infections and inflammatory diseases, extensively

studied diverse cancers, and neurodegenerative disorders such as parkinsons disease, Alzheimer disease and epilepsy (Aggarwal and Harikumar, 2009). Prevalence of Alzheimer disease is 4.4 fold less in India at the age of 70-79 due to extensive use of curcumin as spice (Ganguli et al., 2000).

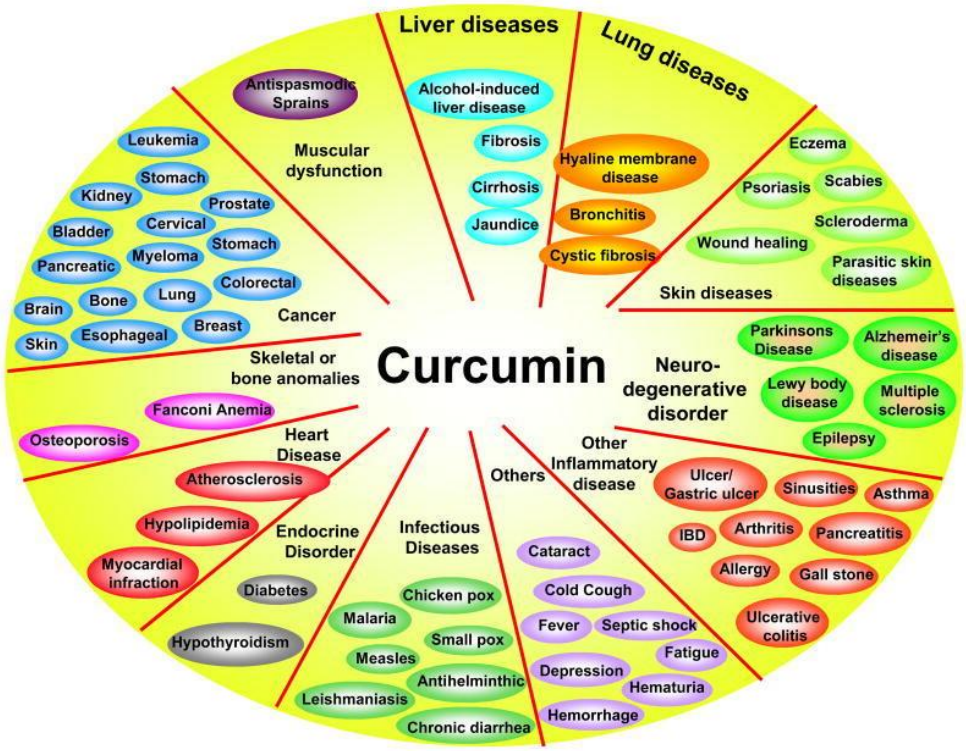


Figure.2.13. Schematic diagram represents potential therapeutic use of curcumin in diverse diseases and disorders.

Since the past few years, curcumin nano-formulation which have high surface to volume ratio, improved solubilization and internalization, superior pharmacokinetics and controlled responsiveness (Bhawana et al., 2011). All these characterstics have increased the interest of researcher to use it as therapeutic agent (Kanai et al., 2012). Accumulating body of evidences depicts that curcumin nano-formulations either shows similar effect compared to free curcumin or shows improved effect due to higher efficacy of nano-formulation (Gupta et al., 2013; Kanai et al., 2012). Although curcumin is therapeutically helpful in many diseases it is not the remedy of everything (Nelson et al., 2017a), moreover, curcumin alone is not found to as efficient as the turmeric, since the function of active compound curcumin thought to be promoted by other constituent compounds of turmeric (Nelson et al., 2017b).

2.15. Curcumin in epilepsy

In previous decade, studies investigated the use of curcumin for the treatment of epilepsy; number of investigations demonstrated the possible therapeutic application of curcumin on different experimental models of epilepsy, such as Noor et al. (2012) showed the antiepileptic effect of curcumin on pilocarpin-induced TLE rat model, same study suggested the therapeutic use of curcumin over valproate, similarly, protective effects of curcumin have been reported against seizure and cognitive impairment in pentylenetetrazol (PTZ) model as well as in electrically induced SE rat model (Mehla et al., 2010). In post-traumatic epilepsy Jyoti et al. (2009) showed the antiepileptic effect on electrobehavioral progression of seizures in $FeCl_3$-induced PTE rat model.

2.16. miRNAs and curcumin

miRNA expression is a type of epigenetic modification in the biological system and accumulating body of evidences suggests that curcumin can act as a epigenetic regulator including DNA mehtylation and regulation of miRNAs (Fu and Kurzrock, 2010; Reuter et al., 2011; Teiten et al., 2013). While DNA methylations as well as histone modifications are involved in maintaining the pattern of gene expression at the time of development, it has been seen that curcumin is involved in reversal of DNA methaylation modification in many disorders including several neurological disorders. Both *in-vitro* as well as *in-vivo* study showed alteration in expression of epigenetic associated DNA methyltransferase 1 (DNMT1) in cancer (Link et al., 2013), A study also suggested involvement of curcumin in histone modifications (Reuter et al., 2011). Growing body of evidences suggested that curcumin alter the expression of miRNAs; miR-125a-5p, 19a, 19b, miR-21 are down-regulated by curcumin, whereas miR-9, miR-27a, miR-29b, miR-34a miR-34c, miR-145, miR-146 and miR-181b were up-regulated by curcumin treatment (Figure.2.14). There are many reports which showed the altered expression of miRNAs in different disorder after curcumin treatment. Li et. al. (2014) reported the alteration of miR-19 in breast cancer, and miR-125a in nasropharengial carcinoma (Jaruga et al., 1998; Saini et al., 2011; Sun et al., 2008; Teiten et al., 2013).

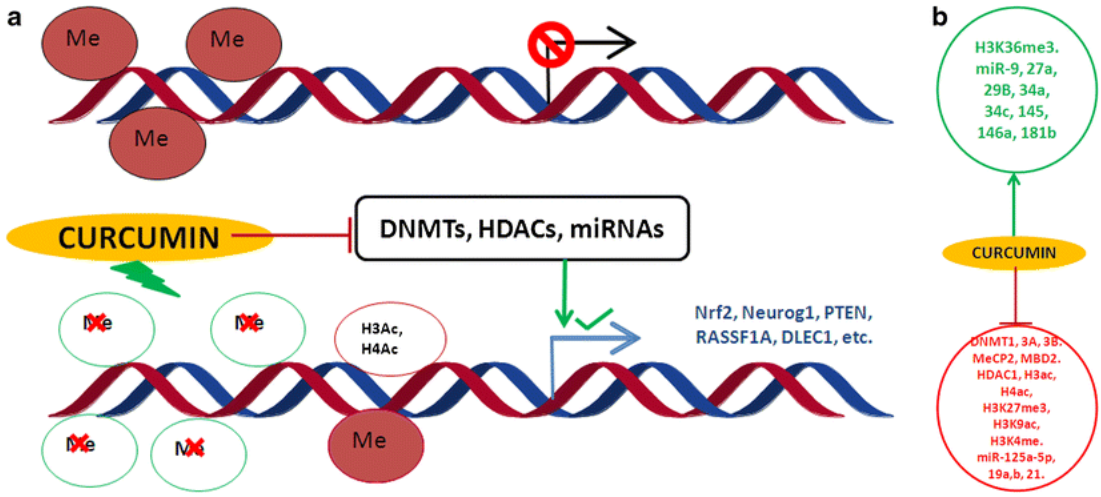


Figure.2.14. Schematic diagram showing that curcumin blocks the epigenetic pathways including DNMTs, HDACs and miRNAs (a). Up-regulated miRNAs are shown in green while down-regulated miRNAs are in red (b) (Boyanapalli and Kong, 2015).

There are many reports on curcumin as an epigenetic regulator in many diseases especially on cancer. However, there is a need of a thorough investigation of curcumin on the alteration of epigenetic mechanism in epilepsy, moreover, effect of curcumin on the regulation of gene expression controlled by miRNAs in the brain, an area which is still unexplored.

Thus, in the study embodied in this thesis, we have investigated the relation of electrobehavioral seizure progression with the epigenetic alteration of miR-3120 and miR-214 in $FeCl_3$-induced PTE model, it is imperative that miRNAs be studied as epigenetic alteration due to PTE. The comparisons were drawn between alteration of miR-3120 and miR-214 after supplementation of curcumin during epileptogenesis, miRNA targets were predicted by using bioinformatics tools, and relative changes of those targets were quantified to find some link between change in miRNAs, and its effect on molecular signal pathways that are incorporated due to epileptogenesis.

AIMS AND OBJECTIVES

3. Aims and objectives

Objective I: To monitor the effect of curcumin on electrobehavioral progression of $FeCl_3$–induced seizures in rats.

1. To monitor Electorcorticogram (ECoG) recordings in epileptic and curcumin fed rats.
2. To investigate behavioural alteration in epileptic and curcumin-fed rats.

Objective II: To investigate changes in the expression of miR-3120 and miR-214 (mirror micro RNA) in epileptic as well as in curcumin-fed (groups 4-6 and 18-20 months) rats brain.

1. To quantify the levels of miR-3120 and miR-214 in epileptic rats.
2. To quantify the levels of miR-3120 and miR-214 in curcumin-fed rats.

Objective III: To analyze major targets of miR-3120 and miR-214.

Objective IV: To study the response of miR-3120 targets to dietary curcumin in epileptic rats.

MATERIALS AND METHODS

4. Materials and methods

4.1. Materials

4.1.1. Chemicals

The following chemicals were obtained from sources mentioned against each chemical:

i. Curcumin, TRI-reagent, taq DNA polymerase, β-mercaptoethanol, N,N,N′,N′-tetramethylenediamine (TEMED), 3,3′-Diaminobenzidine (DAB), bovine serum albumin (BSA) and tris (hydroxymethyl) aminomethane were purchased from Sigma-Aldrich, St. Louis, USA.

ii. Acrylamide, bis-acrylamide, sucerose, glycerol and aceton were purchased from Merck Millipore, Billerica, USA.

iii. Agarose, Triton X-100, Bradford reagent, ammonium persulfate (APS) were purchased from G-Biosciences, St. Louis, USA.

iv. Sodium dodecyl sulfate (SDS), ethylenediamine tetracetic acid (EDTA), acetic acid, sodium hydroxide and paraformaldehyde were purchased from Fisher Scientific International, Waltham, USA.

v. Polyclonal anti-CACNA1A and anti-GABRD antibodies were obtained from Biorbyt Ltd**,** Cambridge, UK.

vi. Anti-mouse IgG (secondary antibody) and β-actin were obtained from Abcam, Cambridge, UK.

vii. Nitrocellulose membrane was purchased from Whatman Gmbh, Dassel, Germany.

viii. TaqMan miRNA reverse transcription kit, cDNA synthesis kit, DNTPs mix and DNA markers were purchased from Applied Biosystems, Foster City, USA.

All other chemicals used in the study were of analytical reagent grade.

4.1.2. Animals

Male Wistar rats of two age groups: 4-6 months and 16-18 months were used. Animals were obtained from the Central Laboratory Animal Resources of Jawaharlal Nehru University, New Delhi, India; and all experiments were carried out as per the experimental protocols approved by the Committee for the Purpose of Control and

Supervision of Experiments on Animals (CPCSEA) and the Institutional Animal Ethical Committee (IAEC) of Jawaharlal Nehru University, New Delhi, India. Animals were housed in pairs in standard laboratory cages of dimensions 8"x 12"x 5" made of polypropylene with stainless steel covering and maintained at 24 ± 4 °C, under light conditions of 12 hours daylight and 12 hours darkness cycles. Each animal was provided with *ad libitum* access to food and water. Each rat was checked for its health status by observing various criteria, such as tail sores, posture hunch, grooming, red nose rim, red eye rims, tumors, teeth etc. (Markowska et al., 1990; Sharma et al., 1993).

4.2. Methods

4.2.1. Experimental setup

Experimentation was carried out in two parts.

i. First part constituted investigation of alternations in behavior, electrophysiology, gene and protein expression associated with $FeCl_3$- induced Epileptogenesis. Groups of randomly selected rats of 4-6 and 16-18 months of age were designated as 1) Untreated controls – maintained on normal rat feed. 2) Saline injected controls- injected with 5µl normal saline intracortically for five minutes and 3) Epileptic rats injected with 5µl $FeCl_3$ intracortically for five minutes. Electrophysiological, behavioral, histological and molecular biology experiments were performed on every animal of each group. Most of the parameters were studied in the cortex and hippocampus of experimental rats and the results obtained were compared with those of age matched controls.

ii. The second part of the work consisted of the evaluation of the effect of dietary curcumin on the expression of miRNA and its targets proteins. Anticonvulsive or anti-epileptic potential of curcumin was determined in two age groups by further observing the effect of curcumin at the miRNA level. Groups of randomly selected rats of 4-6 and 16-18 months of age were designated as 1) Saline injected controls – maintained on normal rat feed. 2) Saline injected curcumin-fed controls - injected with 5µl $FeCl_3$ intracortically for five minutes and 3) Epileptic rats that are supplemented with curcumin. After $FeCl_3$ injection rats from both groups were fed curcumin supplemented diet (1000 ppm) for next 28 days.

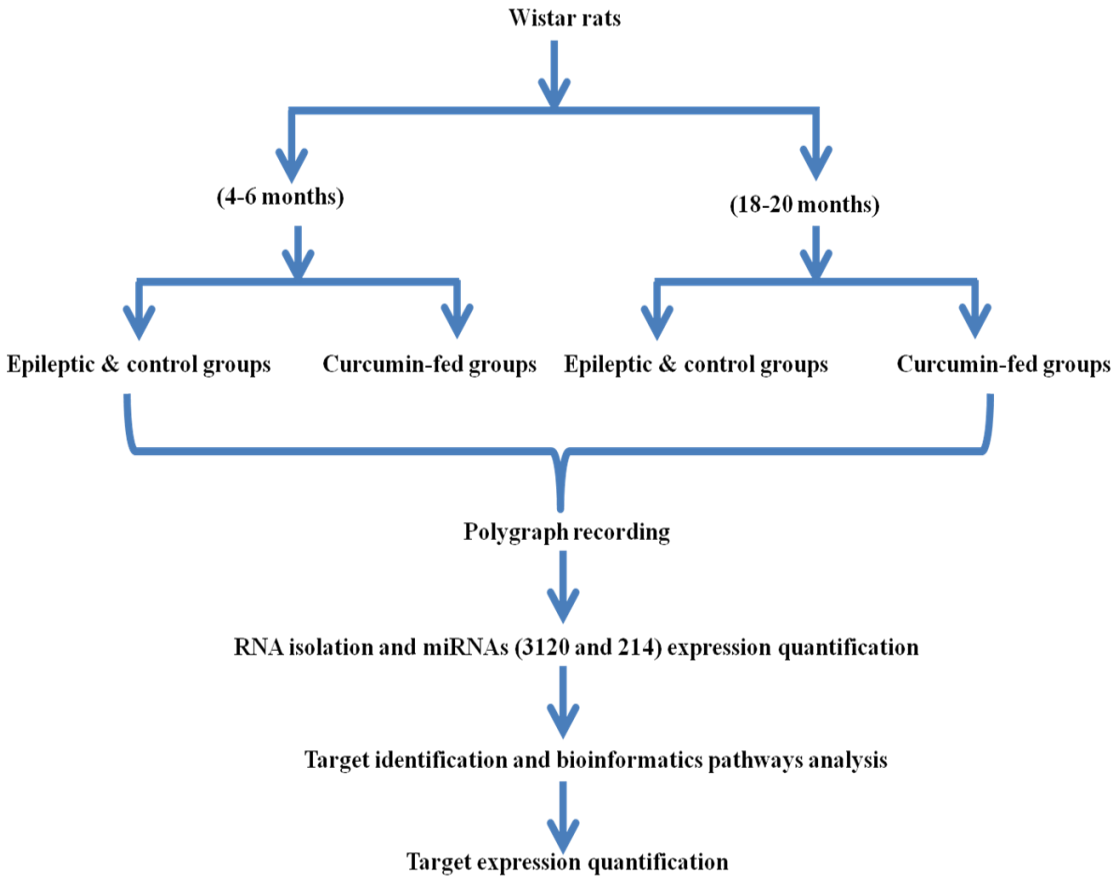


Figure.4.1. Representative summary of work plan and parameters studied.

4.2.2. Electrophysiological experiments

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

i. Rat stereotaxic apparatus (INCO, India)

ii. Accessories: Screw and wire electrodes: For recordings the cortical signals (ECoG), screw electrodes were used. Screw electrodes were connected to a nine-pin socket.

iii. Brain atlas: The stereotaxic coordinates for the electrode implantation were according to the rat brain atlas of Paxinos and Watson (2013) (Figure.4.2).

Surgical procedures

i. Anesthesia: The animals were anesthetized by using 4% isoflurane as an inhalation anesthesia. Isoflurane was filled to minimal level in the vaporizer which was directly connected to oxygen gas supply. Mask delivery method was used for anesthetization of animals, flow rate was maintained at 2-8 l/min

to produce 30-60% of inspired oxygen and oxygen pressure was maintained at 40-55 psi (Grimm et al., 2015).

ii. **Surgery:** After mild anesthesia, 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 were drilled on the surface of the skull marked stereotaxically for the placement of the electrodes and the intracortical injection (Sharma et al., 1993, 2007). Each electrode was connected by wire to an individual pin of nine-pin adaptor/connector. Later, the nine-pin adaptor was affixed onto the surface of the skull with dental acrylic cement to make robust platform.

iii. **$FeCl_3$ injection:** The coordinates for the $FeCl_3$ injection were: antero-posterior, -1.0 mm, lateral 1.0 mm; and ventral (depth) 1.5 mm. A volume of 5µl, 100 mM $FeCl_3$ in physiological saline solution (pH-adjusted) was injected through the burr hole in the somatosensory region of the cortex for five minutes with rate of 1µl/min (saline used as vehicle). After injection, the burr hole was sealed with bone wax (Willmore, 1990).

iv. **Recovery:** The operated rats were provided with optimal post-operative care and habituation before polygraph-EEG recording. Nebasulf an antibacterial sprinkling powder was applied around the wounds to prevent infection. The rats were monitored continuously. In case of low water consumption or dehydration, a 1cc saline or 10% sucrose solution was injected subcutaneously. Each rat was checked for its health status by observing various criteria, such as tail sores, posture hunch, grooming red nose rim, red eye rims, tumors, teeth, etc. (Mishra et al., 2013). Recording was started after complete recovery from the surgery and adequate habituation (five days) in the recording chamber.

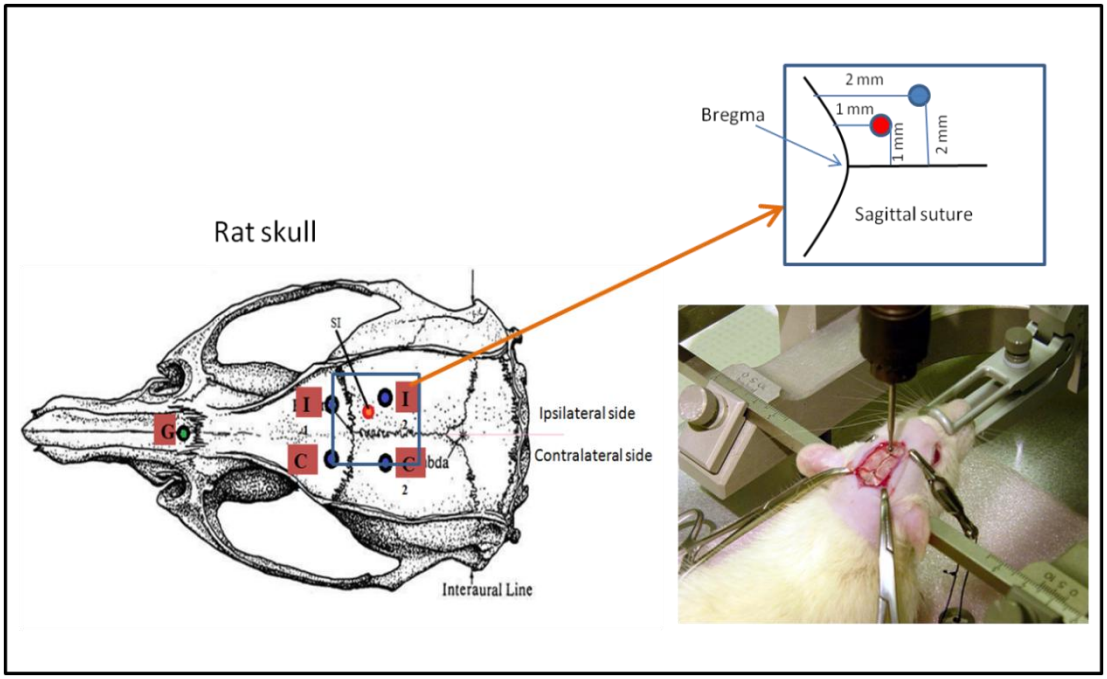


Figure.4.2. Representative picture of epidural electrodes (blue circles) and $FeCl_3$ injection (red circle) site on the rat skull.

Recordings

ECoG was recorded from the ipsilateral and contralateral sides of the cortex. Bipolar ECoG recording were made via the epidural screw electrodes placed in the parietal cortex on the ipsilateral and contralateral sides of the injection site (SI). The ECoG records were obtained from a minimum of six animals from each of the control as well as experimental groups (Sharma et al., 1993). For MUA recordings, composite extracellular signals from the same electrodes which recoreded ECoG were routed through a high impedance probe (Grass HIP 511), amplified and filtered (300 Hz to 10 KHz) by Grass P5IIJ AC preamplifiers, electronically discriminated by using window discriminator (WPI) and displayed on oscilloscope. The TTL-spike pulses from window discriminator were simultaneously recorded on the polygraph. Using Grass integrator preamplifiers, cumulative mathematical integration of EEG traces was also recorded on one of the polygraph channels (Roy and Singh, 1988). The recordings were limited to the awake immobile state i.e. the behavioral state in which a rat sits quietly but remain awake (Sirviö et al., 1989). Thus, all electrophysiological activity remained uncontaminated from movement related changes and artifact since other waking behaviors (Buzsáki et al., 1983; Vanderwolf, 1969) such as waling, turning, rearing posture, grooming etc. were excluded. ECoG and MUA was recorded

using 79-D model P11J (Grass, Massachusetts, USA.) polygraph in 4-6 months rats and using Polyview-16 (Grass, Massachusetts, USA.) in 18-20 months rats (Figure.4.3a,b). The later setup provides an output in computerized readable format by using polyview-16 graphic unit interface (GUI) to read the output signals of the recordings in place of paper output in earlier P11J model of Grass polygraph.

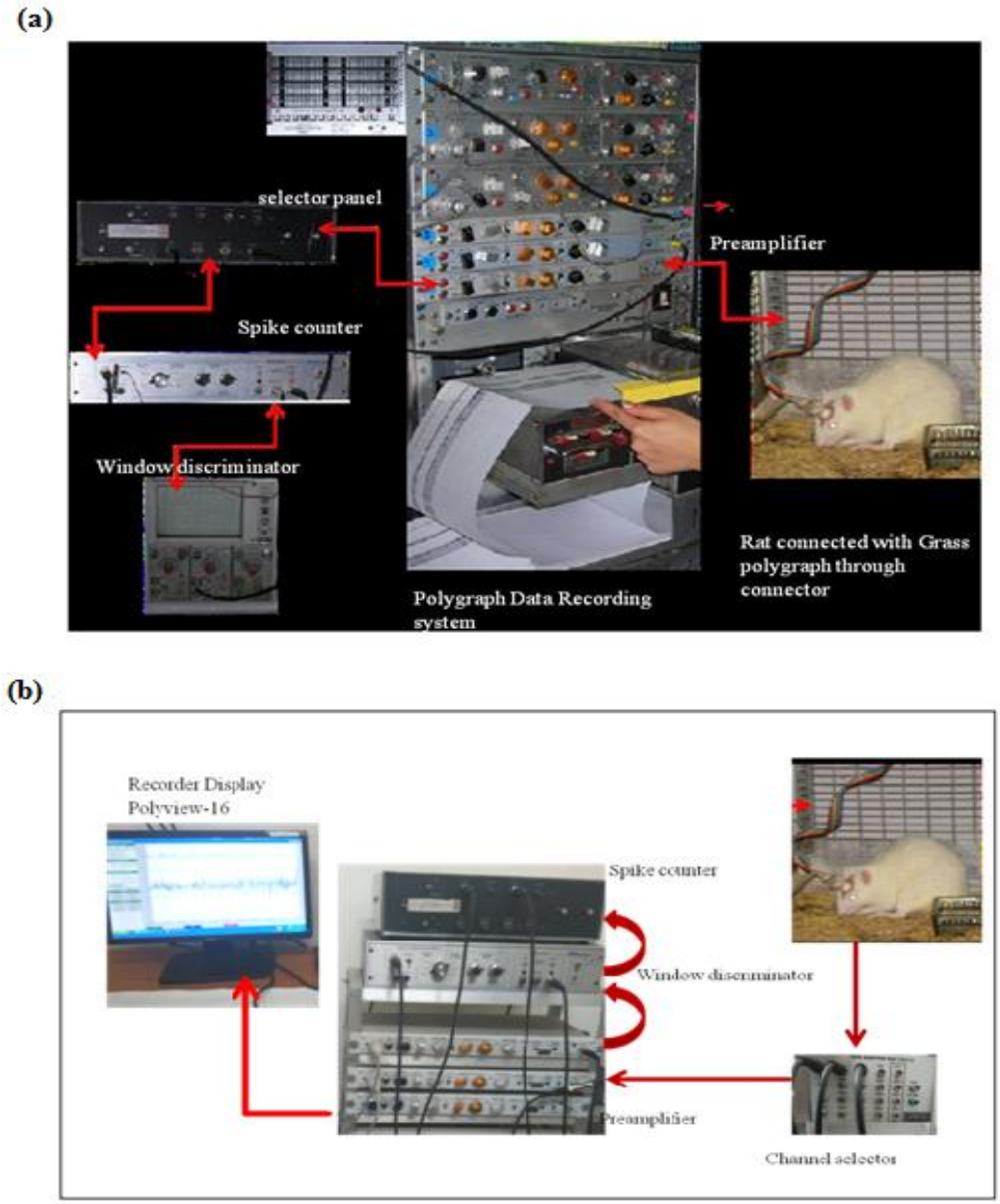


Figure.4.3. Photograph of electroencephalographic recording setup (a) Grass P11J polygraph (b) Grass polygraph polyview-16.

4.2.3. Behavioral studies

i. ***Experimental setup:*** Different groups of rats were investigated for their spatial learning abilities with the help of the Morris water maze test.

ii. ***Morris water maze (MWM) Test:*** The animals were subjected to memory consolidation tests based on a modified version of the MWM test (Morris, 1984). The maze consisted of a black painted circular tank of diameter 168 cm and depth 50 cm, containing four different size, shapes and color maze cues made of thermocol as shown in figure 4.4. The tank was filled with water (24 ±2 ^{o}C) to a level of approximately 30 cm height. The escape platform was positioned at the center of one quadrant and was hidden about 2.0 cm below the surface of water so that the rat could easily escape onto it from swimming in the tank. The tank and visual cues were well illuminated by the room light and kept stable over the learning period. The perimeter of the tank was marked at four places pointing north, south, east and west. Rats selected randomly from the each group were screened for their swimming abilities by recording their respective latencies to reach the platform. (Some researcher paints the platform white and keep it exposed 1.5 cm above the water surface in order to make it easily visible to the animal). The animals were habituated to the experimental conditions prior to the beginning of the experiment by placing them in the water tank without the platform for 60 seconds (for a minimum for 5 days). Those animals exhibiting low swimming speed were excluded from learning and memory tests. After 4 days of testing, the rats were trained to exit the tank onto the 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 our trails. A minimum of eight trials per day was performed, and during each trail, with latency period being 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-trail interval for 60 seconds. Each rat was exposed to the task for five consecutive days (a minimum 20 trials). The location of the platform was fixed during the acquisition period. The MWM training was recorded using a web camera mounted at about 1 m height on the top of the tank.

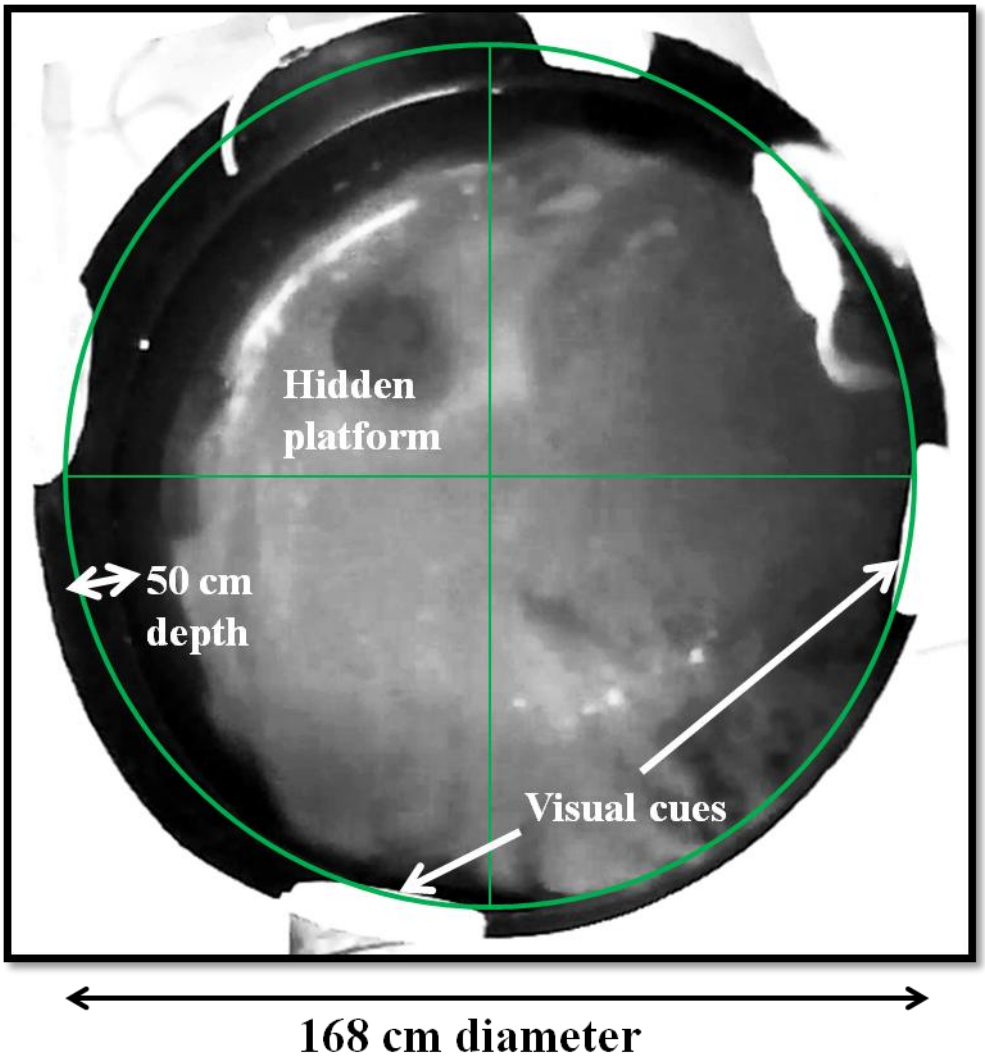


Figure.4.4. Morris water maze test apparatus.

Data was analyzed by the recorded latency period required to reach the platform. The method is useful to assess the rats visuo-spatial learning abilities. The MWM tests were performed between 13:00 hours and 16:00 hours in order to minimize the circadian light/day rhythm-related variations.

4.2.4. Bioinformatics analysis

i. ***Sequence alignment:*** miRNA sequence alignment analysis was done using pairwise sequence alignment tool Basic Local Alignment Search Tool (BLAST) for nucleotides (Needleman and Wunsch, 1970; Tatusova and Madden, 1999) from NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Principle: BLAST works on the computer based algorithms for searching similarity and homology of the query sequence with the given subject

sequence by using either same word search algorithm or using Block Substitution matrix-62 (BLOSUM-62) algorithm for scoring.

Procedure: Query sequence of miR-3120 of rat and subject sequence of miR-3120 of human was extracted from miRBase database and converted to FASTA format. Thereafter, both sequences were added to BLAST search tool on the NCBI website to obtain the alignment scoring results.

ii. ***miRNA target prediction:*** miRNA targets were searched by using miRBase database, miRDB and Diana tools to identify the target mRNAs for miR-3120 using microT algorithm (http://www.microrna.gr/microT-CDS). Target genes reported to be involved in epilepsy are selected from the output of the tool. Further, microinspector webserver tool was used to find the binding site of miR-3120 on the target mRNA, free energy of binding was also calculated by microinspector tool (Papadopoulos et al., 2009; Rusinov et al., 2005) (Figure.4.5).

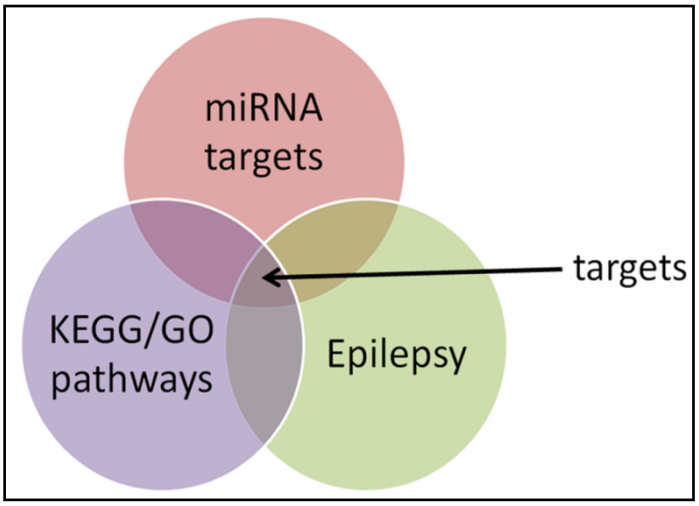


Figure.4.5. Representation of strategy used for target identification of miR-3120 and miR-214 for epilepsy.

iii. ***Pathway analysis of searched targets:*** Involvement of targets either in epileptic pathways or in epilepsy related pathways were analyzed by using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways search tool. Predicted targets of miR-3120 were searched in KEGG system database to find the information about involvement of targets in various pathways. Targets that were involved in pathways already reported to be involved in epilepsy were chosen for further analysis and experimentation (Kanehisa and Goto, 2000).

4.2.5. Animal sacrifice

Animals of each group were sacrificed by cervical dislocation (n=4) for molecular experiments (stemloop miRNA PCR, semi-quantitative PCR and western blotting). The tissues were thoroughly washed with saline to remove blood and stored at -80 C. Transcardial perfusion (n=4) for histological studies (immunohistochemisty) after behavioral experiments was done.

4.2.6. Molecular studies

i. ***RNA isolation:*** After EEG recording and behavioral study, rats were sacrificed by cervical dislocation and their brains were removed. The cortex was dissected out. Brain samples were crushed with liquid nitrogen and mixed with Trizol/TRI-Reagent (Sigma-Aldrich) followed by alcohol precipitation of total RNAs including micro-RNA using isopropanol and 70% ethanol. The palate which was obtained by centrifugation of precipitating RNA was dissolved in RNAase free DEPC-treated water, and the concentration was quantified by using Nanodrop system. Aliquots were made after equilibrating the concentration of RNA and stored at -80^{o} C for further use (Aronica and Gorter, 2007; Mishra et al., 2013).

ii. ***RNA quantification:*** Isolated RNA was quantified by using NanoDrop-2000c quantification system from Thermofischer Scientific, Waltham, USA.

Principle: Nucleic acids absorb UV light at 260nm due to the aromatic base meoieties within their structure. Purines (thymine, cytosine and uracil) and pyrimidines (adenine and guanine) both have peak absorbance at 260 nm, thus making it the standard for quantitating nucleic acid samples. The Beer-Lambert law draws a direct correlation between absorbance and concentration.

Procedure: A small volume of the sample (1-2 µl) was dispensed or loaded on the pedestal and lever arm was closed; because the measurement is volume independent, the sample only needs to bridge the gap between the two optical surfaces for a measurement to be made. Nanodrop software was used for measurement of optical density which it automatically calculated and converted to concentration of RNA in the sample. Purity of RNA was checked by analyzing the ratio of calculated value of absorbance at 260 nm and 280 nm (approximate value 2).

iii. ***miRNA assay by Stemloop-RT-PCR:***

Principle: Stemloop-reverse transcription is similar to conventional cDNA synthesis by reverse transcription. However, stemloop primers were used for reverse transcription reaction which are specific for a target mature miRNA, while the stemloop structure increases the size of primer which increases the specificity of binding and stability after binding to a miRNA. In addition, the product cDNA molecule is long and efficient for further PCR reaction. The resultant PCR product is about 50-60 nt long.

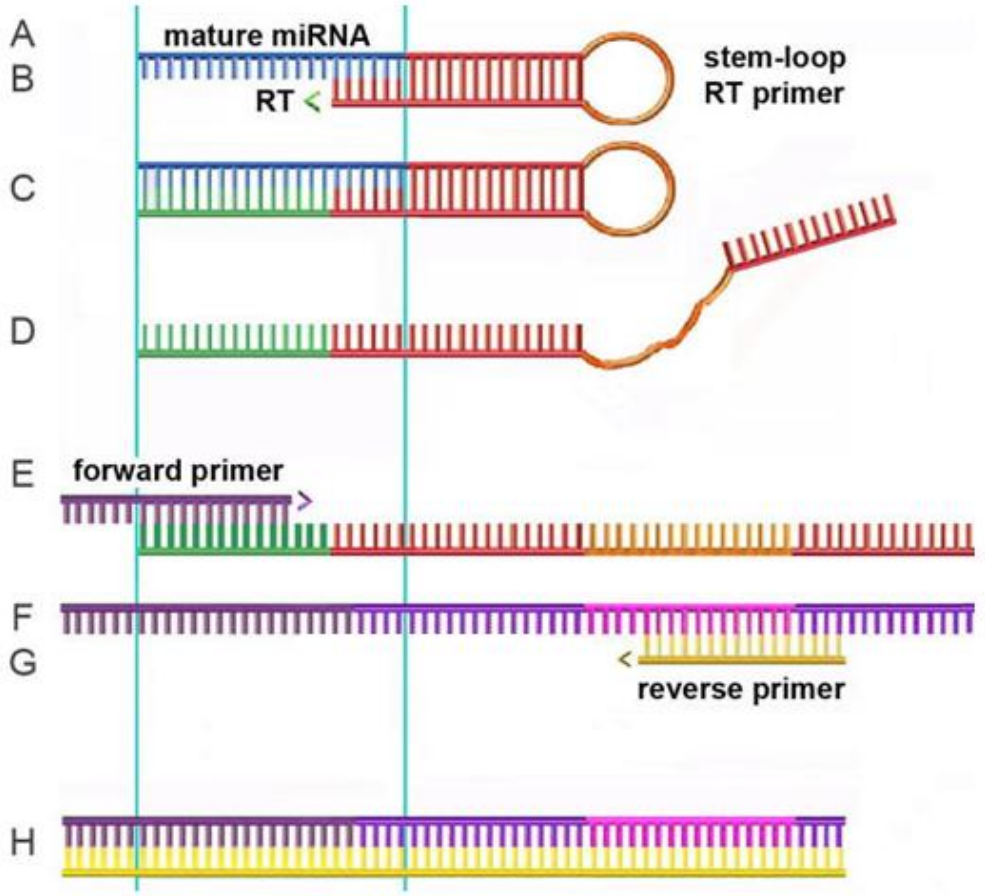


Figure.4.6. Schematic diagram on stem-loop RT-PCR A, Mature miRNA (blue). A-H, Light blue lines show the boundary of the mature miRNA sequence within the RT and qPCR reagent sequences; open arrow-heads indicate directions of polymerization. B, The stem-loop primer 5′ 6 nt annealed with mature miRNA 3′ 6 nt; RT, reverse transcriptase. C and D, First strand cDNA, after polymerization, C, and heat denaturation, D. E, Forward primer with added 5′ nts. F, Second strand cDNA. G, reverse primer. H, PCR product defined by the 5′ termini of the forward and reverse primers (Kramer, 2011).

Components of the kit (TaqMan miRNA reverse transcription kit)

a) miRNA-specific RT primer,
b) U6snRNA (control) primer
c) miRNA-specific forward PCR primer,
d) Specific reverse PCR primer,
e) Multiscribe reverse transcriptase 50 U/µl,
f) 10x reverse transcription buffer, RNase inhibitor 20 U/µl

Procedure:

miRNA Reverse transcription*:* miRNA cDNA synthesis was carried out from the purified and intact total RNA by using the MicroRNA Reverse Transcription kit. Each reaction was of 15 µl which consists of 7 µl master mix, 3 µl primers, and 5 µl RNA samples (Kramer, 2011).

***miRNA-PCR*:** cDNA products (1 µl) were subjected to semi-quantitative PCR analysis on a gradient thermal cycler instrument. PCR cycle comprised of initial denaturation at 94°C for 2 min. The amplification was then carried out for 35 cycles consisting 30 second each at 94°C (denaturation) and 72°C (annealing), 1 minute (extension). Final extension was done at 72°C for 10 minute (Table.4.1). The RT-PCR products were applied to 4% (w/v) agarose gel electrophoresis containing ethidium bromide. PCR products were visualized under UV light and photographed using gel documentation system. The intensities of the bands were quantified densitometrically using ImageJ software.

Table.4.1. Thermocycler program for cDNA synthesis

Step type	**Time (min)**	**Temperature**
HOLD	30	16
HOLD	30	42
HOLD	5	85
HOLD	∞	4

iv. In vitro reverse transcription

Components of the kit

i. Reverse Transcriptase (200 U/µl) in storage buffer : 50 mM Tris-HCl, pH 8.3, 0.1 M NaCl, 1 mM EDTA, 5 mM DTT, 0.1% Triton X-100 and 50% glycerol

ii. RiboLock^{TM} Ribonuclease Inhibitor (20 U/µl) in storage buffer : 20 mM HEPES-NaOH, pH 7.5, 50 mM NaCl, 8 mM DTT, 0.5 mM ELUGENT Detergent and 50% glycerol

iii. 5x Reaction Buffer : 250 mM Tris-HCl, pH 8.3 at 25ºC, 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT

iv. 10 mM dNTP mixture : 10 mM aqueous solution of each dGTP, dATP, dTTP, dCTP

v. Control primer : 15 µl of 10 pmol/µl ($1.7A_{260}$ units/ml) 17-mer aqueous solution

vi. Control RNA : 1.1 kb RNA with 3´-poly (A) tail, 0.5 µg/µl

vii. DEPC-treated Water

Procedure: cDNA synthesis was carried out from the purified and intact total RNA by using the RT-PCR kit. One unit of MuLV RT incorporates 1 nM of dTMP into a polynucleotide fraction (adsorbed on DE-81) in 10 min at 37ºC. One unit of RiboLock^{TM} Ribonuclease Inhibitor inhibits the activity of 5 ng RNaseA by 50%. To the 5 µg of total RNA, 1 µl of Oligo-dT was added in order to make it 12 µl. The contents were mixed gently and microcentrifuged for 3-5 second. The mixture was incubated at 70ºC for 5 minutes, chilled on ice and drops were collected by brief centrifugation. On ice, 5x reaction buffer (4 µl), ribonuclease inhibitor (20U/µl) and 10 mM dNTP (2 µl) were sequentially added and mixed followed by brief centrifugation. The reaction mixture was then incubated at 37ºC for 5 min and reverse transcriptase was added at 200 U/µl. Total reaction volume of 20 µl was incubated at 42ºC for 60 min and the reaction was stopped by inactivating the reverse transcriptase at 70ºC for 10 minutes. The cDNA products thus formed were chilled and stored at 4ºC for amplification by the PCR. Two µl of the cDNA synthesized was used for the PCR amplification in which cDNA represents 1/10th of the total PCR reaction mixture. Under similar conditions, the cDNA of the control

RNA provided with the kit was synthesized so as to monitor the quality and efficiency of the *in vitro* synthesized cDNA from the RNA of the samples.

v. ***Semi quantitative RT-PCR analysis of PTEN:*** For the amplification of specific genes and β-actin, cDNA products (1 µl) were subjected to semi quantitative PCR analysis on a gradient thermal cycler instrument. PCR cycle comprised of initial denaturation at 94°C for 2 minutes. The amplification was then carried out for 30 cycles consisting 30 seconds each for 94°C (denaturation) and 72°C (annealing), 1 minutes (extension). Final extension was done at 72°C for 10 min. β-actin was used as an internal control. Primers were designed with Primer3 software using sequences data available on National Centre for Biotechnical Information (NCBI) database (Table.4.2). The RT-PCR products were applied to 1.2% (w/v) agarose gel electrophoresis containing ethidium bromide. PCR products were visualized under UV light and photographed using gel documentation system. The intensities of the bands were quantified densitometrically using ImageJ software.

Table. 4.2. Sequences of PCR primers used for the amplification of cDNAs (Mishra et al., 2013; Rahal and Simmen, 2010).

Primer	**Sequence (Forward, Reverse)**
Pten	5'CAATGTTCAGTGGCGGAACTT3'
	5' GGCAATGGCTGAGGGAACT3'
GAPDH	5'ACCACAGTCCATGCCATCAC3'
	5'CACCACCCTGTTGGCTGTAGCC3'

vi. ***Protein isolation:*** Tissue samples were homogenized in 50 mM Tris (pH 7.4) with a potter-elevehjem type homogenizer fitted with Teflon plunger. The homogenate was diluted 1:10 (with Tris, pH 7.4, buffer) and centrifuged at 6000 rpm for 5 minutes in a refrigerated centrifuge (Sorvall RCS or RC5C). The resulting pellet (P1), consisting of nuclear and cellular material, was discarded. The supernatant (S1), containing mitochondria, synaptosomes, micorsomes and cytosol, was futher ultracentrifuged at 25,000 rpm for 25

minutes to form mitochondrial pellet (P2). The resulting supernatant (S2) was used as such as cytosolic fraction.

vii. ***Protein estimation:*** Protein estimation was performed by Bradford's method (Bradford, 1976) using BSA.

Principle: The commassie brillian blue dye binds to the anionic form of the arginine tryptophan, histidine, and phynalanine residues in the protein, which have an absorbance maximum at 595 nm (blue). This absorbance, can thus, be measured spectrophotometrically, higher the absorbance, greater in the amount of protein in the sample.

Apparatus: Procedure: Different concentrations (1-10 µg in steps of 2 µg) of BSA were taken to generate a standard curve. For, the estimation of protein from the brain tissue homogenate samples, 3 µl of homogenate and, 47 µl of distilled water, and 250 µl of Bradford's reagent (Sigma Aldrich) were added to each well of a 90 well plate. The absorbance was measured spectrophotometrically, and the quantitation was done by comparing with the standard BSA curve.

viii. ***SDS-PAGE***

Principle: Separation of charged molecules in an electric field is based on the relative mobility of similar negatively charged species (due to presence of SDS) which is related to frictional resistance.

Material: 30% acrylamide, 10% SDS, 10% APS, TEMED, 1.5 M Tris, pH 8.8 (resolving), 1.0 M Tris, pH 6.8 (stacking gel), 5x SDS running buffer, Coomassie Blue stain, SDS sample loading buffer, 10% acetic acid.

Procedure: Polyacrylamide gel was prepared according to standard protocols for 8% and 12% gel concentrations and samples were loaded. Protein samples were prepared in SDS and β-mercaptoethanol and loaded to gel to run either at constant current of 25mA or constant voltage of 60-80V; after that gel was further used for transferring of proteins to PVDF membrane for Western Blotting.

ix. ***Western blotting***

Principle: SDS-PAGE followed by Western blotting in an analytical protein detection technique. The SDS denatures the protein, which are separated by

the number of polypeptides on a PAGE gel. These separated proteins can then be transferred onto a membrane and the desired protein can be detected and quantitated by specific anti-bodies (Burnette, 1981).

Apparatus: Bio-Rad PAGE apparatus was used to separate the denatured proteins.

Procedure: Proteins (10-30 µg) from the tissue homogenate was separated on 8% SDS- PAGE for CACNA1A and 12% SDS-PAGE for GABRD proteins, transferred to PVDF membrane (Whatman, Sigma-Aldrich) for 3 hours at 4 °C. Overnight incubation with primary antibodies used for detection (anti-CACNA1A, anti-GABRD and anti-β-actin) was performed at 4°C. This was followed by incubation with secondary antibody for 1 hour at room temperature and detected using 3,3 diaminobenzidine (DAB). Blocking (skimmed milk) and buffer washing were performed after every antibody step.

4.2.7. Histological Experiments

i. ***Tissue Processing:*** The animals were deeply anesthetized with a ketamine (50mg/kg i.p.). Each rat was transcardially perfused with physiological saline solution and then fixed with a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Perfusion was performed by infusing the saline solution into the left ventricle of the animal's heart, as the left ventricle channels blood to the systemic circulation through the aorta. Simultaneously, an incision was made in the right atrium, which receives blood from the entire body via the inferior and superior vena cava, to wash out all the blood and thereafter, the perfusate from the system. For the rats, 200ml of saline solution (approximately four times the total blood volume) was used over a period of 6-8 minutes to wash out all the blood. Thereafter, 200-300 ml of fixative was perfused for 15 minutes. Post perfusion, the brain was removed and fixed in 10% formalin solution for 7 days. Following formalin-fixation, the brain was put in gradients of sucrose (10% to 30%) for sucrose embedding. Sucrose is a cryopreserving and thus protects the tissue integrity upon cryosectioning. Cryosectioning was performed using Leica (CM 1860 UV) crytome, sections 15 µm in thickness were cut and mounted on gelatin-coated slides. The sections were stored (mounted on gelatin slides) at -20 °C till further processing/ staining.

ii. ***Immunohistochemistry (IHC):***

Principle: IHC is a technique used to detect antigens (e.g. proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. In the present study, Streptavidin-biotin-complex staining method was employed. Here, the primary antibody is specific to the protein of interest in the biological tissue; the secondary antibody is specific for the primary antibody and is biotinylated i.e. bound to biotin.

Biotin is a small molecule (244 gm/mol), in the IHC context the, its valeric acid side chain is modified to conjugate to secondary antibody. Streptavidin is the avidin from *Steptomyces avidinii*. It consists of 4 identical subunits, each capable of binding one biotin molecule. Therefore, one avidin molecule can bind 4 biotin molecules and hence, 4 secondary antibodies. Streptavidin has an advantage over other avidin molecules: it does not glycosylate easily, as opposed to avidin, which has very high glycosylation. Thus, it makes streptavidin less prone to non-specific binding. When the streptavidin-biotin complex comes in contact with 3,3-diaminobenzidine (DAB), it turns brown, indicating the presence of the desired protein.

Voltage gated Calcium channel alpha 1 a subunit (CACNA1A) and GABA receptor delta subunit (GABRD) IHC was performed to show the localization in the cell, and show the regions of neurons in which there is increase in the expression of these proteins.

Procedure: Cryosections were used for immunohistochemical localization of CACNA1A and GABRD positive neurons. Briefly, sections were taken out from -20°C and left at room temperature (37 °C) for 1 hour, followed by denaturation in 1% triton-100, which is a mild detergent. Thereafter, to inhibit any endogenous peroxidase, the sections were incubated in the dark in 1% hydrogen peroxidase activity, the sections were incubated in 10% normal goat serum (NGS) at room temperature for 90 minutes to prevent non-specific binding. The sections were then incubated in primary antibody (1:1000 for anti-CACNA1A, anti-GABRD), overnight at 4°C in humid chamber. The slides were again normalized to room temperature for 1 hour after incubation with primary antibody, before incubation with primary antibody, before

incubation with biotinylated secondary antibody (IgG 1: 100) at room temperature for 2 hours. The slides were next incubated in streptavidin-peroxidase (1:100) for 2 hours at room temperature. A 10-15 minutes incubation was then given in DAB (0.25% in 1% H_2O_2), or till the sections turn brownish in color. The slides were then washed in running tap water, followed by two washed in dH_2O. After every step, the slides were washed thrice with PBS. Finally, the slides were dehydrated by ascending grades of alcohol, cleared with xylene and mounted with DPX.

4.2.8. Statistical analysis

Data were expressed as mean ± standard error of mean (SEM). Statistical comparison was performed by one way ANOVA followed by students-t-test analysis for observing the effect of each day of trails in Morris water maze. Calculated probabilities of < 0.05 were considered to be of significance and < 0.01 highly significant. Students t-test was performed for all pair wise analysis western blotting.

RESULTS

5. Results

5.1. Objective I: To monitor the effect of curcumin on electrobehavioral progression of $FeCl_3$–induced seizures in rats

5.1.1. Electrocorticogram (ECoG) recordings

(a) Epileptic rats

In the present study, we generated PTE model by steriotaxically injecting $FeCl_3$ (dissolved in physiological saline) into the sensorimotor cortical brain region of 4-6 and 18-20 month rats. The occurrence of seizures was monitored using encephalographic ECoG. The animals that were administered intracortical $FeCl_3$ injections progressively developed epileptiform activity. Distinct chronic epileptiform activity on ECoG began to appear around the 7^{th} day in rats of both age groups (Figure.5.1). The epileptiform activity was spontaneous, recurrent, and consisted of isolated spikes, polyspikes, and spike-waves complexes. Behavioral seizure activity was also concomitant with ECoG paroxysms, progressed with time, and consisted of more facial automatisms, head nods following pauses in behavior, steadfast posture, tonic flexing concurrent with biting, and chewing of hindlimb extremity. Furthermore, the epileptiform activity was quantified by multiple unit action potentials (MUA). The MUA recordings clearly showed the progressive development and build-up of the epileptic ECoG activity. Figure.5.1 shows that the MUA count increased by about 3, 9, and 11 folds on the 7^{th}, 21^{st}, and 28^{th} day, respectively, of the ECoG recording as compared with their respective controls in the 4-6 month rats. Similarly, about 5, 7, and 16.5 fold increase in MUA count was observed on the 7^{th}, 21^{st}, and 28^{th} day, respectively, in the 18-20 month rats. The control animals (which received an intracortical saline injection instead of $FeCl_3$) of both age groups did not show any electrographic seizure activity (Figure.5.1).

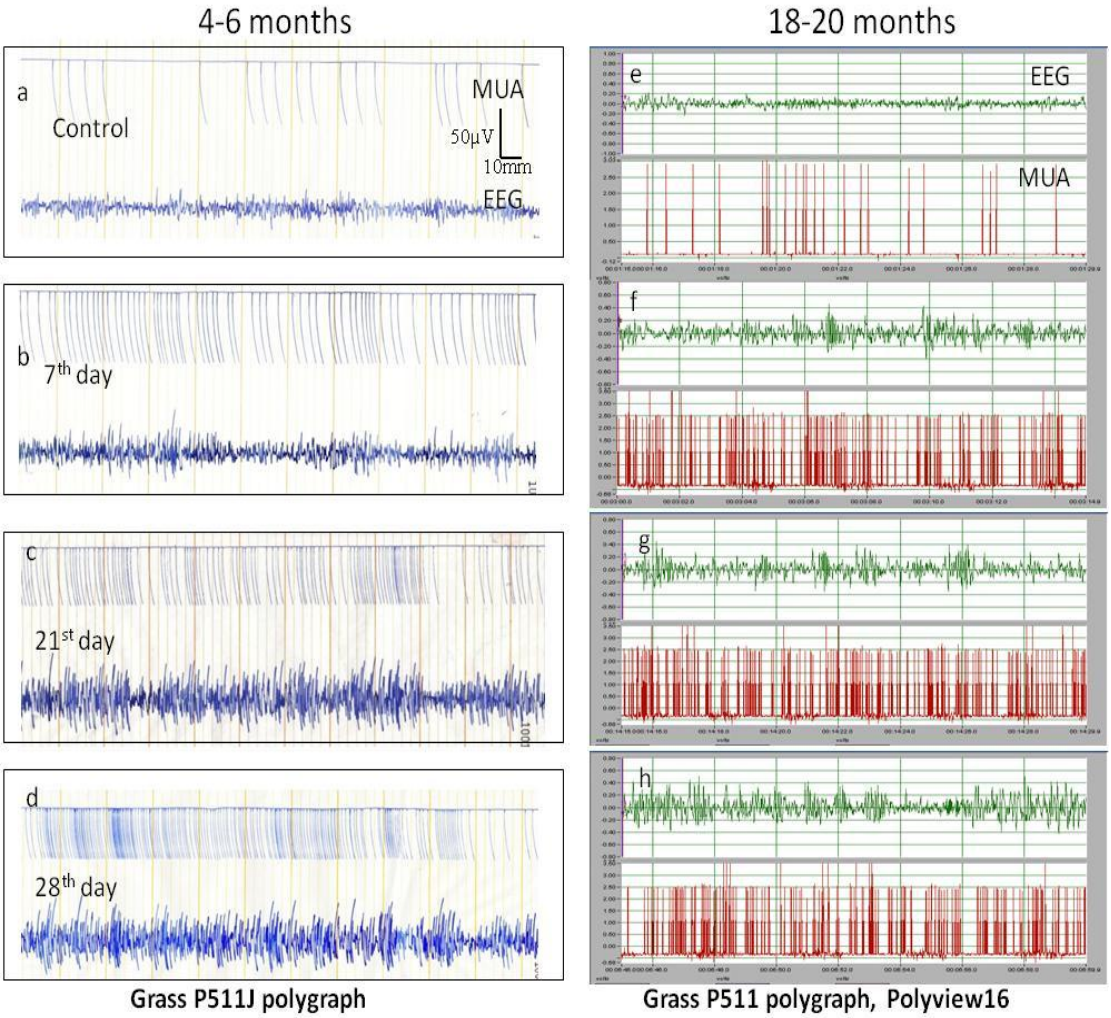


Figure.5.1. Representative samples of polygraph recordings (ECoG and MUA) from the somatosensory region of cortex in epileptic rats of two different age groups showing epileptogenesis (a) control, (b-d) day 7, 21, and 28 epileptic 4-6 month old rats (e) control (f-h) day 7, 21, and 28 epileptic 18-20 month old rats.

(b) Curcumin-fed rats

Thereafter, we monitored the epileptiform activity in curcumin-fed rats and the results demonstrated that curcumin supplementation suppressed the behavioral seizures as well as epileptiform activity. Curcumin-fed epileptic rats showed significant decreases in epileptiform activity in both age group rats as compared to their epileptic controls. Figure.5.2 depicts that the corresponding MUA counts of 4-6 month old, curcumin-fed, epileptic rats were progressively decreased by 1.25, 1.9, and 20 folds on the 7^{th} , 21^{st}, and 28^{th} day, respectively, as compared to their age match epileptic controls. Similar results were also found in 18-20 month old rats, where MUA count was decreased by 1.3, 1.4, and 1.8 folds on the 7^{th}, 21^{st}, and 28^{th} day, respectively, as compared to age matched epileptic controls. Moreover, results showed that MUA count of curcumin-fed epileptic rats was higher in the 18-20 month old rats on day 21 when compared with 4-6 month old rats. However, on day 28, the MUA count did not show any significant difference in the rats of both age groups after curcumin feeding (Figure.5.3).

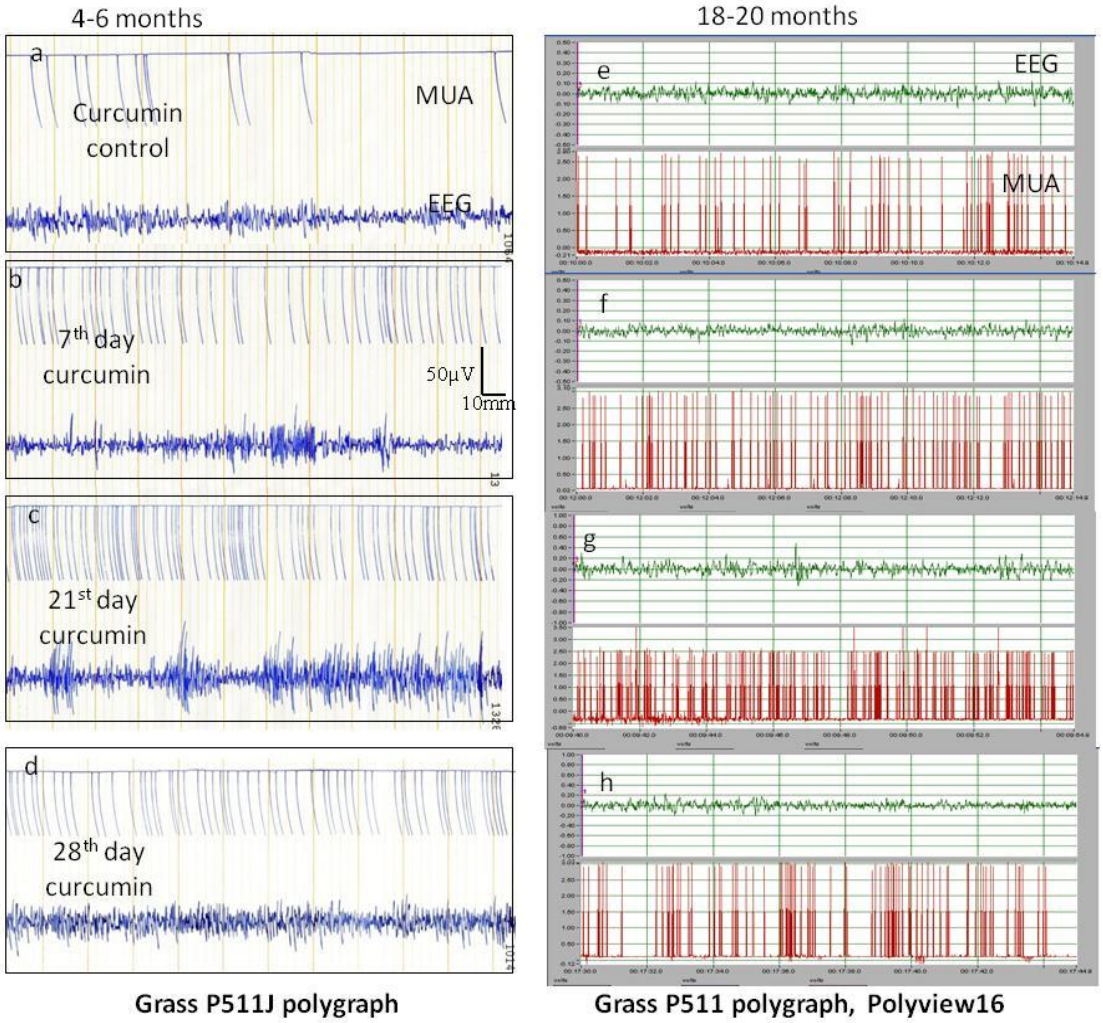


Figure.5.2. Representative samples of polygraph recordings (ECoG and MUA) from the somatosensory region of cortex in curcumin-fed rats of two different age groups showing suppressed epileptic seizures (a) control, (b-d) day 7, 21, and 28 epileptic 4-6 month old rats (e) control (f-h) day 7, 21, and 28 epileptic 18-20 month old rats.

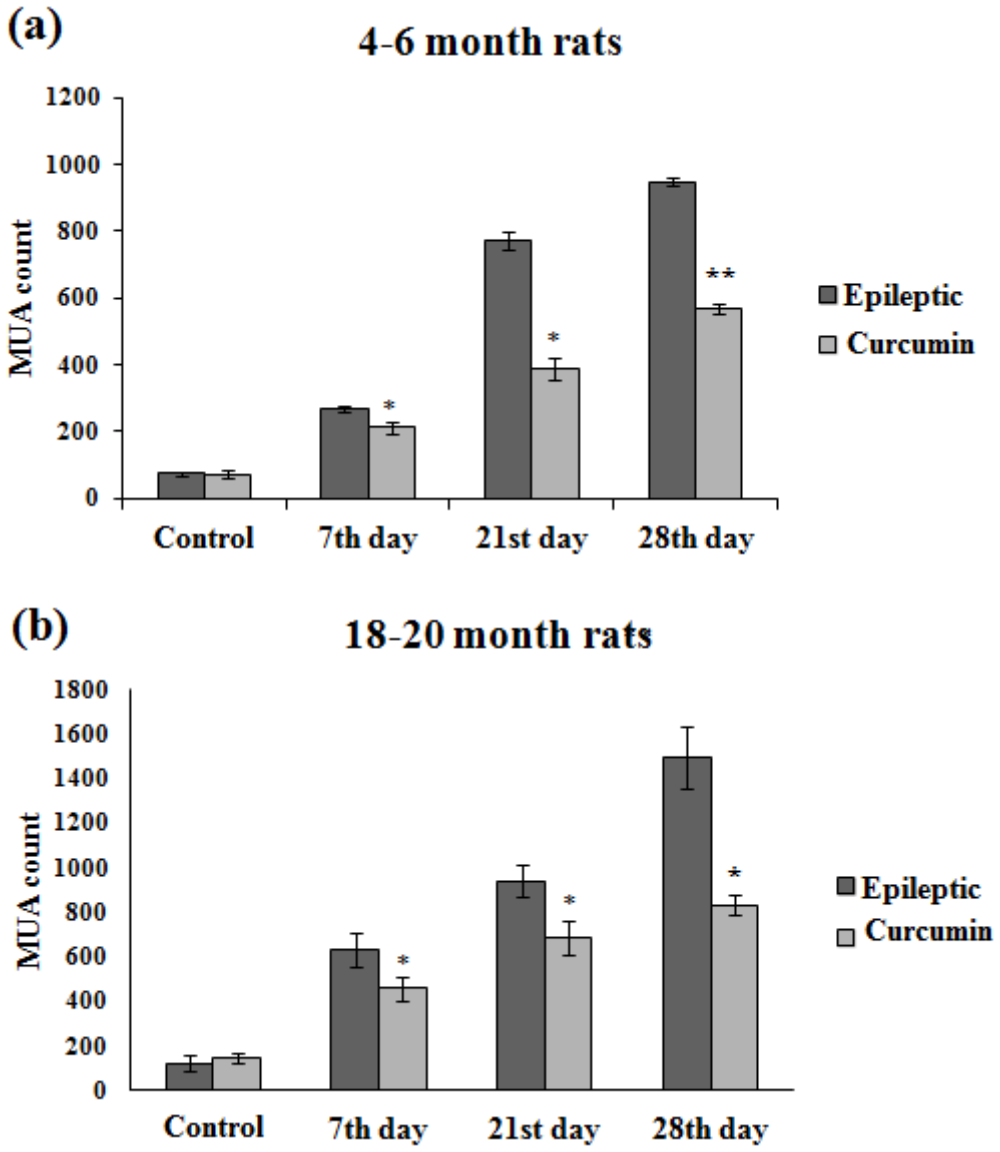


Figure.5.3. Effect of curcumin treatment for 7, 21, and 28 days on MUA in epileptic rats; each bar represents mean ± SEM of 4 rats (a) 4-6 month old (b) 18-20 month old. Statistical comparison is with their respective controls. *p< 0.05, **p< 0.01.

5.1.2. Morris Water Maze:

The Morris water maze test (cue-based spatial learning test) results are expressed as the average latency period of each rat from all the groups to reach the fixed hidden platform over each of the five trial days. We found that all the experimental rats learned to escape from swimming by searching for the hidden platform using visual cues. As depicted in Figure.5.4 epileptic animals from both age groups showed altered latency to find hidden platform. The $FeCl_3$-induced epileptic rats displayed a significantly longer latency to find the hidden platform compared to control in both 4-6 month (Figure.5.4a) and 18-20 month (Figure.5.4b) old groups. Whereas, curcumin supplementation reduced the latency period to find the hidden platform as compared to epileptic rats in both age groups. Hence, our results clearly illustrate that curcumin feeding significantly rescued rats from PTE-induced impairment in learning and memory.

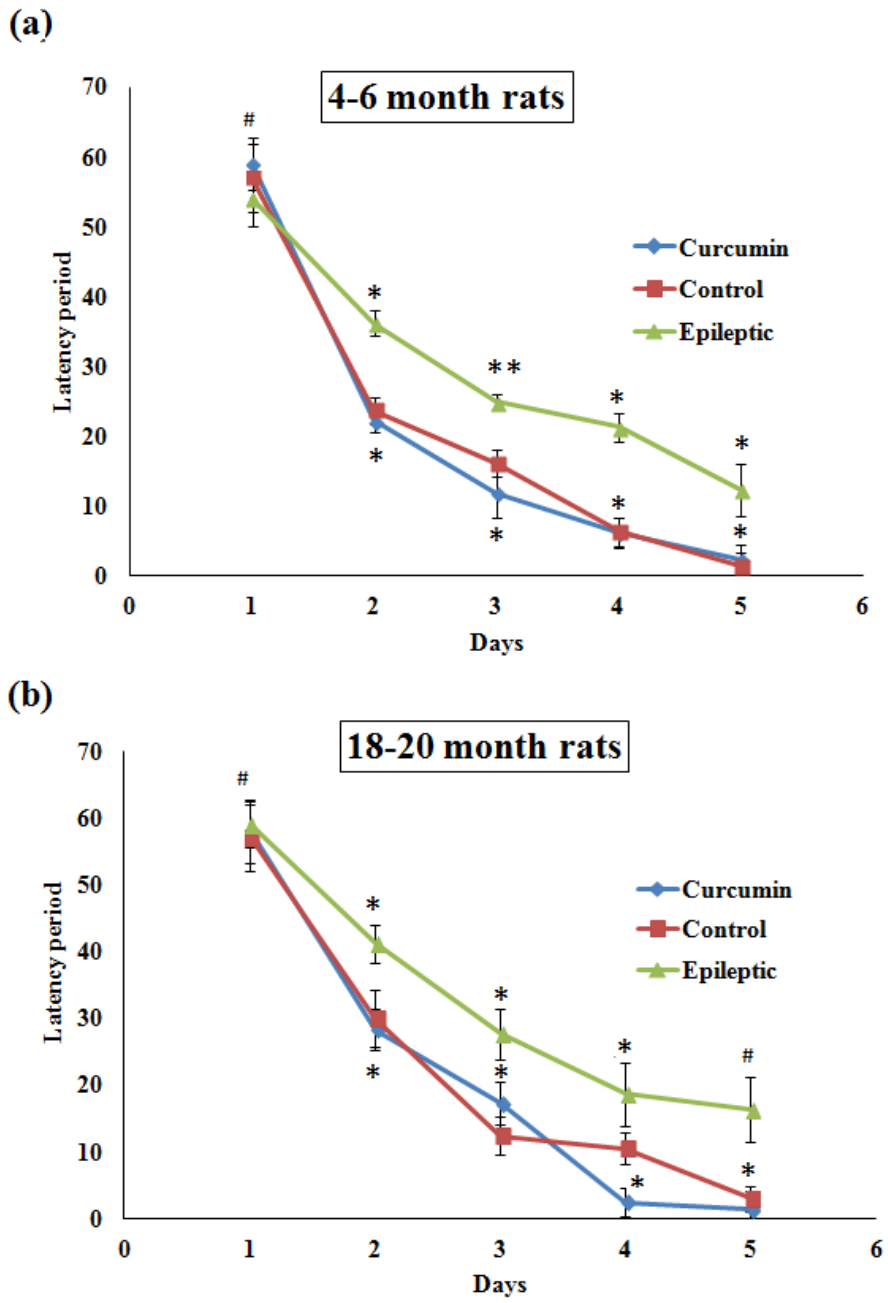


Figure.5.4. Comparison of control, epileptic, and curcumin-fed rats with respect to their respective latencies to find the hidden platform in the Morris water maze test (a) 4-6 month and (b) 18-20 month old rats. Each data point represents the mean latency ± SEM of 4 rats. The significance in the case of the epileptic rats is with respect to sham controls and for curcumin-fed rats is with respect to epileptic controls: *p< 0.05 **p< 0.01 ; # not significant.

5.2. Objective II: To investigate changes in the expression of miR-3120 and miR-214 (mirror micro RNA) in epileptic as well as in curcumin-fed rats brain.

5.2.1. Expression of miR-3120 and miR-214 in the cortex of $FeCl_3$-induced epileptic rats

To understand the difference of miRNA expression in two different age groups, miRNA quantification was done using semi-quantitative RT-PCR. The expression of miR-3120 was determined in the cortex and hippocampus of 4-6 month and 18-20 month old rats. As depicted in Figure.5.5, there was a higher expression of miR-3120 in the cortex of 4-6 month old rats as compared to 18-20 month old rats (*$p<0.05$). In addition, results also showed that the expression of miR-3120 was higher in the hippocampus as compared to the cortex (Figure.5.6).

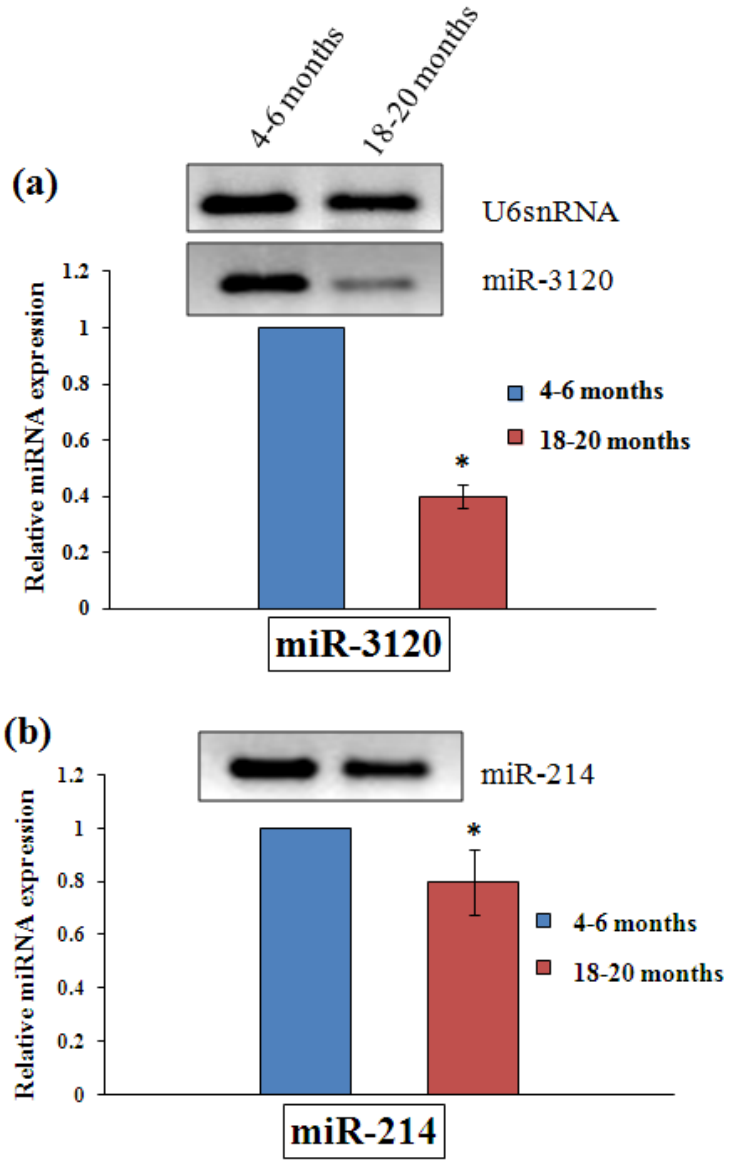


Figure.5.5. Relative expression of miR-3120 and miR-214 in the cortex of (a) 4-6 month and (b) 18-20 month old rats, as measured by semi qRT-PCR analysis. Each bar represents mean ± SEM of 4 rats *p< 0.05.

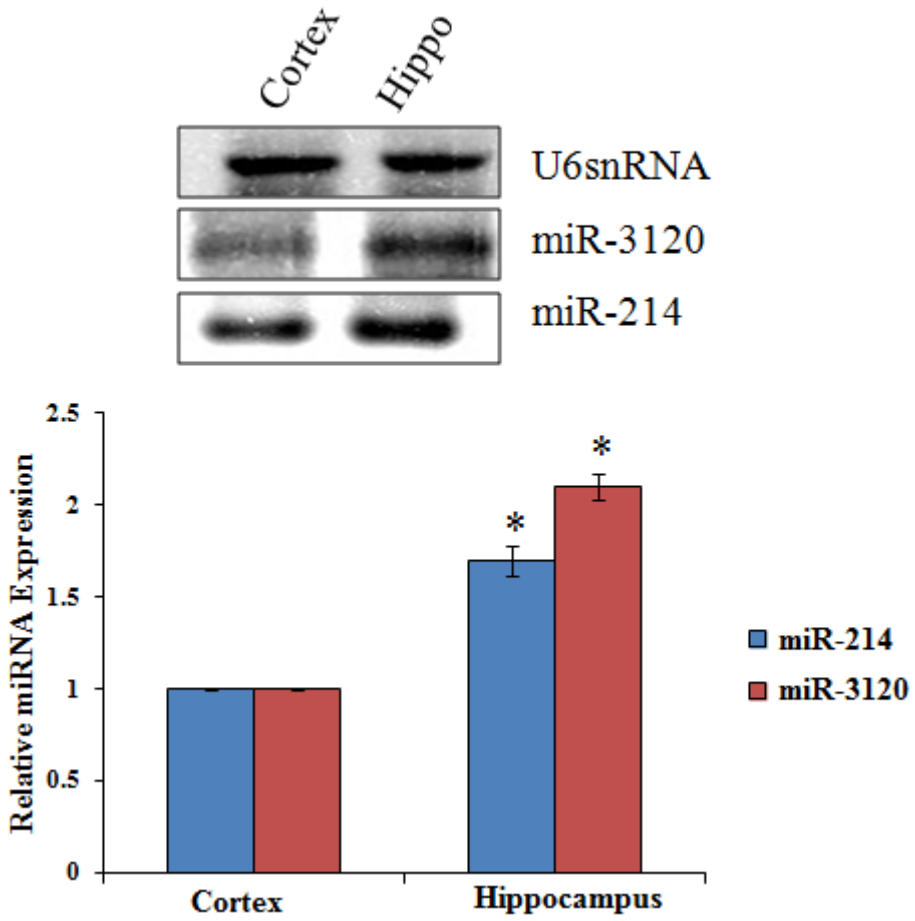


Figure.5.6. Relative expression of miR-3120 and miR-214 in the cortex and hippocampus of rats, as measured by semi qRT-PCR analysis. Each bar represents mean ± SEM of 4 rats. *$p<0.05$, significant difference.

(a) miR-3120

Further, the level of miR-3120 was determined in the cortex of $FeCl_3$-induced epileptic rats at days 7, 14, 21, and 28 after induction of epilepsy. Figure.5.7 shows that the expression of miR-3120 was progressively reduced in $FeCl_3$-induced epileptic 4-6 month old rats. At 7, 14, 21, and 28 days, we found that levels of miR-3120 were significantly decreased by 1.25, 2.25, 2.5, and 3.35 folds with the progression of electrographic seizure when compared with their age matched sham controls (Figure.5.7a; *p< 0.05) . Similarly, in 18-20 month old rats, the levels of miR-3120 were estimated at the 7^{th} and 28^{th} day. Results showed a 4.5 fold decrease in the expression of miR-3120 on day 28 (*p<0.05), however, no significant change was found on day 7 when compared with their respective controls (Figure.5.7c).

(b) miR-214

In addition, expression of miR-214 was determined in the cortex of $FeCl_3$-induced epileptic rats at days 7, 14, 21, and 28 after induction of epilepsy. Figure.5.7b shows that the level of miR-214 was reduced progressively in $FeCl_3$-induced epileptic 4-6 month old rats. At 7, 14, 21, and 28 day, we found that levels of miR-214 were significantly decreased by 1.17, 2.8, 5.55, and 50 folds with the progression of electrographic seizure at 7, 14, 21, and 28 day when compared with their age matched sham controls (Figure.5.7b; *p< 0.05). In 18-20 month old rats, the levels of miR-214 were also measured on the 7^{th} and 28^{th} day. There was 5.5 fold decrease in the expression of miR-214 on day 28; however, there was no significant change on day 7 when compared with their respective age matched controls (Figure.5.7d; *p< 0.05).

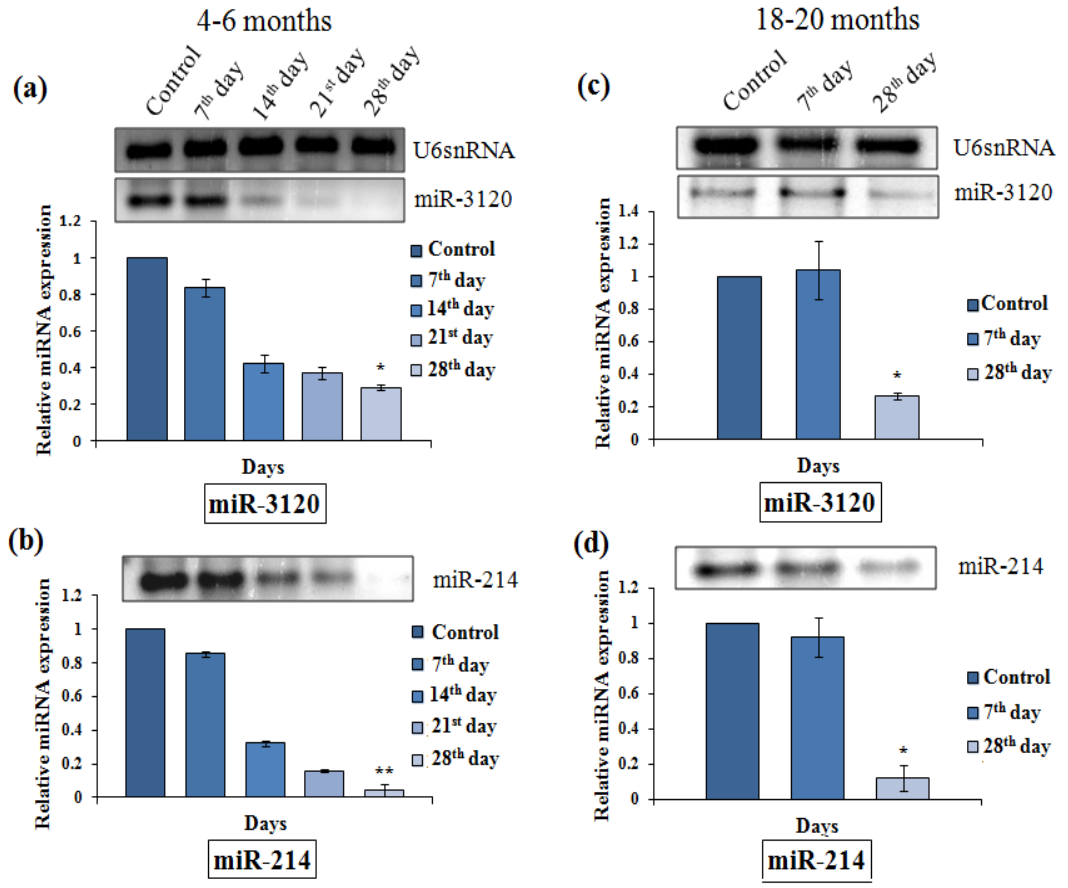


Figure.5.7. Relative expression of miR-3120 and miR-214 in the cortex of epileptic rats measured by semi qRT-PCR analysis. Each bar represents mean ± SEM of 4 rats. Statistical comparison of values at day 7, 14, 21, and 28 of epileptogenesis are with respect to controls. *$p<0.05$, **$p<0.01$, significantly different from control group.

5.2.2. Effect of curcumin on the expression of miR-3120 and miR-214 in the cortex of $FeCl_3$-induced epileptic rats

(a) miR-3120

To evaluate the effect of dietary curcumin supplementation on the expression of miR-3120, RT-PCR analysis was done. Level of miR-3120 was determined in curcumin-fed $FeCl_3$-induced epileptic rats on the 7^{th} and 28^{th} day, in the 4-6 month and 18-20 month old rats. In the 4-6 month old group, we observed that there were no significant change in the expression of miR-3120; however, significantly increased level of miR-3120 was found on the 28^{th} day after curcumin supplementation (Figure.5.8a; *$p< 0.05$). Results showed an approximate 2 fold increase in the expression of miR-3120 on day 28 when compared with the age matched epileptic controls. This clearly indicates that curcumin partially countered the epileptogenesis-associated decline in miR-3120 levels in their cortex of 4-6 month old rats (Figure.5.9a; *$p< 0.05$).

In the 18-20 month old rats, the expression pattern of miR-3120, as measured on the 7^{th} and 28^{th} day, was found to be similar to that of the 4-6 month old rats. As evident from Figure. 5.8c, there was not significant change in expression of miR-3120 on day 7; however, after the 28-day curcumin supplementation, a significant elevation in the expression of miR-3120 was observed. Figure. 5.9b depicts that the level of miR-3120 was increased about 4.5 folds, indicating that curcumin partially countered the epileptogenesis-associated decline in miR-3120 expression in their cortex of 18-20 month old rats (*$p< 0.05$).

(b) miR-214

Furthermore, the level of miR-214 was also estimated in curcumin-fed $FeCl_3$-induced epileptic rats on the 7^{th} and 28^{th} day in 4-6 month old rats. Results demonstrated that there was no significant change in the expression of miR-214 on day 7; however, significantly higher level of miR-214 was detected on day 28 after curcumin supplementation (Figure.5.8a; *$p< 0.05$). We found an approximate 14 fold elevation in the expression of miR-214 on day 28 when compared with the aged matched epileptic control animals. This clearly indicates that curcumin partially countered the epileptogenesis-associated decline in miR-214 levels in the cortex of 4-6 month old rats (Figure.5.9b; *$p< 0.05$).

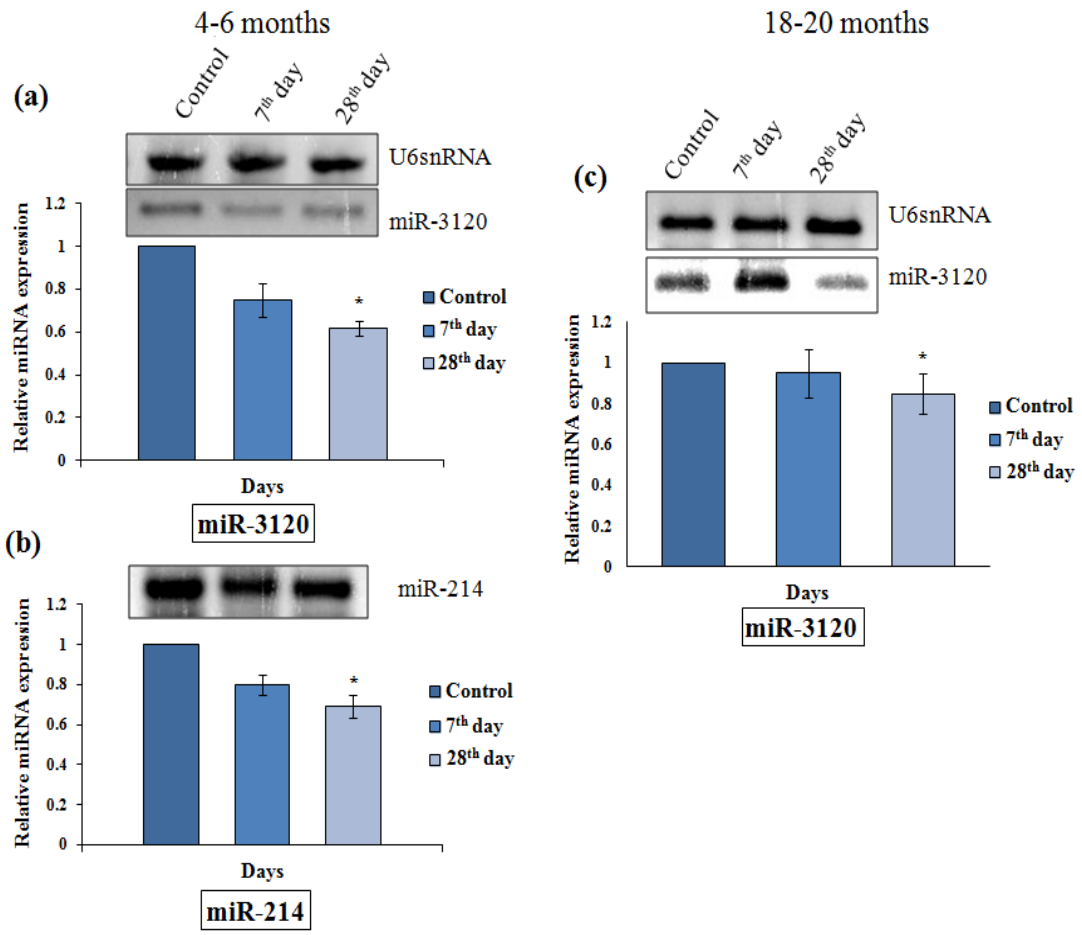


Figure.5.8. Effect of curcumin on relative miR-3120 (a, c) and miR-214 (b) expressions in the cortex of $FeCl_3$-induced epileptic rats, as measures by semi qPCR. Each bar represents the mean ± SEM of 4 rats. (*$p<0.05$)

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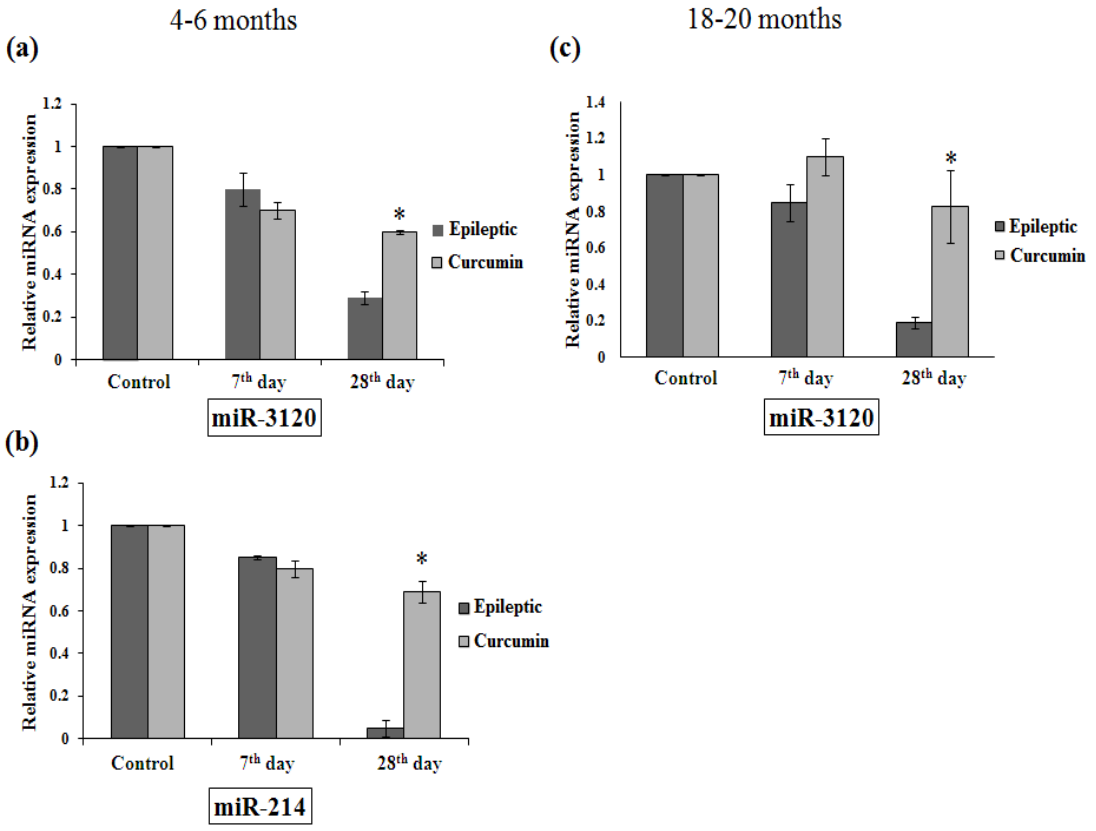

Figure.5.9. Effect of curcumin supplementation for 7 days and 28 days on miR-3120 (a, c) miR-214 (b) expressions in the cortex of $FeCl_3$-induced epileptic rats. Each bar represents the mean ± SEM of 4 rats. Statistical comparisons of each group is with respect to epileptic controls. *$p<0.05$ significantly different from epileptic control group.

5.2.3. Expression of miR-3120 and miR-214 in the hippocampus of $FeCl_3$-induced epileptic rats

(a) miR-3120

In addition to the cortex, expression of miR-3120 was also determined in the hippocampus of $FeCl_3$-induced epileptic 4-6 month old rats at days 7, 14, and 28 after induction of epilepsy. Figure.5.10 demonstrate that in the hippocampus the expression of miR-3120 was reduced progressively similar to its expression in the cortex of 4-6 month old $FeCl_3$-induced epileptic rats at 7, 14, and 28 day. We found that level of miR-3120 was significantly decreased by 1.25, 1.8 and 4.3 folds with the progression of seizure at 7, 14, and 28 day, respectively, in 4-6 month old epileptic rats when compared with their age matched sham controls (Figure. 5.10a; *$p< 0.05$). In 18-20 month old rats, the levels of miR-3120 were measured on the 7^{th} and 28^{th} day. There was an approximate 3 fold decrease in the expression of miR-3120 on day 28; whereas, no significant change was found on day 7 in epileptic rats compared with their age matched sham controls (Figure. 5.10c; *$p< 0.05$).

(b) miR-214

Moreover, expression of miR-214 was determined in the hippocampus of $FeCl_3$-induced epileptic rats at days 7, 14, and 28 after induction of epilepsy in 4-6 month old rats. Figure.5.7b shows that the level of miR-214 was reduced progressively in $FeCl_3$-induced epileptic rats at 7, 14, and 28 day. We found that level of miR-214 was significantly decreased by 1.2, 1.8, and 12 folds at 7, 14, and 28 day, respectively, when compared with their age matched sham controls (Figure.5.10b; *$p< 0.05$).

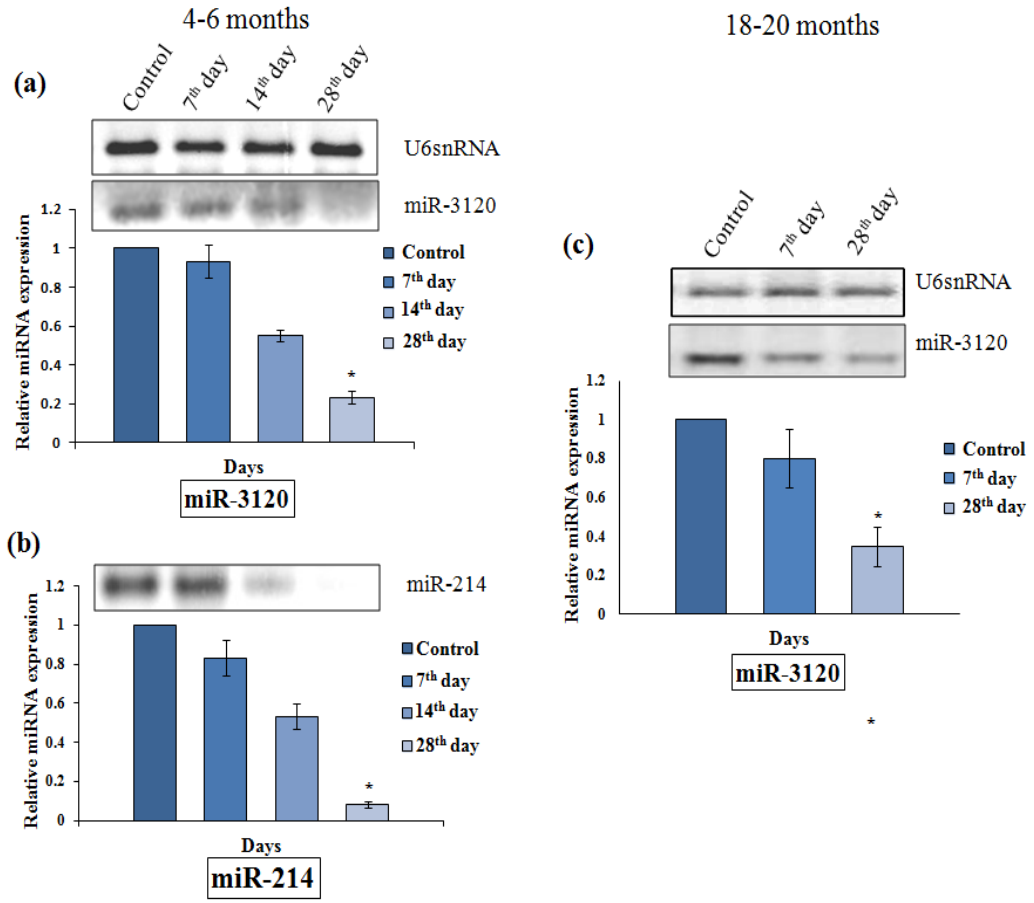

Figure.5.10. Effect of curcumin on relative miR-3120 (a, c) and miR-214 (b) expression in the hippocampus of $FeCl_3$-induced epileptic rats, as measures by semi qPCR.

5.2.4. Effect of curcumin on the expression of miR-3120 in the hippocampus of $FeCl_3$-induced epileptic rats

The effect of dietary supplementation of curcumin on the expression of miR-3120 was evaluated in the hippocampus and the level of miR-3120 was determined in curcumin-fed $FeCl_3$-induced epileptic rats on the 7^{th} and 28^{th} day in 4-6 month and 18-20 month old rats. In the 4-6 month old group, albeit, on 7^{th} day, there were no significant change in the level of miR-3120, significantly higher level of miR-3120 was detected at day 28 after curcumin supplementation (Figure.5.11 a) (*$p< 0.05$). Results demonstrated about 4.4 folds elevation in the expression of miR-3120 was determined on day 28 when compared with their respective epileptic controls animals. This clearly indicates that curcumin partially countered the epileptogenesis-associated decline in miR-3120 levels in the hippocampus of 4-6 month old rats (Figure.5.12a; *$p< 0.05$).

Similarly, in the 18-20 month old rats, the level of miR-3120 was measured on the 7^{th} and 28^{th} day. The level of miR-3120 showed similar pattern of expression in these groups also (Figure.5.11b) as there was no significant change in the expression on day 7; however, after 28 days of curcumin treatment, a significant elevation in the expression of miR-3120 was observed. Figure.5.12 depicts that the level of miR-3120 was increased about 2 folds. This result also indicates that curcumin partially countered the epileptogenesis-associated decline in miR-3120 levels in the hippocampus of 18-20 month old rats (Figure.5.12b; *$p< 0.05$).

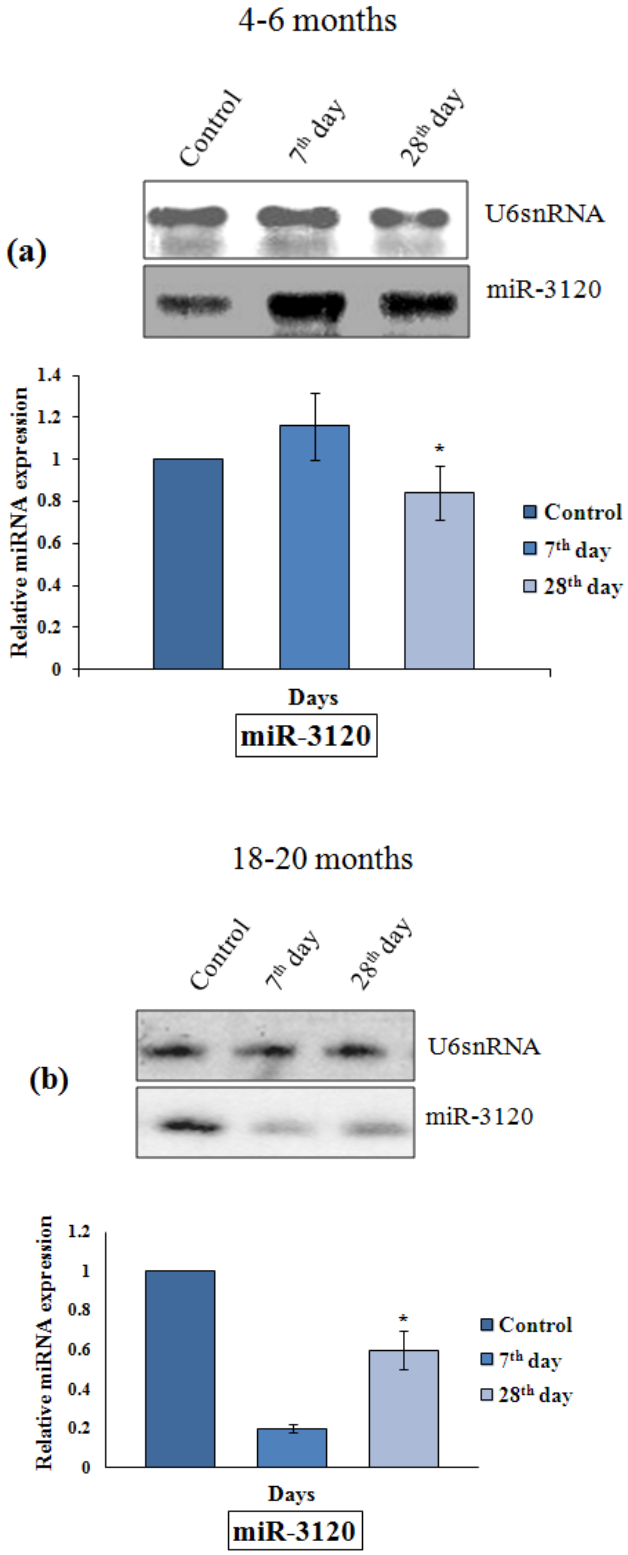

Figure.5.11. Relative expression of miR-3120 in the hippocampus of epileptic rats, as measured by semi qRT-PCR. Each bar represents the mean ± SEM of 4 rats. Statistical comparison of values at day 7 and 28 in epileptic rats with respect to the controls (n=4, *p<0.05 [ANOVA]). Decrease in miRNAs expression with the epileptogenesis is evident.

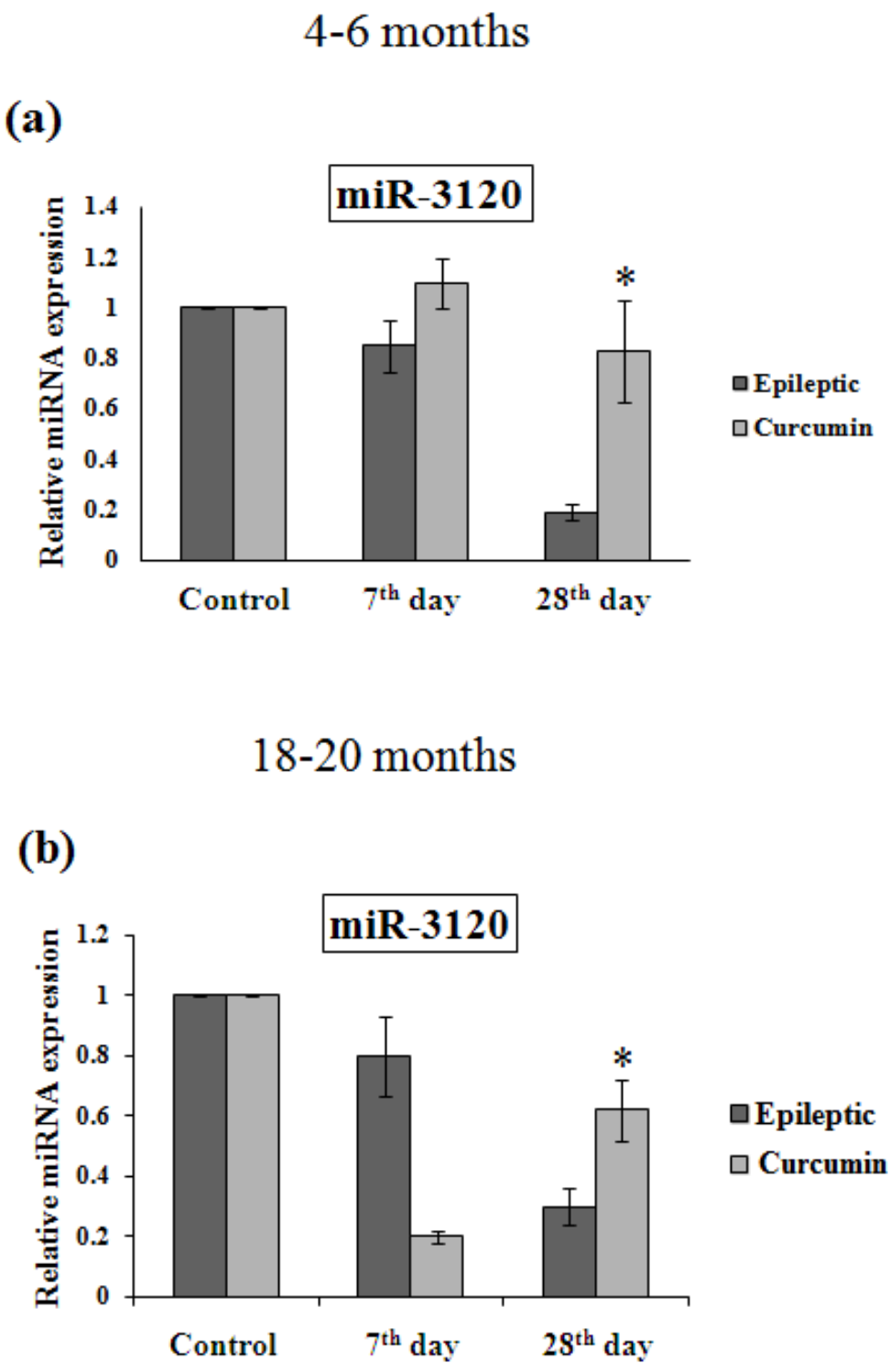

Figure.5.12. Effect of curcumin treatment for 7 days and 28 days on miR-3120 (A) miR-214 (B) expression in the cortex of $FeCl_3$-induced epileptic rats. Each bar represents the mean ± SEM of 4 rats (*$p < 0.05$).

5.3. Objective III. To analyze major targets of miR-3120 and miR-214

5.3.1. Sequence alignment

Pairwise sequence alignment has been used to identify similarity between two nucleotide sequences. Sequence homology of miR-3120 and miR-214 of human and rat was checked by BLASTn. Sequences of both miRNAs were retrieved from mirbase database and submitted to BLAST. Sequence alignment results are depicted in Figure.5.13 and show that both miR-3120 and miR-214 of rat and human have great sequence similarity with 69% homology in miR-3120 when gaps are included, however, when we exclude gaps of lengthy sequence of rat the homology increases to 96% for miR-3120 between rats and human. Similarly, miR-214 shows 98% sequence homology between rat and human.

5.3.2. miRNA target prediction

A single miRNA can target various mRNA transcripts for either translation repression or degradation. We used the computational algorithms miRDB, mirbase, and Diana databases to identify epilepsy-related potential target genes of miR-3120 based on seed-sequence homology with 3′ UTRs of mRNA transcripts. We relied on a consensus-based approach by considering only those target genes that were similarly predicted by two or more of these algorithms. Microispector tool was also used in parallel manner to understand the binding site as well as the free energy of binding of these targets with the miR-3120. Figure.5.14 shows various predicted targets, their binding sites, and the binding energy of the target mRNAs to miR-3120. Table-5.1 illustrates the targets and their binding energy with the miR-3120. Lower the binding energy, greater will be the binding affinity. Thus, a negative value above 20 Kcal shows that all the targets given in Table 5.1 showed a high binding affinity with the miR-3120.

5.3.3. Pathways analysis of targets using web-tools

In addition, KEGG webserver database was used for further filtering of the predicted targets to either specifically related to epilepsy or at least the related pathways of neurological disorders similar to epilepsy. Table-5.2 and 5.3 show the various pathways of the predicted targets that are related to epilepsy. Table-5.2 shows pathways in which targets of miR-3120 that are involved in mTOR, MAPK, and apoptosis pathways that have already been reported to be involved in epilepsy. Similarly, Table-5.3 shows pathways in which targets of miR-214 are involved similar to miR-3120 target's pathways and these were chosen for further analysis.

(a)

```
#=======================================
#
# Aligned_sequences: 2
# 1: hsa-mir-3120
# 2: rno-mir-3120
# Matrix: EDNAFULL
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 117
# Identity:      81/117 (69.2%)
# Similarity:    81/117 (69.2%)
# Gaps:          36/117 (30.8%)
# Score: 405.0
#
#
#=======================================

hsa-mir-3120       1 ----------------------GUCAUGUGACUGCCUGUCUGUGCCUGCUG     29
                                            |||||||||||||||||||||||||||||||
rno-mir-3120       1 GGUUGUCAUUCAGGCUGGGUUGUCAUGUGACUGCCUGUCUGUGCCUGCUG     50

hsa-mir-3120      30 UACAGGUGAGCGGAUGUUCUGCACAGCAAGUGUAGACAGGCAGACACAUG     79
                     |||||||||||||||||||||||||||||||||||||||||||||||||||||
rno-mir-3120      51 UACAGGUGAGCGGAUGUUCUGCACAGCAAGUGUAGACAGGCAGACACAUG    100

hsa-mir-3120      80 AC----------------     81
                     ||
rno-mir-3120     101 ACAACUCUGUCCAUCCA    117


#---------------------------------------
#---------------------------------------
```

(b)

```
#=======================================
#
# Aligned_sequences: 2
# 1: hsa-mir-214
# 2: rno-mir-214
# Matrix: EDNAFULL
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 110
# Identity:     108/110 (98.2%)
# Similarity:   108/110 (98.2%)
# Gaps:           0/110 ( 0.0%)
# Score: 532.0
#
#
#=======================================

hsa-mir-214        1 GGCCUGGCUGGACAGAGUUGUCAUGUGUCUGCCUGUCUACACUUGCUGUG     50
                     |.||||.||||||||||||||||||||||||||||||||||||||||||||
rno-mir-214        1 GUCCUGGAUGGACAGAGUUGUCAUGUGUCUGCCUGUCUACACUUGCUGUG     50

hsa-mir-214       51 CAGAACAUCCGCUCACCUGUACAGCAGGCACAGACAGGCAGUCACAUGAC    100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
rno-mir-214       51 CAGAACAUCCGCUCACCUGUACAGCAGGCACAGACAGGCAGUCACAUGAC    100

hsa-mir-214      101 AACCCAGCCU    110
                     ||||||||||
rno-mir-214      101 AACCCAGCCU    110


#---------------------------------------
#---------------------------------------
```

Figure.5.13. Sequence alignments showing alignment of (a) miR-3120 and (b) mir-214 of human and rat using BLASTn.

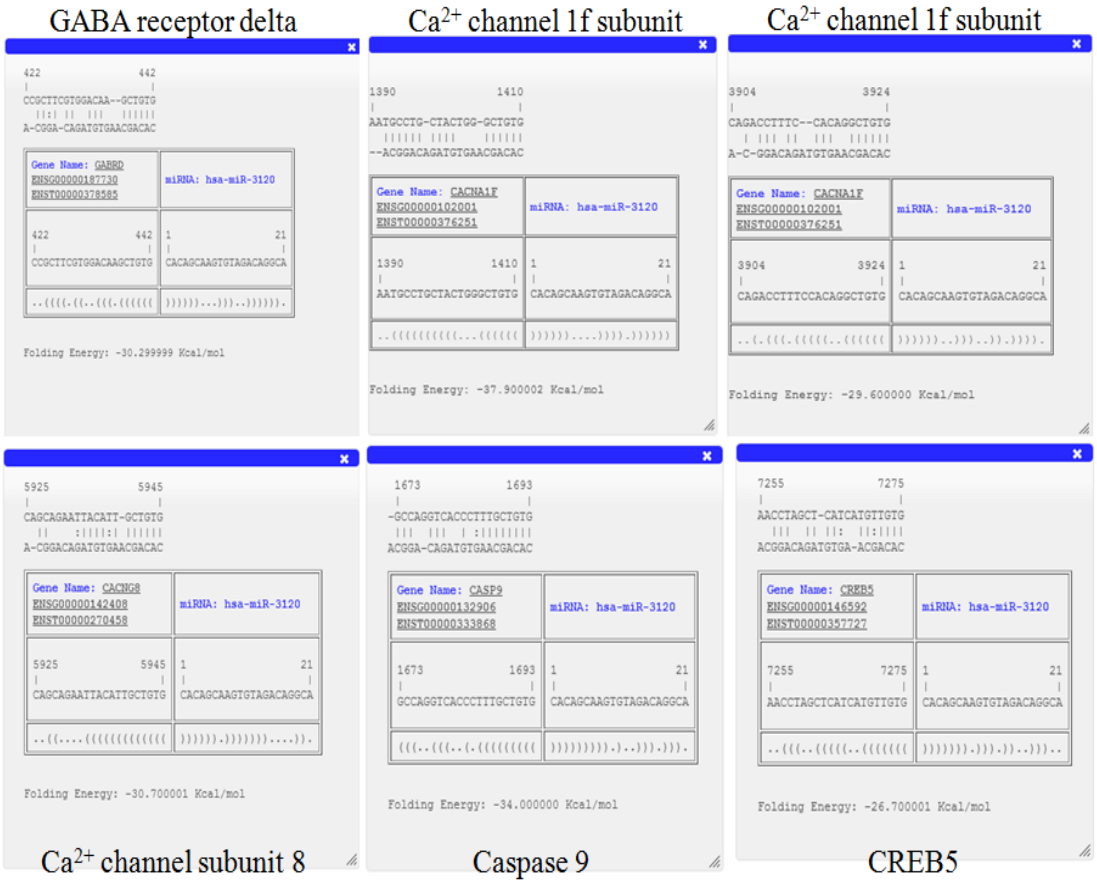

Figure.5.14. Binding sites and free binding energy of miR-3120 with its putative targets.

Table.5.1. Binding sites of miR-3120 on the predicted targets and free binding energy

Targets	**Binding site on RNA**	**Free energy of Binding(kcal)**
GABRD	422-442	-30.29
Calcium channel subunits	1390-1040,3904-3924	-37.90, -29.60
CREB5	7255-7257	-26.70
Caspse9	1673-1693	-34.00
Pten	118-139, 4035-4041	-24.00

Table.5.2. Major pathways that are associated with a set of targets of miR-3120 genes

Pathways	**Pathway ID humans**	**Pathway ID rats**
MAPK signaling pathway	hsa04010	rno04010
mTOR signaling pathway	hsa04150	rno04150
cAMP signaling pathway	hsa04024	rno04024
AMPK signaling pathway	hsa04152	rno04152
PI3K-Akt signaling pathway	hsa04151	rno04151
TNF signaling pathway	hsa04668	rno04668
Apoptosis	hsa04210	rno04210
Cholinergic synapse	hsa04725	rno04725
Dopaminergic synapse	hsa04728	rno04728
GABAergic synapse	hsa04727	rno04727

Table.5.3 Most common pathways associated with a set of targets of miR-214 genes

Pathways	**KEGG pathway ID**	**KEGG pathway ID in rat**
P53 signalling pathway	hsa04115	rno04115
mTOR signalling pathway	hsa04150	rno04150
MAPK signalling pathway	hsa04010	rno04010
Axon guidance pathway	hsa04360	rno04360
Glioma signalling	hsa05214	rno05214
Insulin signalling	hsa04910	rno04910

5.4. Objective IV. To study the response of miR-3120 targets to dietary curcumin in epileptic rats.

5.4.1. Expression of CACNA1A and GABRD in the rat brain

5.4.1.1. Immunohistochemistry (IHC)

(a) CACNA1A

IHC study was performed to check the expression and localization of CACNA1A in the neurons. In the cortex, CACNA1A was expressed in all types of neurons and more specifically in the membrane, where pyramidal neurons showed a high expression in 4-6 month old rats. However, the protein was evenly distributed in the cortex of 18-20 month aged rats. $FeCl_3$-induced epilepsy reduced the expression of CACNA1A in 4-6 month old rats as compared to their respective age matched controls (Figure.5.15). Similarly, 18-20 month old, $FeCl_3$-induced epileptic rats also showed the decreased expression of CACNA1A in the cortex of (Figure.5.15).

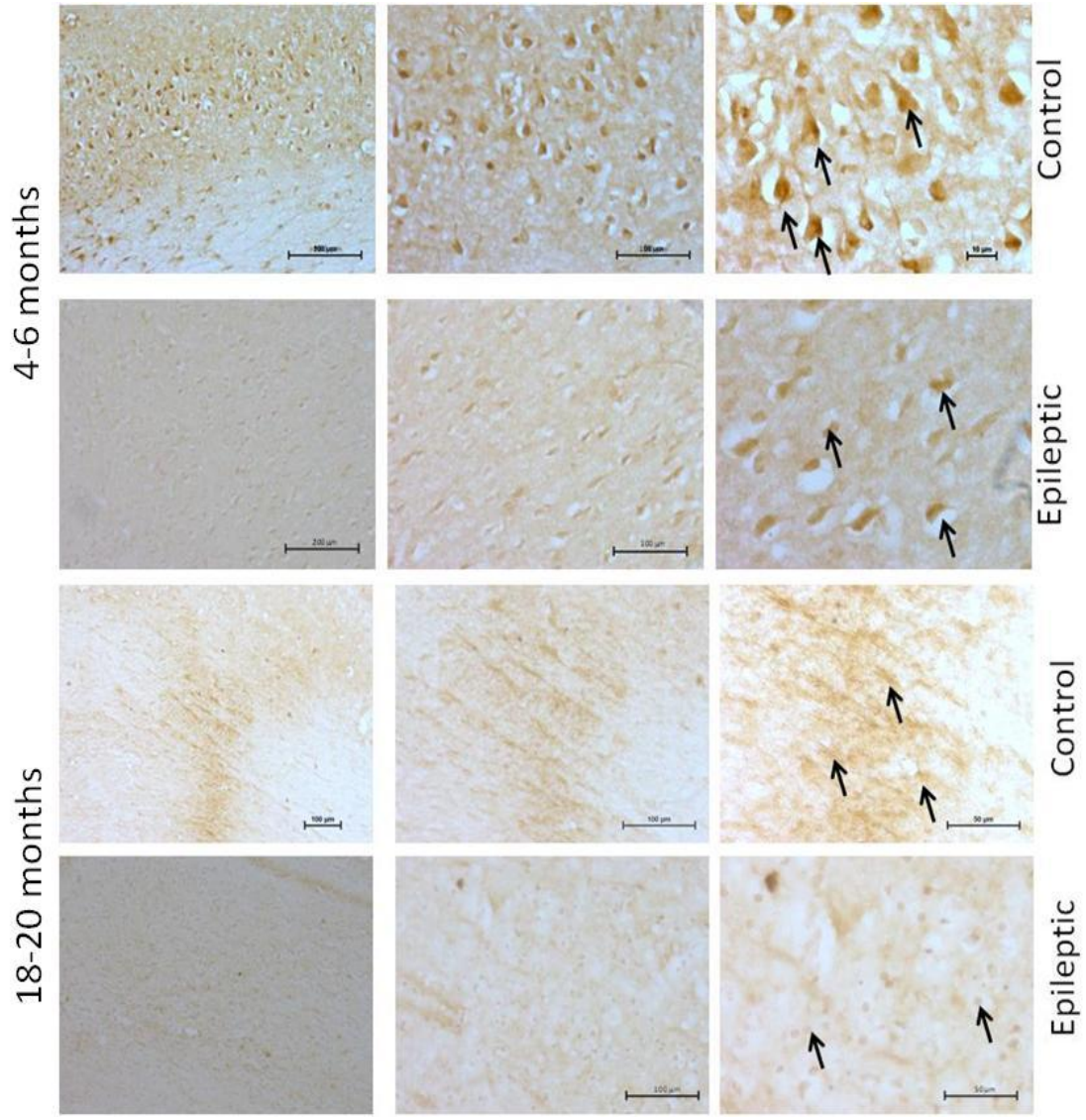

Figure.5.15. Representative photographs (10, 20 and 40x) showing IHC of CACNA1A in the cortical sections of $FeCl_3$-injected 4-6 month and 18-20 month old rats. There was decreased expression of CACNA1A in the cortex of epileptic rats as compared to controls in both groups.

In addition, effect of dietary supplementation of curcumin on the expression of CACNA1A protein was evaluated in 4-6 month old curcumin-fed rats and we found elevated expression of CACNA1A in the cortex after the 28-day curcumin supplementation in 4-6 month old rats (Figure.5.16). The 18-20 month old rats also showed a similar pattern of increased expression of CACNA1A protein after the 28-day curcumin supplementation (Figure.5.16).

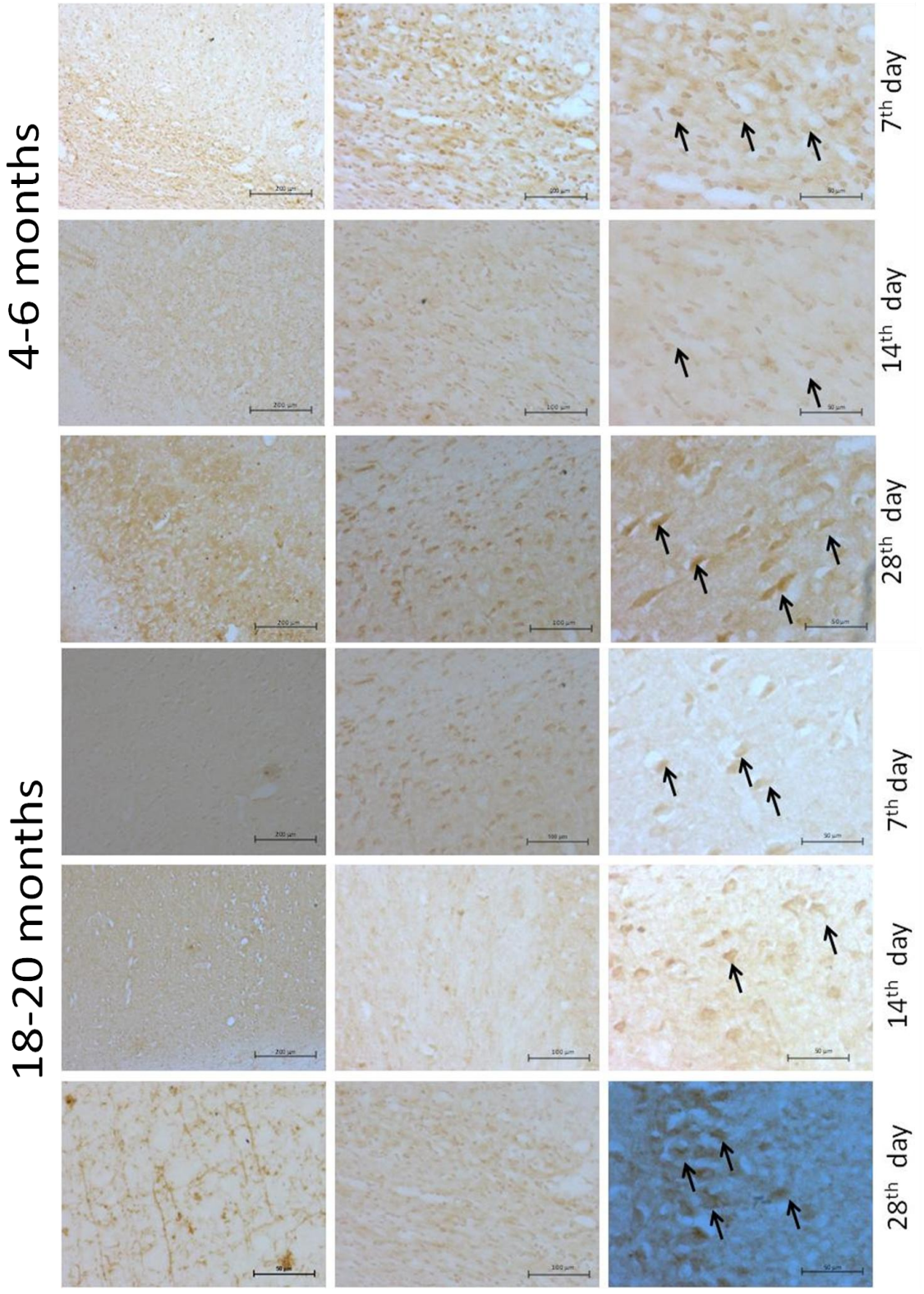

Figure.5.16. Representative photographs (10, 20 and 40x) showing effect of dietary curcumin in IHC of CACNA1A in the cortex of 4-6 month and 18-20 month old epileptic rats. There was elevated expression of CACNA1A in the cortex of epileptic rats after 28 days of curcumin supplementation as compared to epileptic rats.

(b) GABRD

Further, IHC study of GABRD was performed to check the expression and localization of GABRD in the neurons and the results showed that GABRD was densely localized in the neocortex region. Epileptic rats showed decreased expression of GABRD in the cortex as compared to their age matched control rats. Figure.5.17 demonstrates that the level of GABRD was reduced in epileptic 4-6 month old rats as compared to the controls. Similarly, 18-20 month old rats showed decrease in the protein expression of GABRD in the cortex when compared with their age matched controls (Figure.5.17).

Moreover, IHC study of GABRD after supplementation of the dietary curcumin for 28 days showed elevated expression of GABRD on the 28^{th} day in the cortical region of 4-6 month old rats; however, no significant change was found on the 7^{th} and 14^{th} day as compared to their age matched epileptic controls (Figure.5.18). Similarly, in 18-20 month old rats, there was increased expression of GABRD protein after the 28-day curcumin supplementation, whereas, no significant change was found on the 7^{th} and 14^{th} day as compared with their age matched epileptic controls (Figure.5.18).

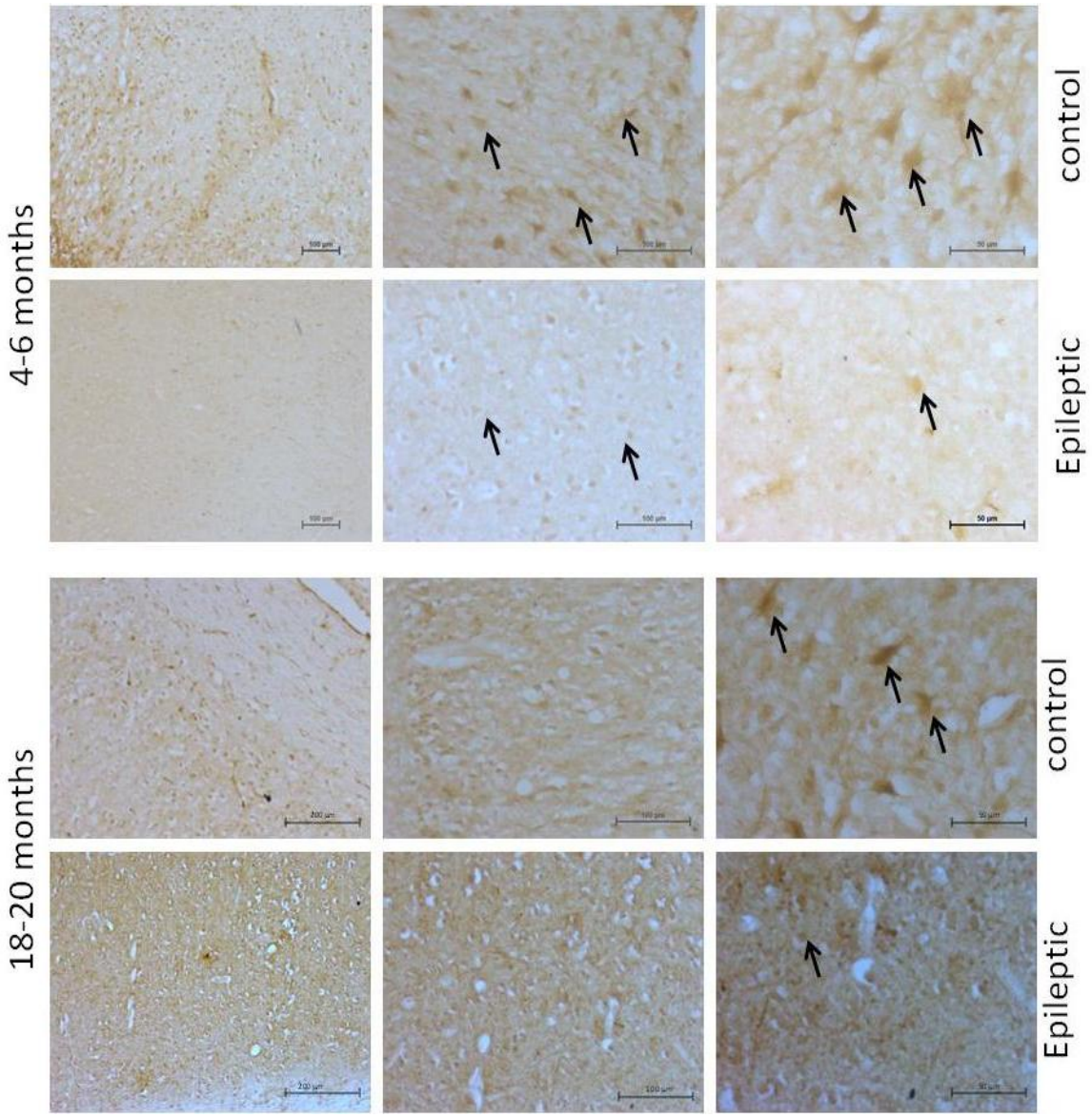

Figure.5.17. Representative photographs (10, 20 and 40x) showing IHC of GABRD in the cortical sections of $FeCl_3$-injected 4-6 month and 18-20 month old rats. There was decreased expression of GABRD in the cortex of epileptic rats as compared to the controls.

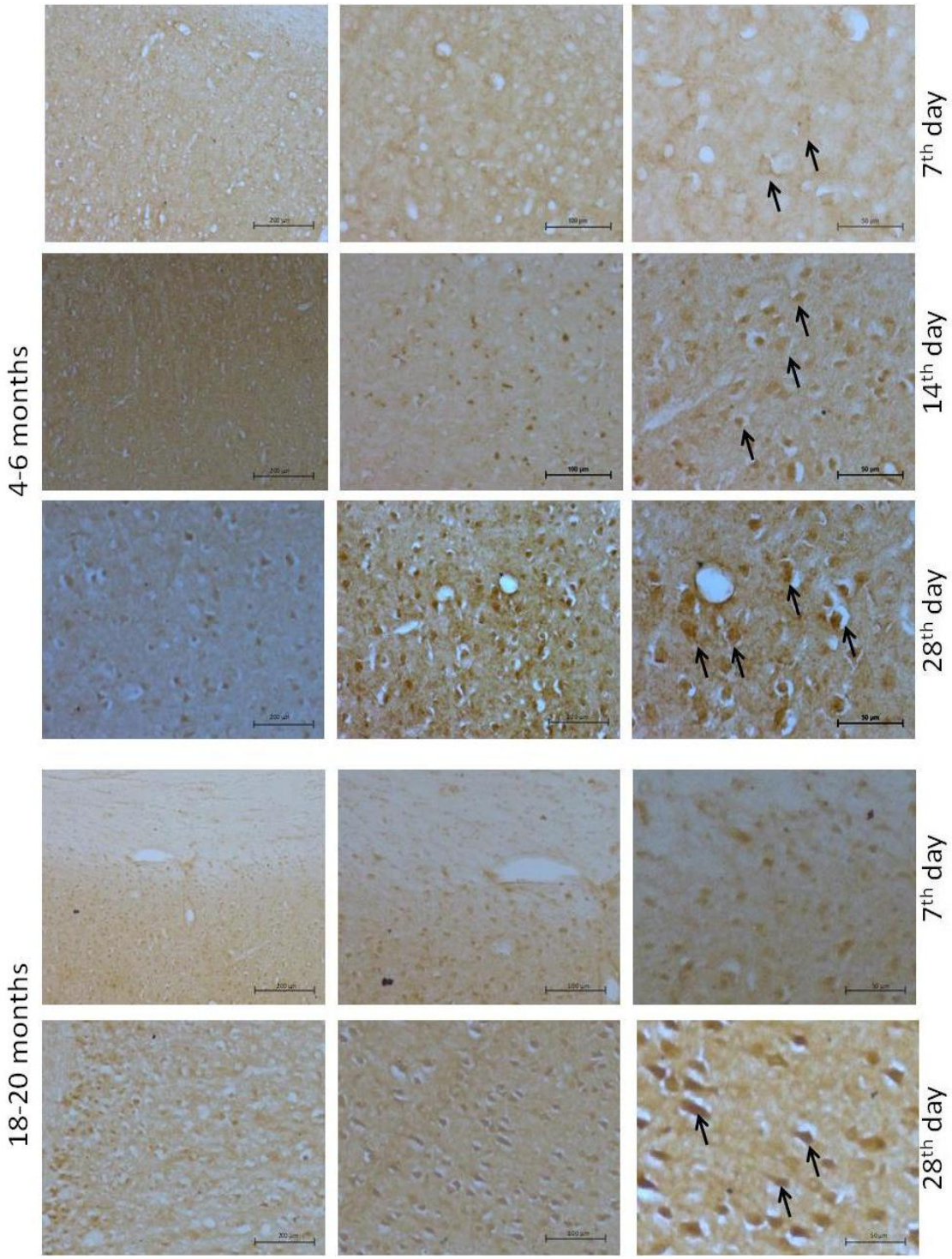

Figure.5.18. Representative photographs (10, 20 and 40x) showing effect of dietary curcumin in IHC of GABRD in the cortex of 4-6 month and 18-20 month old epileptic rats. There is increased expression of GABRD in the cortex of curcumin-fed as compared to $FeCl_3$-injected rats.

In addition to the cortex, IHC study of GABRD was also performed in the hippocampus and IHC micrographs showed that GABRD was densely localized in the CA1 and CA3 region of the hippocampus. Epileptic rats showed decreased expression of GABRD in the hippocampus, similar to its expression in the cortex, as compared to their age matched control rats. Figure.5.19 demonstrates that the level of GABRD was reduced in epileptic 4-6 month old rats. Similarly, 18-20 month old rats showed decrease in expression of GABRD in the hippocampus as compared with their aged matched controls (Figure.5.19).

Furthermore, the results demonstrate that curcumin supplementation increased the expression of GABRD as evident from the IHC micrographs. Dietary supplementation of curcumin for 28 days showed elevated expression of GABRD on the 28^{th} day in the hippocampus of 4-6 month old rats; however, there was no significant change on the 7^{th} day as compared to their age matched epileptic controls (Figure.5.20). Similarly, in 18-20 month old rats, there was increased expression of GABRD protein on the 28^{th} day after the 28-day curcumin supplementation, whereas, there was no significant change on the 7^{th} day when compared with their age matched epileptic controls (Figure.5.20).

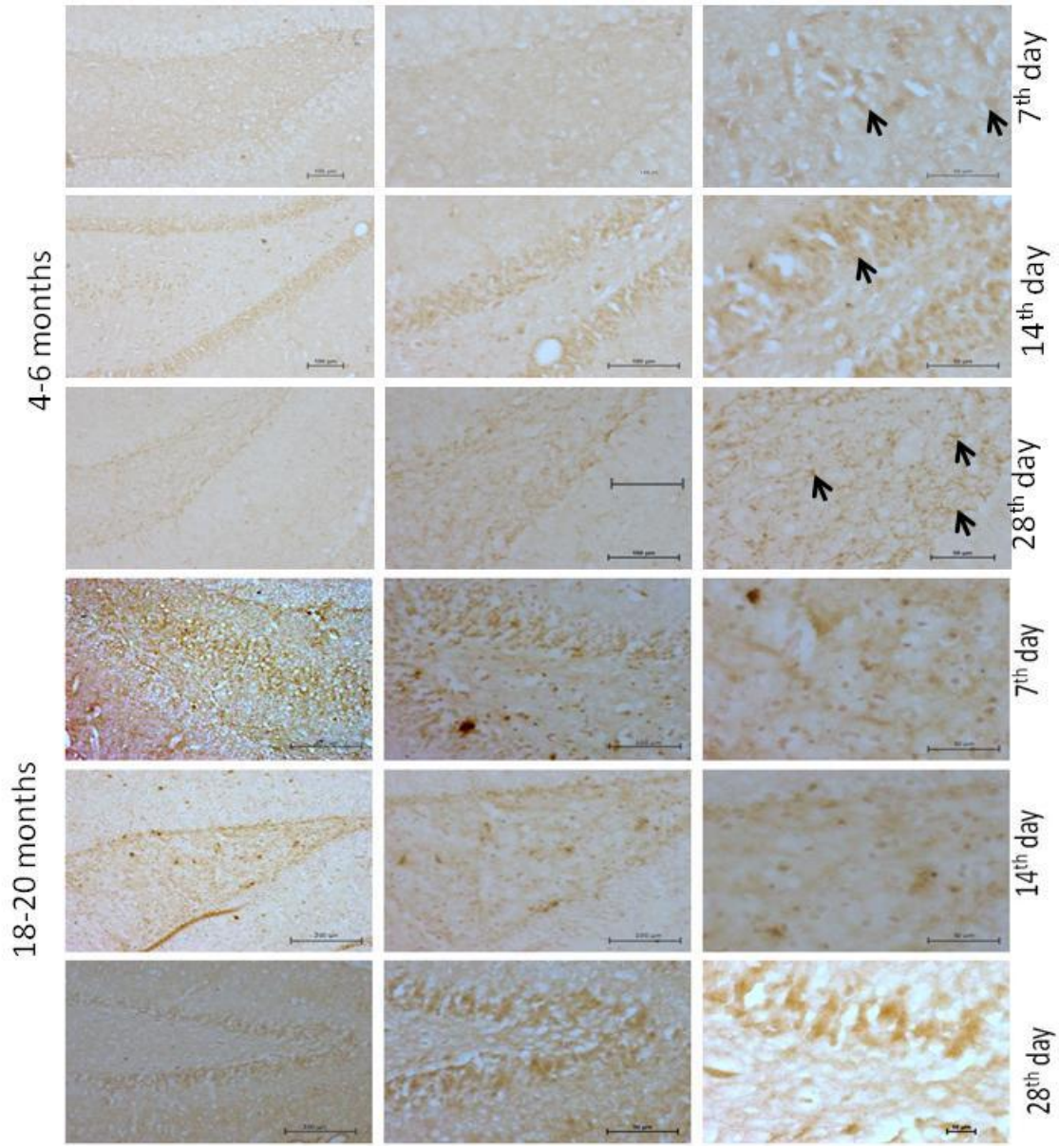

Figure.5.19. Representative photographs (10, 20 and 40x) showing IHC of GABRD in the hippocampus of $FeCl_3$-injected 4-6 month and 18-20 month old rats. There was decreased expression of GABRD in the hippocampus of epileptic rats as compared to the controls.

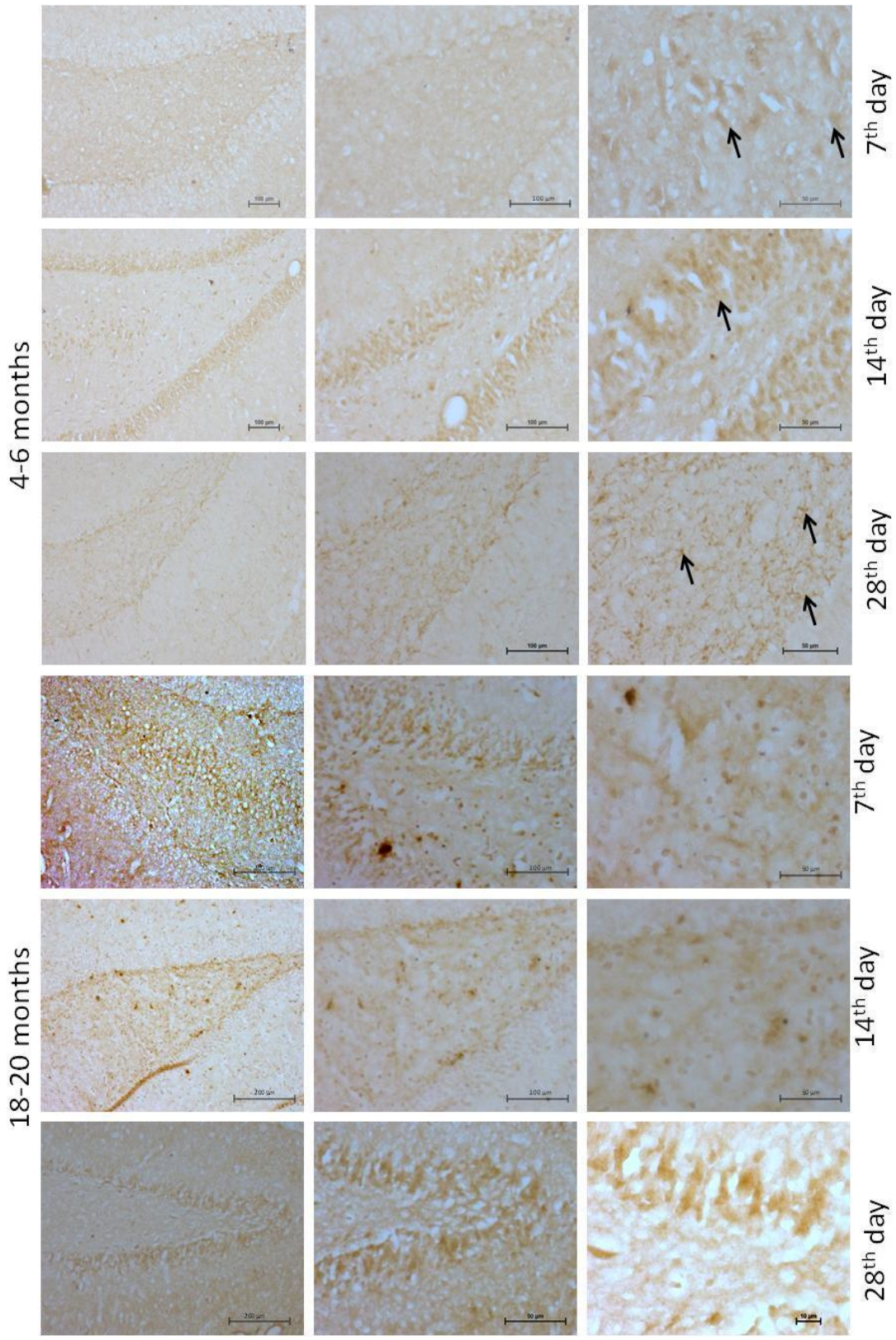

Figure.5.20. Representative photographs (10, 20 and 40x) showing effect of dietary curcumin in IHC of GABRD in the hippocampus of 4-6 month and 18-20 month old epileptic rats. There was higher expression of GABRD in the hippocampus of curcumin-fed rats compared to $FeCl_3$-injected rats.

5.4.1.2. Western Blotting

(a) Epileptic

i. CACNA1A

Further, to validate the IHC results, western blot analysis of CACNA1A and GABRD was performed on the 7th, 14th, and 28th day. Similar to the results of IHC, western blotting results also showed decreased expression of CACNA1A in the cortex of $FeCl_3$-injected 4-6 month and 18-20 month old rats as compared to their respective age matched controls. The downregulation of CACNA1A protein in the cortex was 1.6 and 2 folds on the 14th and 28th day of $FeCl_3$-induced epilepsy, respectively, in 4-6 month old rats (Figure.5.21 a and c; *p< 0.05). Similarly, 18-20 month old rats also showed a decrease of 1.4 and 1.6 folds on the 14th and 28th day of $FeCl_3$-induced epilepsy, respectively, in18-20 month old rats; however, there was no significant change found on day 7 in both of the age groups.

ii. GABRD

In addition to this, we have also carried out western blot analysis of GABRD and the result showed decreased protein expression of GABRD in the cortex of $FeCl_3$-injected 4-6 month and 18-20 month old rats as compared to their respective age matched controls. The CACNA1A protein in the cortex was downregulated 2 and 5 folds on the 14th and 28th day of $FeCl_3$-induced epilepsy, respectively, in 4-6 month old rats (Figure.5.21b; **p< 0.01). Similarly, 18-20 month old rats also showed a 3.5 and 4.8 fold decrease in its expression on the 14th and 28th day of $FeCl_3$-induced epilepsy, respectively, in 18-20 month old rats; however, there was no significant change on day 7 (Figure.5.21d; *p< 0.05).

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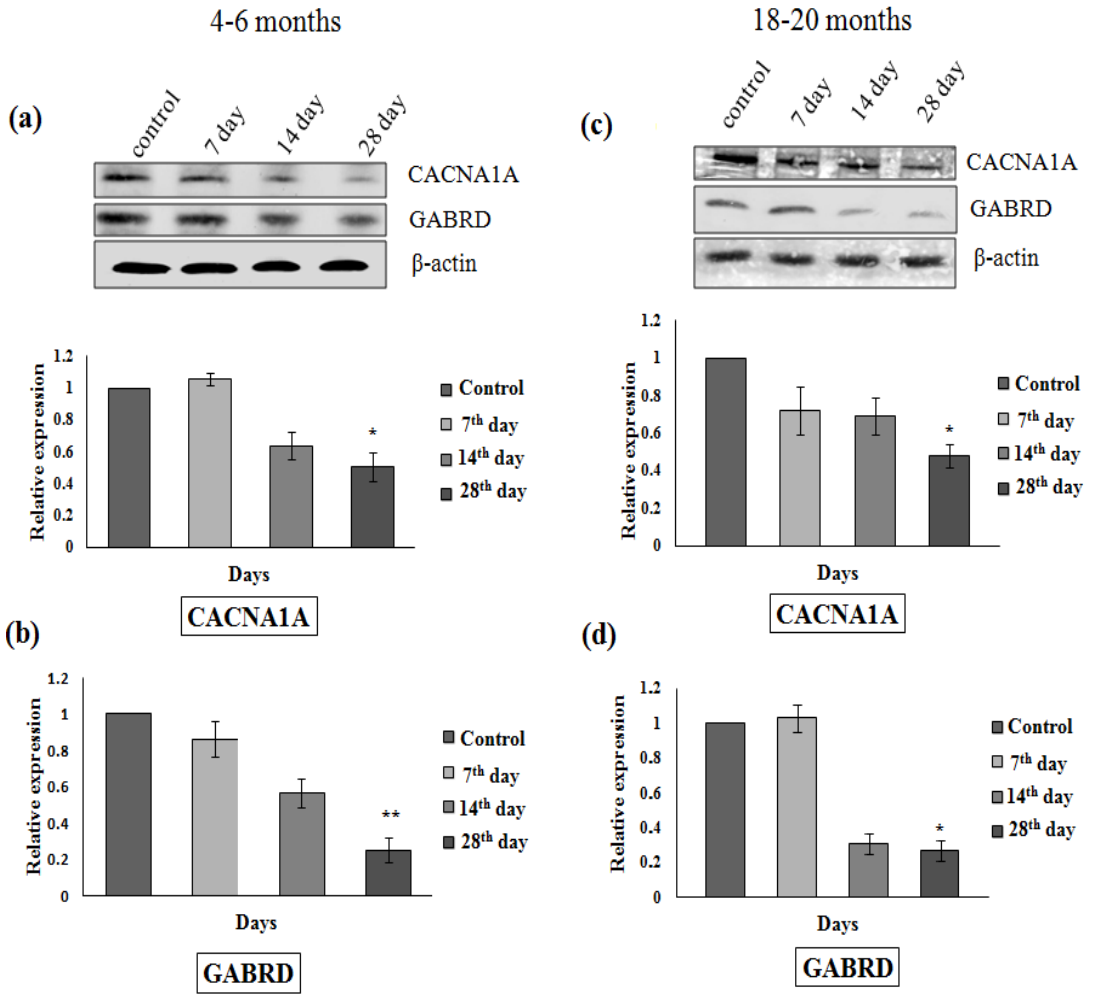

Figure.5.21. Western blots showing protein expression in 4-6 month and 18-20 month old rats. Relative expression of CACNA1A (a, c) and GABRD (b, d) in the cortex of epileptic rats, as measure by densitometry analysis. Each bar represents mean ± SEM of 4 rats. Statistical comparison of values at day 7, 14, and 28 of epileptogenesis are with respect to controls. *p<0.05, **p< 0.01. significantly different from control group.

(a) Curcumin supplementation

i. CACNA1A

Moreover, to evaluate the effect of dietary curcumin supplementation and to validate the IHC results, western blotting analyses of CACNA1A and GABRD were done. Expression of CACNA1A was determined in curcumin-fed $FeCl_3$-induced epileptic rats on the 14^{th} and 28^{th} day in 4-6 month and 18-20 month old rat groups. Curcumin supplementation increased the expression of CACNA1A on the 28^{th} day after epilepsy induction (Figure.5.22a; *$p< 0.05$). Results showed an approximate 2 fold increase in the expression of CACNA1A on day 28 when compared with their age matched epileptic controls; however, there was no significant change on the 14^{th} day. This clearly indicates that curcumin partially countered the epileptogenesis-associated decline in CACNA1A levels in the cortex of 4-6 month old rats (Figure.5.23; *$p< 0.05$).

Similarly, in 18-20 month old rats, the expression of CACNA1A was measured on the 14^{th} and 28^{th} day. The expression pattern was found to be similar to that observed in the 4-6 month old rats. As evident from Figure.5.22c, there was no significant change in the expression of CACNA1A protein on day 14; however, after the 28-day curcumin treatment, a significant elevation in the expression of CACNA1A was observed. Figure.5.23 depicts that the level of CACNA1A was increased about 2 folds, indicating that the curcumin partially countered the epileptogenesis-associated decline in CACNA1A expression in the cortex of 18-20 month old rats (*$p< 0.05$).

ii. GABRD

Expression of GABRD was determined in curcumin-fed $FeCl_3$-induced epileptic rats on the 14^{th} and 28^{th} day in 4-6 month and 18-20 month old rats. Expression of GABRAD was determined in curcumin-fed $FeCl_3$-induced epileptic rats on the 14^{th} and 28^{th} day in 4-6 months as well as in 18-20 months old rat groups (Figure.5.22b; *$p< 0.05$). Results showed an approximate 4.5 fold increase in the expression of GABRD on day 28 when compared with their age matched epileptic controls; however, there was no significant change on the 14^{th} day. This clearly indicates that curcumin partially countered the epileptogenesis-associated decline in GABRD levels in the cortex of 4-6 month old rats (Figure.5.23; *$p< 0.05$).

Similarly, in 18-20 month old rats, the expression of GABRD was measured on the 14^{th} and 28^{th} day. The expression pattern was found to be similar to that of the 4-6 month old rats. As evident from Figure.5.22d, there was no significant change in the expression of GABRD protein on day 14; however, after the 28-day curcumin treatment, there was a significant elevation in the expression in GABRD. Figure.5.23 depicts that level of GABRD was increased about 2 folds, indicating that curcumin partially countered the epileptogenesis-associated decline in GABRD expression in the cortex of 18-20 month old rats (*$p < 0.05$).

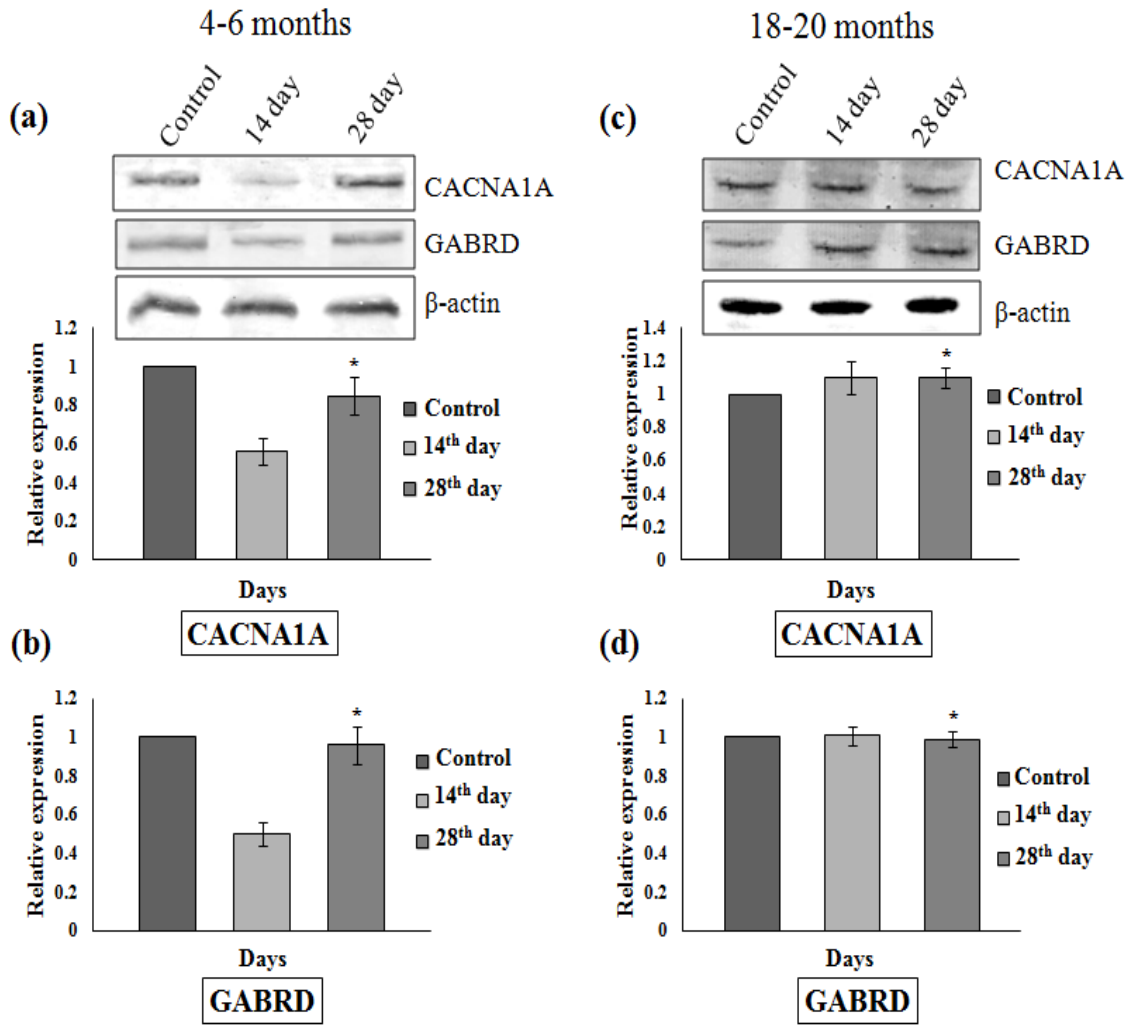

Figure.5.22. Western blots showing protein expression in 4-6 month and 18-20 month old rats. Relative expression of CACNA1A (a, c) and GABRD (b, d) in the cortex of curcumin-fed epileptic rats was measure by semi qRT-PCR analysis. Each bar represents mean ± SEM of 4 rats. Statistical comparison of values at day 14 and 28 of epileptogenesis are with respect to controls. *p<0.05, significantly different from control group.

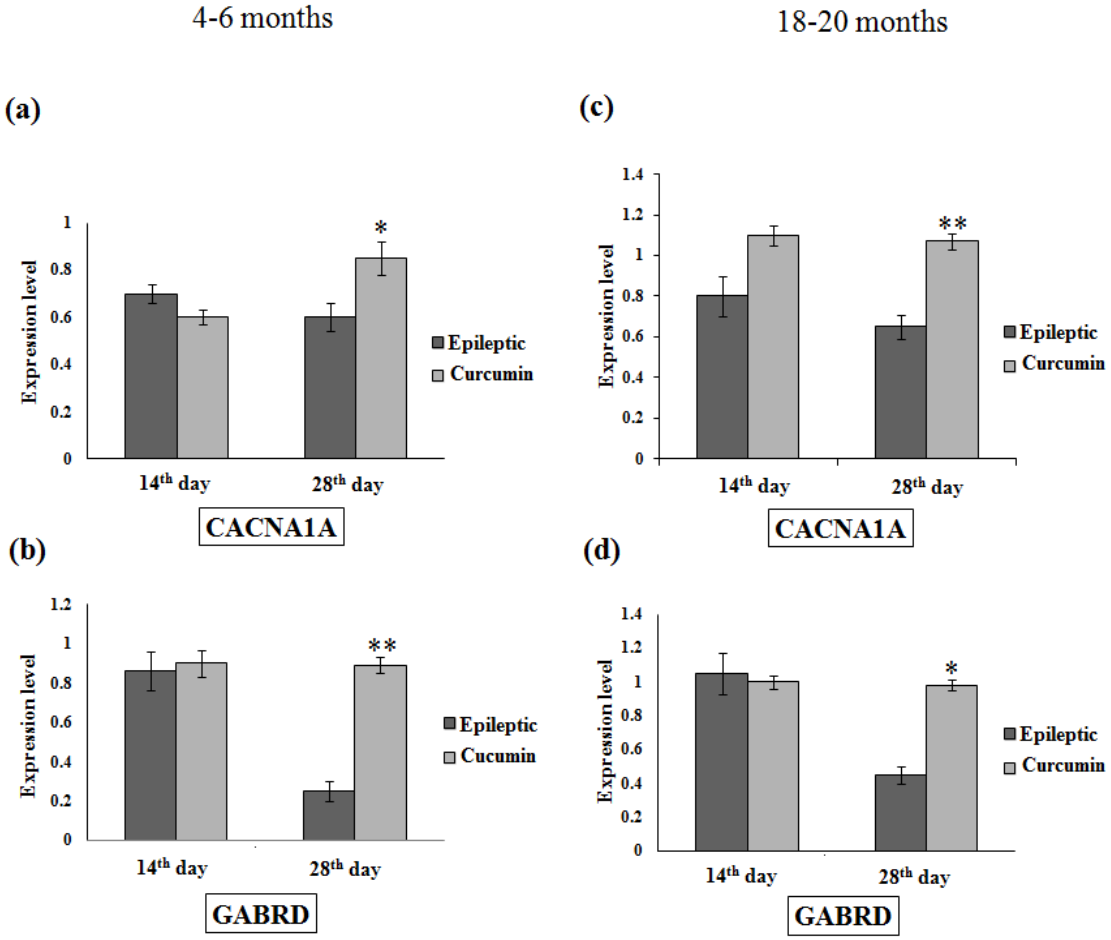

Figure.5.23. Effect of curcumin supplementation for 14 and 28 days on the protein expressions of CACNA1A and GABRD in the cortex of $FeCl_3$-induced epileptic rats. Each bar represents the mean ± SEM of 4 rats. Statistical comparison is between each treatment group and their respective epileptic controls. Increased expression after curcumin supplementation (28-day) was evident.(*p< 0.05 **p< 0.01)

5.4.2. Expression of *Pten* as a target of both miR-3120 and miR-214

As evident from numerous earlier studies, *Pten* has also been reported as a target of miR-214. Our results from in-silico analyses showed that miR-3120 also targets the *Pten* gene and it may alter its expression. Hence, we checked whether any alteration in the expression of miRNAs affects the expression of *Pten*, by using semi-quantitative Reverse-transcriptase PCR at days 7 and 28 after induction of epilepsy. Results showed that *Pten* expression was significantly increased with the progression of electrographic seizure activity as on the 28^{th} day there was a 1.4 fold increase in *Pten* expression (Figure.5.24). However, curcumin treatment significantly decreased the expression of *Pten* gene (mRNA) in $FeCl_3$-induced epileptic rats. Seven-days treatment resulted in non-significant (Figure.5.25) decrease. However, the 28-days treatment produced a significant decline in the expression and restored the level of expression near to the control level. Thus, curcumin treatment prevented the epileptogenesis-associated rise in *Pten* gene expression (Figure.5.26).

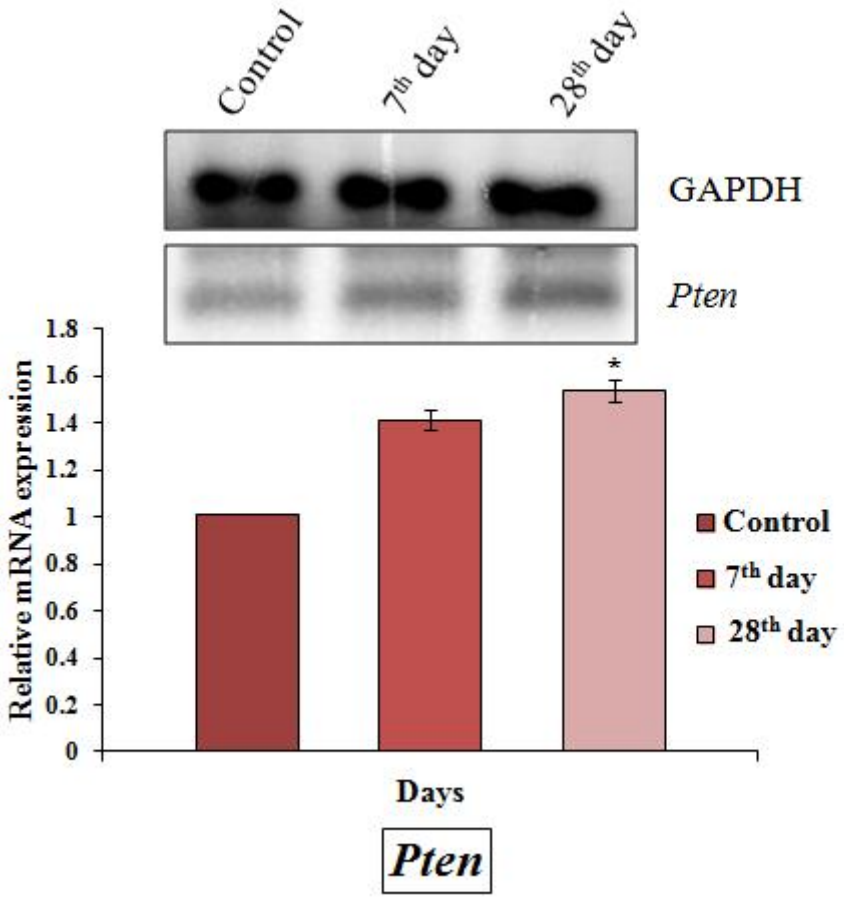

Figure.5.24. Relative mRNA expression of *Pten* in the cortex of $FeCl_3$-induced epileptic rats, as measured by semi qRT-PCR. Each bar represents the mean ± SEM of 4 rats. Statistical comparison of values at day 7 and 28 in epileptic rats as compared to their controls. (*$p < 0.05$)

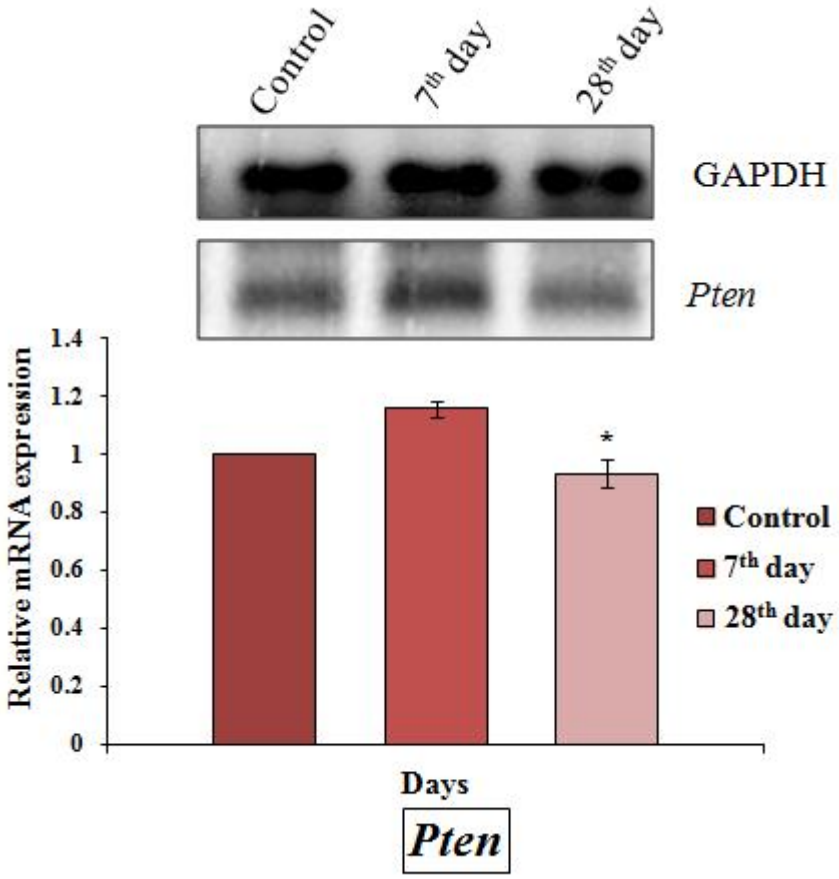

Figure.5.25. Relative mRNA expression of *Pten* in the cortex of curcumin-fed $FeCl_3$-induced epileptic rats, as measured by semi qRT-PCR. Each bar represents the mean ± SEM of 4 rats. Statistical comparison of values at day 7 and 28 in epileptic rats as compared to their respective controls. (*$p < 0.05$)

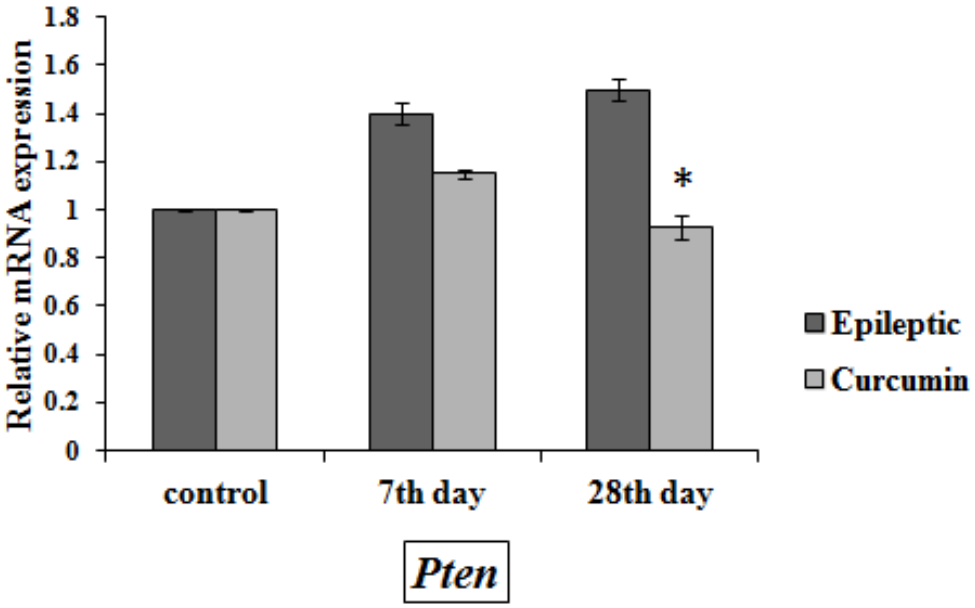

Figure.5.26. Effect of curcumin supplementation for 7 and 28 days on the expression of *Pten* gene in the cortex of $FeCl_3$-induced epileptic rats. Each bar represents the mean ± SEM of 4 rats. *$p < 0.05$, significantly different from epileptic group.

DISCUSSION

6. Discussion

The present study was executed to investigate the anti-epileptic potential of curcumin in $FeCl_3$-induced PTE model with special emphasis on role of miRNAs in the regulation of molecular mechanism of epilepsy. It has been reported that rats given intracortical injection of $FeCl_3$ showed development of epileptiform electrographic activity (Willmore et al., 1978), suggesting occurrence of $FeCl_3$ induced seizures by $FeCl_3$. Hence, we have selected the $FeCl_3$-induced PTE model of epilepsy by injecting $FeCl_3$ in the rat brain. In this epilepsy a spontaneous epileptogenic focus develops that spreads from the site of its origin to entire brain (Moriwaki et al., 1992; Sharma and Singh, 1999; Sharma et al., 2007). It has been reported that seizure activity is generated by activation and recruitment of different brain regions including the hippocampus and cortex (Bouilleret et al., 2000; Handforth and Ackermann, 1995). Moreover, seizure activity of cortical region is governed by the hippocampus CA1 area (D'Ambrosio et al., 2005, 2004).

In the present study we have quantified field potential activity (ECoG) and multiple unit action potentials simultaneously for the verification of epileptic-activity associated increase in neuronal firing at the recording sites. The MUA potential recordings ensured that the recorded ECoG represented real epileptogenic signals, rather than any accidentally recorded potential spreads from nearby brain regions. In this study, ECoG recordings as well as MUA potential in $FeCl_3$-induced epilepsy demonstrated the increased hyperexcitation of neurons as evident from increased epileptiform electrographic activity and MUA count in epileptic rats. The results obtained from ECoG and MUA study were in accordance to some previous studies executed on $FeCl_3$-induced epileptogenesis (Das et al., 2017; Jyoti et al., 2009; Mishra et al., 2013; Zou et al., 2017). Further, we have also checked the effect of curcumin supplementation on the $FeCl_3$-induced epilepsy with respect to ECoG and MUA. Curcumin supplementation suppressed epileptiform electrographic activity from the cortex and hippocampus on the ECoG and EEG recordings, suggesting curcumin counter balance the generation of seizures. The pattern of electrographic epileptic activity recorded and quantified was similar to that described in some earlier studies (Willmore, 1990; Willmore et al., 1978). Antiepileptic potential of curcumin was is already reported in many studies where its suppressive effect on

ECoG and MUA was demonstrated in $FeCl_3$-induced PTE model (Drion et al., 2016; Noor et al., 2012; Jyoti et al., 2009).

It has been evident from existing literature that most of the PTE studies were done either on young or randomly selected rats based on their weights (Kabuto et al., 1998)) Willmore et al. (1981) used rats weighing 250-300 gm for their studies for epilepsy where others have used rats of 350-450 gm. However, there are few reports which demonstrated the age dependent seizure susceptibility (Coenen and Van Luijtelaar, 1987; Jensen et al., 1992). Therefore, in the present study we studied epilepsy-induced epileptiform electrographic activity alterations in 4-6 months and 18-20 months old rats as well as studied the response of dietary curcumin in the light of electrophysiology recordings.

Seizures induction causes epileptogenesis-associated behavioural alterations in humans (Dinkelacker et al., 2003; Williams, 2003) and various experimental models (Pineda et al., 2014; Powell et al., 2014; Umpierre et al., 2014). Epilepsy-related memory impairment have been widely exhibited in human patients (Giovagnoli et al., 1997; Thompson and Corcoran, 1992) and animal model epilepsy such as electrical-kindling (Becker et al., 1992; Genkova-Papazova and Lazarova-Bakarova, 1995; Gilbert et al., 1996; Letty et al., 1995), PTZ-induced kindling (Hamm et al., 1995; Lamberty and Klitgaard, 2000; Omrani et al., 2007), PTE animal models and fluid percussion injury (Browne et al., 2006; Hamm et al., 1992). Head trauma also causes the cognitive dysfunction which can occur in both severe injury as well as in mild to moderated head trauma (Capruso and Levin, 1992; Guthrie et al., 1999). Therefore, in the current study behavioural studies would be of great significance to validate the ECoG and EEG recordings results. We used MWM-test to assess the cognitive status in epileptic and curcumin supplemented epileptic rats. We found that epileptic rats have higher latency to reach the platform as compared to controls, indicating epilepsy-coupled cognitive deficit. The perceived learning and memory impairment could be the result of manifestation of seizure (Gilbert et al., 1996) in the cortex as well as in hippocampus of epileptic rats. Moreover, the study demonstrated that curcumin supplementation counter balance the epilepsy induced cognitive defects in rats. This is evident from MWM-test results where curcumin feeding significantly improved latency in curcumin-fed rats as compared to epileptic controls. Our results are in concordance to several earlier studies that have showed

enhanced memory after curcumin feeding in rodents with various neurological disorders (Choudhary et al., 2013; Jyoti et al., 2009; Yow et al., 2017). Other earlier studies have reported curcumin efficacy in delaying (Sumanont et al., 2007) or completely preventing the onset of convulsive seizures (Shin et al., 2007) in KA-induced epilepsy in epileptic rats.

Epilepsy is a neurodegenerative disorder regulated by several complex factors including epigenetic alteration of gene expression (Henshall and Kobow, 2015), where alteration of miRNAs level is one of most prominent phenomenon for being as a therapeutic target in future (Henshall, 2014). There are many miRNAs that have been indicated to be involved in the pathogenesis of epilepsy (Li et al., 2014). For example, miR-124, miR-134, miR-196 appear to be involved in glioma-related seizures (Peng et al., 2013). In addition, up-regulation of miR-146a was found in animal models (Hu et al., 2011; Omran et al., 2012) and human epilepsy (Aronica et al., 2010). An another study showed that miRNAs are implicated with astrocyte-mediated inflammation in pilocarpin-induced SE model (Peng et al., 2013). Similarly, up-regualtion of miRNA-21-5p and miR-132 reported to be involved in pathogenesis of epielepsy (Jimenez-Mateos et al., 2011; Peng et al., 2013). Few recent studies showed that expression of miR-146a, miR-34a and miR-210 was altered in pilocarpine-induced status epilepticus. miRNAs-23 a/b and let-7e also appears to be involve in TLE (Song et al., 2011) and miR-219 seems to be involved in kainic-acid induced seizures, as well as in TLE (Zheng et al., 2016). Numerous studies have also demonstrated dysregulation of various miRNAs during post-traumatic epileptogenesis and after neuronal injury (Liu et al., 2010). Recently, Li et al. (2014) have reported effect of epileptogenesis on the miRNAs related epigenetic regulation and found altered of miR-3120 and miR-214. We have also checked the effect of $FeCl_3$-induced epileptogenesis on the expression of miR-3120 and miR-214. Data obtained in the present study showed time dependent decrease in the levels of miR-3120 and miR-214 in the cortex as well as in hippocampus of $FeCl_3$-induced epileptic rats suggesting that these miRNAs may also be involved in the pathogenesis of epilepsies. Silencing of miRNA (by antagomir) such as miR-219 has been found to induce seizure like EEG in normal mice (Zheng et al., 2016). Similarly, miR-214 have been shown to induce the oxidative stress after alcohol treatment in rat liver cells (Dong et al., 2014) indicating the role of miR-214 in oxidative stress-mediated mechanism.

Therefore, decreased levels of miR-3120 and miR-214 in the cortex and hippocampus of $FeCl_3$-induced epileptic rats indicated involvement of these miRNAs in $FeCl_3$-induced epileptogenesis. In addition, miR-214 have been reported to be involved in stress-induced neuronal cell death through PTEN and (Yan et al., 2013) p53 mediated mechanisms (Rahal and Simmen, 2010). Numerous reports have demonstrated increased (Gorter et al., 2014; Risbud and Porter, 2013) as well as decreased expression (Kan et al., 2012; Kretschmann et al., 2014) of miR-214 in various epilepsy models. Moreover, decrease in miR-214 level have been reported in the case of SE mice model (Schwarzenbach et al., 2012). Similarly, any alteration in the expression of these miRNAs by the supplementation of curcumin was also quantified to assess the miRNAs related epigenetic dysregulation during epileptogenesis. We found significant increases in the expression of miR-3120 as well as in miR-214 in the curcumin-fed rats when compared with epileptic rats. Results are in agreement to several earlier studies where altered expression of miRNAs was observed by the supplementation of curcumin (Dahmke et al., 2013; Reuter et al., 2011; Saini et al., 2011; Sun et al., 2008; Teiten et al., 2013). Moreover, some studies demonstrated that curcumin treatment even ameliorates damage at the chromatic level by attenuating histone modifications in KA-induced SE (Sng et al., 2006; Teiten et al., 2013). Similarly, it has been reported to alter the expression of numerous miRNAs in different pathological disorders (Dahmke et al., 2013; Reuter et al., 2011; Saini et al., 2011). Thus, in this study miR-3120 and miR-214 were observed to be up-regulated in curcumin-fed rats indicating that curcumin counter balanced the epigenetic changes in post-traumatic epileptogensis.

The miRNAs show broad spectrum of expression in the brain, yet many miRNAs showed the region specific differential expression, miR-195, miR-497 and miR-30b were shown higher expression in cerebellum (Tusher et al., 2001), miR-34a, miR-451, miR-219, miR-338, miR-10a and miR-10b were reported to be raised in medulla, miR-7 and miR-7b were found to be enriched in hypothalamus (Farh et al., 2005). Same reports demonstrated that miR-218, miR-221, miR-26a, miR-128 enriched in hippocampus, suggesting the region specific function of miRNAs in the brain (Farh et al., 2005; Hohjoh and Fukushima, 2007). Therefore, in the present study, we also quantified the level of miR-3120 and miR-214 in the cortex and hippocampus region of brain of control rats, and a higher expression of both miRNAs

was observed in the hippocampus indicating about the link of miR-214 as well as miR-3120 with learning and memory.

miRNAs are the regulator of gene expression, and are involved in epileptogenesis by regulating various signalling pathways including hyperexcitation of neurons and apoptosis (Lippi et al., 2016; Qureshi and Mehler, 2010; Wang et al., 2012). There are several reports suggesting that *in-silico* target prediction and miRNA identification in a particular biological process can help to investigate the role of a miRNA in a specific cellular mechanism (Kretschmann et al., 2014; Redell et al., 2011). Hence, in this study we predicted the putative targets of miR-3120 using various bioinformatics tools. Quantification of efficient binding energy of miR-3120 to the predicted sites of putative targets associated with generation of epilepsy was done to ensure the significance of the role miR-3120 in the epileptogensis. We found several targets of miR-3120 with efficient binding energy and selected three most important targets (PTEN, CACNA1A and GABRD) known to be associated with epilepsy. . Signaling pathways regulated by these targets was observed using KEGG pathway analysis webtool. Some earlier reports have also showed that neuronal cell death is mediated by PTEN regulated signaling pathway (Zhu et al., 2006). Similarly, CACNA1A and GABRD are also reported of be involve in synapses and excitability of neurons by regulating neurotransmitter levels inside the cell (Caddick et al., 1999; Dibbens et al., 2004). These proteins are also trigger hyperexcitation of the neurons and cause epileptic bursts later (Mark et al., 2011; Zamponi et al., 2010). Therefore, this study indicated the link of miR-3120 expression with the hyperexcitation, neurotoxicity and further neuronal cell death due to epilepgenesis mediated signaling cascades.

Role of channel proteins in hyperexcitation of neurons during seizures is an essential phenomenon in which, voltage gated Ca^{2+} channels play a pivotal role. There are various known voltage gated calcium channels such as L type Calcium channels consisting of Cav1.1- Cav1.4, P/Q type includes Cav2.1- Cav2.3, similarly N and R type comprise Cav3. All have been reported to be involved in the generation of epilepsies, for example several studies showed the up-regulation of L-type voltage gated calcium channel during epilepsy in humans as well as in animal models (Zamponi et al., 2010). Jouvenceau et al. (2001) showed that voltage gated Ca^{2+} channel Cav2.1 subunit-A (CACNA1A) gene of P/Q type calcium channel mutation

leads to epilepsy in humans. Similarly, another study by Guerin et al. (2008) showed the presence of mutation in CACNA1A gene, and caused seizure in human patient (Candece et al., 2016; Damaj et al., 2015). Earlier reports also showed the involvement of Cav2.1 as well as its subunit CACNA1A in epilepsy (Mark et al., 2011). CACNA1A have been reported to be down-regulated in KA-induced SE model (Wen et al., 2009). Another study of Lv et al. (2015) demonstrated the association of CACNA1A with epilepsy in Chinese patients.

Accumulating body of literature suggests down-regulation of CACNA1A during epileptogenesis (Jouvenceau et al., 2001; Peng et al., 2004). Studies also demonstrated that the mutation of CANA1A leads to epileptic encephalopathy (van den Maagdenberg et al., 2004), in the light of results from above given studies and predicted targets of miR-3120, protein expression of CACNA1A was also quantified using IHC and western blot analysis Protein expression of CACNA1A was down regulated in the cortex and hippocampus of epileptic rat's brain. Since, our bioinformatics results along with existing knowledge suggested that CACNA1A are the putative target of miR-3124 but decreased miRNA as well as protein expression of CACNA1A suggesting that involvement of multi-factorial effect at translational and posttranslational levels to regulate the expression of CACNA1A.

Earlier reports have shown that curcumin can suppress the activity of calcium channel and decrease the glutamate release through the voltage gated calcium channels, hence, generating neuroprotective effect on the neurons (Shah et al., 1999). We also verified the effect of dietary curcumin supplementation on the expression of CACNA1A and found increased expression of CACNA1A in the curcumin-fed epileptic rats. The present results showed that supplementation of curcumin normalized the epileptogenesis-mediated down-regulation of CACNA1A. Interestingly, expression of miR-3120 and miR-214 were also found to be up-regulated suggesting involvement of deferent regulatory pathway that affect the expression of CACNA1A.

Synaptic excitability of neurons depends upon neurotransmitter receptors on the membrane of neurons including both excitatory and inhibitory. GABA is an inhibitory neurotransmitter which binds to two different GABA receptors (GABA-A and B) (Bowery et al., 1987). Both of the receptors are activate by different mechanisms of inhibition such as GABA-A receptor allostericaly binds with GABA

and releases Cl^- ions through its pore (Bowery et al., 1987; Lerche et al., 2013, 2001). However, GABA-B receptor is linked via G-protein coupled receptor and can stimulate opening of K^+ channels to release of K^+ and reduces the frequency of action potential to equilibrium (Bormann, 1988). There are many reports of both of GABA receptors, showing to be associated in many neurological disorders and considered as a therapeutic target (Brickley and Mody, 2012; Stellwagen et al., 2005; Ting Wong et al., 2003). Crino et al. (2001) showed the involvement of GABA-A receptor subunit mRNA in cortical dysplasia. Another study demonstrated the selective inhibition of neurons through GABA-A receptor δ subunit (GABRD) mediated mechanism (Stell et al., 2003). Similarly, a report on extrasynaptic GABA-A receptor, provided evidence of its involvement in central nervous system disease (Brickley and Mody, 2012).

Similar to CACNA1A, GABRD was also reported be involve in the development of different types of epilepsies (Belelli and Lambert, 2005; Brooks-Kayal and Russek, 2012; Dibbens et al., 2004). Maljevic et al. (2006) showed that the mutation of GABAA receptor α1-subunit is implicated with absence epilepsy. Some other reports demonstrated that human epilepsies are associated with GABRD (Berkovic et al., 2006). Therefore, in the light of *in-silico* analysis of miR-3120 and previously reported evidence, expression or GABRD was quantified and our study demonstrated the down-regulation of GABRD protein shows the involvement of GABRD in $FeCl_3$-induced post traumatic epileptogenesis. Though, a recent study showed the increase in GABRD mRNA in Mg^+ induced epilepsy model (Yu et al., 2017). GABRD protein is down-regulated resembles the earlier studies on protein level of GABRD in brain during epilepsy (Peng et al., 2004). This indicate that during epileptogenesis the protein level of GABRD is controlled by other factors which caused the down-regulation of GABRD protein independent of decreased levels of miR-3120. Moreover, ethanol model have shown the potential changes in δ, α4 and γ2 subunits (Cagetti et al., 2003) that displayed a decreased inhibition and threshold for PTZ-induced seizure model (Kang et al., 1998; Kokka et al., 1993; Liang et al., 2004). Hence, decreased level of GABA receptor subunits is linked with increased susceptibility to seizures.

The present study further demonstrated the effect of dietary curcumin on GABRD protein expression. Increase in the expression of GABRD protein showed

counteracting effect of curcumin. In contrast, miR-3120 expression was found to be up-regulated with the curcumin supplementation as well which indicated multi-factorial effect of the antiepileptic action of curcumin on GABRD which either can be direct or indirectly effecting the GABRD protein level.

Epileptogenesis causes neurodegeneration mainly through intrinsic and extrinsic pathways of apoptosis (Henshall, 2007; Henshall and Simon, 2005). In PTE apoptosis has already been reported to be implicated with PTEN mediated pathway, also co-related with DNA damage due to ROS and RNS stress (Rahal and Simmen, 2010; Zhao et al., 2004; Zhu et al., 2006). Moreover, there are many reports showed *Pten* gene induced seizures in mice (Backman et al., 2001). In addition to apoptosis, PTEN is also reported to be involved in mTOR pathway by inhibiting the hyperactivation of mTOR (Park et al., 2010; Pun et al., 2012; Zhou et al., 2007). Although, earlier reports have demonstrated that the hyperactive mTOR ameliorate the cell survival by inhibiting apoptosis, there are numerous reports showing that mTOR activation also reduces the activity of various transcription factors. Due to involvement of PTEN in various neurodegenerative disorders such as Alzheimer's, Parkinson's disease and motor neuron disorders (Ismail et al., 2012), it is considered as the molecular target in neurological disorders (Ismail et al., 2012; Namikawa et al., 2000; Rickle et al., 2006). As PTEN prevent the activation of mTOR its down-regulation causes epileptogenesis (Meng et al., 2013). In previous section of *in-silico* study we demonstrated *Pten* as a target of miR-3120, similarly, earlier literature showed that *Pten* is regulated by miR-214 (Yang et al., 2008; Zhao et al., 2015). In the present study, quantification of PTEN, a predicted target of miR-3120 and reported target of miR-214 was done to understand the regulation of apoptosis by these two miRNAs through PTEN regulatory pathways as reports have already shown the involvement of PTEN in neuronal degeneration during apoptosis (Schwarzenbach et al., 2012; Zhao et al., 2004; Zhu et al., 2006). The observation of increased *Pten* expression during epileptogenesis seems rather unexpected. Increased expression of *Pten*, however, could be a consequence of the depression of miR-3120 and miR-214 expressions, since these two miRNAs are known to target *Pten* gene (Scott et al., 2012; Sharma et al., 2015) to ameliorate the hyper-activation of mTOR and inhibition of apoptosis *Pten* expression elevates as well. However, the possibility of other mechanism for elevation of *Pten* also exists. The present data further showed that

curcumin stimulated the expression of miR-214 and miR-3120, and reduced the expression of *Pten* gene in $FeCl_3$-induced epileptogenesis showing that the antiepileptic action of curcumin is mediated by elevation of miR-3120, miR-214 and down-regulation of *Pten* gene. Curcumin's antiseizure action is also supported by the findings that it inhibits mTOR signalling (Meng et al., 2013). Thus curcumin's antiepileptic action involved elevation of miR-3120 and miR-214 and may involve inhibition of mTOR signalling.

Epilepsy is third widespread neurological disorder in elderly (Tallis et al., 1991). Thus, it is believed that aging of the nervous system may influence seizure susceptibility (Hauser et al., 1993). Studies on various models of experimental epilepsy have shown alteration of seizure susceptibility/ severity with age (Darbin and Naritoku, 2004; Holtkamp et al., 2004). Various *in-vivo* studies demonstrated the increased (Dawson and Wallace, 1992; Kliouеva et al., 2001) susceptibility for seizures with aging in different experimental models of epilepsy. Interestingly, vulnerability of brain to develop PTE in an age dependent manner is still unknown. It is apparent from the present study that 18-20 months rats were more susceptible for development of seizures as compared to 4-6 months rats. Similarly, the attenuation of seizure by antiepileptic action of curcumin is slower indicated the higher vulnerability of seizures in this age group rats. Earlier studies have also reported the alteration in synaptic connection with aging (Norris et al., 1998; Ouanounou et al., 1999). Jyoti et al. (2009) showed that 18-20 months rats are more susceptible to seizures, along with shorter latency in $FeCl_3$-induced epilepsy. Results obtained from the present study also demonstrated reduction of miRNAs levels in 18-20 months rats indicated the link of seizure vulnerability with age related miRNA expression; however, a thorough investigation is required to reach a concrete conclusion.

The oxidative stress after neuronal insult is a major cause of pathogenesis of epilepsy. Thus, stress-mediated signals have been seen to be up-regulated in the aged rats higher than the younger rats (Li et al., 2009),thus, we also quantified the expression of miR-3120 and miR-214 in the cortex of both age groups and found the higher expression of both miRNAs in 4-6 months rats when compared with 18-20 months rats which are considered as aged animals. This indicates the relation of miR-214 and miR-3120 with aging of the brain as well. However, further studies are warranted for a concrete conclusion.

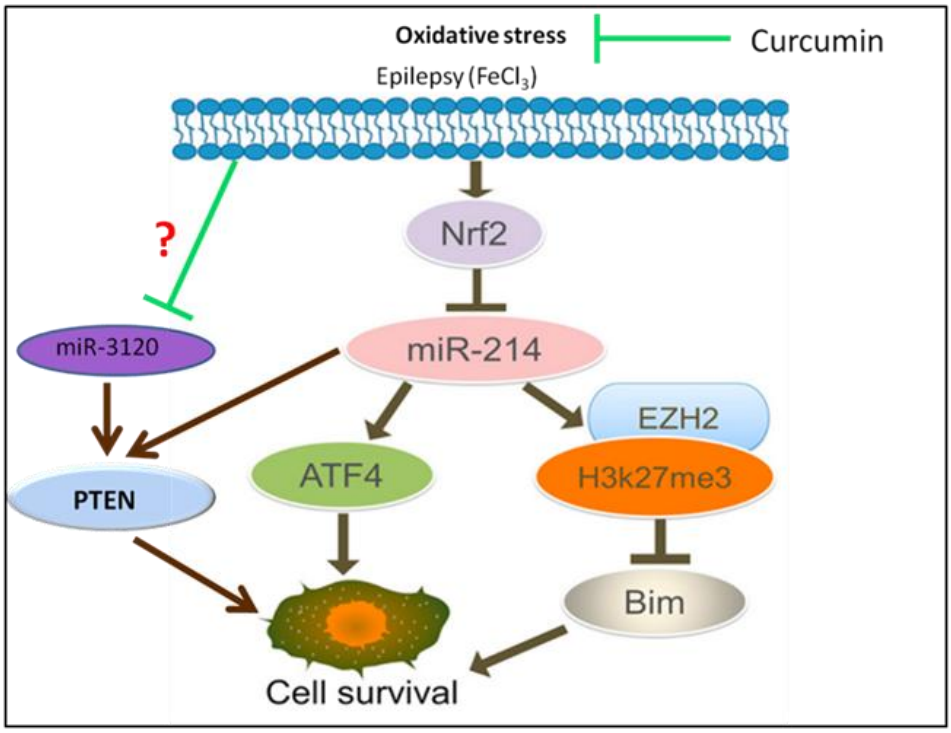

Figure.6.1. Schematic diagram represents possible mechanism of miR-214 and miR-3120 in post-traumatic epileptogenesis.

Therefore, possible mechanism of action of both miRNAs during epileptogenesis is associated with PTEN mediated cell survival pathway. Since, miR-3120 and miR-214 is regulating the expression of *Pten* gene, the neuronal degeneration through apoptosis seems to play an important role during epileptogenesis (Figure.6.1).

SUMMARY AND CONCLUSION

7. Summary and conclusion

The present work focused on to study the expression of miR-3120 as well as on miR-214 through epigenetic effect of $FeCl_3$-induced experimental model of epilepsy. Moreover, the study investigated the antiepileptic potential of curcumin in this epilepsy. Epilepsy was induced by $FeCl_3$ injection in two different age groups of rats. The development and progression of epileptiform activity was monitored electrophysiologically by recording the electroencephalographic (EEG) and multiple-unit activities (MUA) in the $FeCl_3$-induced epileptic and curcumin-fed animals.

1. The epileptic seizure activity in rats and was attenuated in the curcumin-fed epileptic rats as evident from EEG recordings and MUA counts. Supplementation of curcumin for 28 days was most effective compared with other three treatments durations.
2. Epilepsy-related behavioral alterations; (like learning and memory) were assessed by the Morris water maze test in both the epileptic and curcumin treated animals. The result demonstrated that the curcumin supplementation countered epileptogenesis-associated cognitive defects.
3. MiR-3120 and miR-214 levels were quantified in the cortex of two age groups of rats. It was found that 4-6 months rats exhibited higher expression of miRNAs when compared to 18-20 months age group indicating the age dependent decline in the expression of both miRNAs.
4. Levels of both miRNAs were also checked in the cortex and hippocampus of brain. It was found that there was higher expression of miR-3120 and miR-214 in the hippocampus as compared to cortex.
5. Changes in miR-3120 and miR-214 levels were studied during $FeCl_3$-induced epileptogenesis. The results derived from the experiments clearly showed that expression of both miRNAs significantly declined in $FeCl_3$-induced epileptic rats. Moreover, in time dependent quantification of miRNAs expression, levels of miRNAs were found to be the least on 28-day after $FeCl_3$ seizure induction. The levels of both miRNAs were increased after curcumin supplementation. Feeding of curcumin for 28-days seems to be most effective. Increased miRNAs levels after curcumin supplementation indicated that curcumin influenced the expression miRNAs as well as countered the epileptogensis.

6. *In-silico* analysis of miR-3120 and miR-214 was done. Targets of miR-3120 were predicted. *Pten*, CACNA1A and GABRD were found to be major putative targets of miR-3120 among them *Pten* is common target of both miRNAs. Free energy of binding between miR-3120 and targets was calculated. Putative pathways of targets were analyzed using KEGG and these targets were found to be implicated in epilepsy-associated pathways.
7. The expression of CACNA1A was checked in the brain of epileptic and control rats. Expression of CACNA1A was found mainly in pyramidal neurons, the decrease in levels of CACNA1A was found in epileptic rats. However, there was elevated expression of CACNA1A after dietary supplementation of curcumin indicating that curcumin supplementation countered the expression of CACNA1A in the epileptic rat brain.
8. The expression of GABRD was checked in epileptic and control rats. Expression of GABRD was found majorly in neocortex region of brain, the decrease in levels of GABRD was found in epileptic brain. However, there was elevated levels of GABRD when dietary supplementation of curcumin was given to rats indicating the curcumin supplementation countered the expression of GABRD in the rat brain.
9. The mRNA expression of *Pten* gene in epileptic and curcumin treated animals was also measured. The level of *Pten* was found to be up-regulated in $FeCl_3$-induced epileptic rat. However, dietary curcumin supplementation partially countered the epileptogenesis related up-regulation of *Pten* gene in the brain.

The present study leads to the conclusion that miR-3120 as well as miR-214 are linked with the epileptogenesis, as they are alter during epilepsy. The altered expression of *Pten* is regulated by the miR-3120 and miR-214. However, the expression of CACNA1A and GABRD was found to be down-regulated with the down-regulation of both miRNAs indicating the change in expression of both the proteins might be regulated at the translation level but not regulated by miRNAs. All these molecular changes occurring in the generation of epilepsy are countered by dietary curcumin-supplementation, on miRNAs as well as on their target proteins. Though, there are some contradictions in regulation of CACNA1A and GABRD protein expression, the curcumin always seems to inhibit the epileptogenesis associated cellular changes. Thus, in future curcumin might gain

attention as a therapeutic agent against epilepsy. However, further research is required to unravel its exact molecular mechanism.

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PUBLICATIONS

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CONFERENCES, WORKSHOPS AND PRESENTATIONS

1. Poster presentation at "Science Day 2016" organized by Jawaharlal Nehru University (JNU) , New Delhi, sponsored by Department of Science and Technology, Government of India: at JNU convention centre-JNU; 26th February, 2016.
2. Poster entitled, "Curcumin resist the effect of post traumatic epilepsy on miR-3120 expression" presented at "31th International Epilepsy Congress (IEC)" organized by International League Against Epilepsy (ILAE) and International Bureau of Epilepsy (IBE) in Istanbul, Turkey, 5th – 9th September, 2015.
3. Poster entitled, "Characterization of miR-3120 in the experimental model of epilepsy in response to dietary curcumin" in "first IBRO/APRC School" organized by International brain research organization (IBRO) in Chandigarh, 2nd- 9th November, 2014.
4. Poster entitled, "Characterization of miR-3120 in experimental model of epilepsy" in International neurobiology conference: Brain plasticity and Neurological disorders organized by Department of zoology, Rawenshaw University, Cuttack, Odisha, 9th-11th November,2013.
5. Participated in "Research proposal oral presentation" in "Biosparks 11th National science meet" organized by School of Life Science, Jawaharlal Nehru University, New Delhi, 15th – 16th February, 2013.
6. Attended Conference, in Recent advances in "27th Annual meeting of Society of Neurobiology, India (SNCI) and International conference on Molecular mechanism in neurological disorders", organized by All India Institute of Medical Sciences, New Delhi, 21st- 23rd February, 2013.
7. Attended workshop, of "Hands on training on Laboratory animals", organised by Central laboratory animal resources, Jawaharlal Nehru University, New Delhi, 28th October, 2015.

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RESEARCH ARTICLE

ALTERED EXPRESSION OF MIR-214, MIR-3120 AND PTEN IN IRON-INDUCED EXPERIMENTAL EPILEPSY MODEL OF POST-TRAUMATIC EPILEPSY AND THE EFFECT OF CURCUMIN.

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Abstract

MicroRNAs are known to be involved in the pathogenesis of epilepsies. The objective of the present study was to investigate the changes in expression of miR-3120, miR-214 and Pten gene in iron-induced experimental epilepsy model of post-traumatic epilepsy. In addition, the role of these miRNAs and Pten was also studied in the antiseizure action of curcumin. The results showed that in iron-induced epileptogenesis, expression of miR-3120 and miR-214 is reduced and Pten gene expression is upregulated. The results suggest that these miRNAs may be involved in the pathogenesis of epilepsy. Curcumin's antiseizure effect is mediated by an increase in the expression of miR-3120 and miR-214 and downregulation of Pten gene expression.

Introduction:-

MicroRNAs (miRNAs) are endogenous non-coding small RNAs. They regulate gene expression by inhibiting/degrading protein coding mRNAs. However, miRNAs can also upregulate translation (Cao et al., 2006; Kosik, 2006). miRNAs have been found to participate in the molecular mechanisms of many pathophysiological processes including those of neurological disorders. miRNA-214 targets Huntington's gene (Sinha et al., 2011). It is also involved in NMDA receptor-related memory processes (Wang et al., 2014). miRNA-3120 is a brain-specific miRNA which is involved in uncoating of vesicles (Scott et al., 2012) and is also involved in learning. miRNA-214 has been reported to modulate NMDA receptor-mediated neurobehavioral dysfunction (Wang et al., 2014).

Changes in the expression of miRNAs occur in epilepsies as miRNAs target a variety of pathways i.e. inflammation, apoptosis, dendritic growth, and spine dynamics, neurites growth, Ca^{2+}- calmodulin-dependent protein kinase-II and thus NMDA receptors. miRNAs are thus seem to be involved in the pathogenesis of epilepsies. For example, miRNA-146a was shown to be upregulated in human temporal lobe epilepsy (TLE), miR-219 was found to decrease in kainic acid model of epilepsy and in the CSF of epilepsy patients, and silencing of miR-219 was found to induce seizures (Dogini et al., 2015). Several miRNAs were found altered in a lithium-pilocarpine model of status epilepticus. miRNA-214 was found to be down-regulated in mesial temporal lobe epilepsy patients (Li et al., 2014). Brain-specific miR-219 and 134 were found to be significantly upregulated with seizures (Li et al., 2014), miRNAs have even been considered as biomarkers of epilepsy as different miRNAs may be involved in different epilepsies (Li et al., 2014).

Iron-induced experimental epilepsy in rodents models the human clinical post-traumatic epilepsy (Willmore et al., 1978). This experimental model has often been used to investigate the mechanism of epileptogenesis and

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pharmacology of epilepsy (Willmore, 1990). In iron- induced epileptogenesis, glutamatergic mechanisms have been implicated. For example glutamate transporters are down-regulated, glutamate receptors are upregulated and extracellular glutamate levels increase in iron-induced epileptogenesis (Ueda et al., 2001). In the present study, this model was adopted to further investigate the possible involvement of miRNAs in the pathogenesis of epilepsy as miRNAs may show differential responses in different epilepsies/models (Li et al., 2014), Glutamatergic mechanisms are involved in iron-induced epilepsy (Mishra et al., 2013; Ueda and Willmore, 2000) and miRNAs are involved in the regulation of glutamatergic mechanisms (Harraz et al., 2012; Kawashima et al., 2010; Morel et al., 2013). In the present study, we explored the correlation between miRNA-214, miRNA-3120 and development of epileptiform activity in iron-induced epileptogenesis by investigating their expression profiles in epileptogenic tissue.

In addition, expression status of Pten gene (Backman et al., 2001) during iron-induced epileptogenesis was also studied. Specific disruption of Pten gene is known to result in epileptogenesis (Backman et al., 2001; Meng et al., 2013), and miR-214 and miR-3120 both targets Pten gene (Yang et al., 2008)). Pten epileptogenesis is mediated by PTEN inhibition of mTOR signalling. It is the hyperactivation of mTOR that is epileptogenic (Meng et al., 2013). mTOR pathway has been found to mediate temporal lobe epilepsy (Zeng et al., 2009). Therefore, it is of interest to also investigate the Pten gene expression in epileptogenesis.

The second aim of the present study was to determine whether curcumin's anti-epileptic effect (Jyoti et al., 2009) involves curcumin's action on miRNAs. Curcumin is diferuloylmethane. It is obtained from the rhizome of the plant *Curcuma longa* and is a common agent used as a spice in Indian food. Curcumin has been termed as a cureamine and experimentally it has been found to have multiple pharmacological and therapeutic properties of possible clinical importance (Satoskar et al., 1986; Strimpakos and Sharma, 2008). Neurologically it has been found to be a neuroprotective agent (Cole et al., 2007), and exerts antiepileptic action in experimental epilepsy (Noor et al., 2012). It significantly attenuates electrographic and behavioral seizures and their biochemical measures in iron-induced experimental epilepsy (Jyoti et al., 2009). Curcumin has been shown to alter expression of several miRNAs i.e. miR-103, 140, 146a, 148a, 199a, 21, 22, 204, 98, 7 in a variety of experimental conditions (Sun et al., 2008). In the current study we, therefore investigated whether the antiepileptic action of curcumin is mediated by its action on the expression miRNA-214 and miR-3120 and Pten gene in iron-induced experimental epilepsy. Curcumin appears to be an inhibitor of mTOR signalling (Meng et al., 2013) and therefore curcumin's and pten gene's influence on Pten gene is of interest.

Methods:-

Materials:-

Electrodes and wires used in electrophysiology and stereotaxy were made of stainless steel. Curcumin and chemicals used were obtained from Sigma-Aldrich chemical company USA. Curcumin was mixed with standard diet ingredients at a concentration of 1000 ppm and food pellets were made.

Animal treatment plan:-

Rats of six to eight months of age (male Wistar) were used for this study. Animals were housed in pairs in standard laboratory cages and maintained at 23± 4^{o}C, under 12 hour light /12 hour dark cycle. All the experimental protocols were approved by the committee for Purpose of Control and Supervision of Experimental Animals (CPCSEA) and the Institutional Animal Ethical Committee (IAEC) of Jawaharlal Nehru University, New Delhi, India. Animals were grouped as follows. To characterize the epileptiform electrical activity and to ascertain the effect of curcumin on it (in our set of animals), a group of animals (n=10) were made epileptic by intracortical injection of $FeCl_3$ (procedure described below). In five of these animals, development and progression of epileptiform activity was studied and in the other five animals effect of curcumin was assessed, five animals were used as controls in which intracortical injection of saline was given instead of $FeCl_3$. In these animals, electrocorticographic activity was recorded as in $FeCl_3$-injected animals.

For studying changes in miRNAs, animals were grouped as follows: Group I (n=12) consisted of controls which received an intracortical injection of saline (in place of $FeCl_3$) and had electrodes implanted for electrocorticography. These animals served as controls for the next two groups of animals. Group II (n=16) consisted of iron- induced epileptic rats. These rats were made epileptic by intracortical injection of $FeCl_3$ (procedure described below). In these animals, miRNA were estimated at various time points and had electrodes implanted for electrocorticography. Group-III (n=8) animals consisted of iron-induced epileptic rats with electrodes implanted for electrocorticography that were fed curcumin. Curcumin treatment was started on the very day

intracortical injection of $FeCl_3$ was given. In these animals, miRNAs estimations were done to determine the effect of curcumin.

Surgical procedure and recordings:-

Electrodes were implanted stereotaxically under anesthesia with 4% isoflourine.Six burr holes of 0.5 mm diameter (one for iron injection and other five for epidural electrodes placement) were drilled at the surface of the skull at stereotaxically marked sites. $FeCl_3$ (5µl containing 100mM $FeCl_3$ dissolved in physiological saline with flow rate of 1µl/min for 5 mins. Coordinates for $FeCl_3$ injection were antero-posterior= -1.0mm; lateral= 1.0mm and ventral (depth) =1.5mm in somatosensory region of cortex with the help of injector cannula. After injection, burr hole was sealed with bone wax (Mishra et al., 2013). For electrographic recording, four stainless-steel epidural screw electrodes were implanted at the coordinates, 2mm posterior and anterior to bregma, and 2 mm lateral in the somatosensory cortex. One screw electrode was placed on frontal sinus as animal ground. Each electrode was connected with a wire to each individual pin of a 9 pin adaptor. Later, 9 pins adapter was fixed to the surface of the skull using adherent dental acrylic cement to create a stout platform. After surgery, Animals were kept individually and allowed to recover and habituate for 1 week before recordings were started. Operated rats were given proper post-operative care (Jyoti et al., 2009). Curcumin treatment was started on the very day intracortical injection of $FeCl_3$ was given.

After 3 days of post-operative recovery, rats were prepared for electrocorticogram recordings. Recordings were made using Grass polygraph recorder as described previously (Mishra et al., 2013). Electrophysiological recordings were obtained from wake and conscious unrestrained animals and the occurrence of epileptiform electrographic activity was assessed during passive/quite and wakefulness condition. Simultaneous recording of ECoG and multiple unit activity potentials (MUA) was done to verify that there was an epileptic- activity associated increase in neuronal firing. MUA were amplified and filtered (300Hz to 10KHz) by Grass P511J preamplifiers, electronically discriminated using a window discriminator (WPI) and displayed on an oscilloscope. The standard EEG waves and MUA pulses were recorded as described in previous studies (Mishra et al., 2013). MUA potential counts were used to quantify the seizure activity.

RNA isolation and quantification:-

Rats were sacrificed by cervical dislocation and their brains were removed. The cortex was dissected out. Brain samples were crushed with liquid nitrogen and mixed with Trizol/TRI-Reagent (Sigma-Aldrich) followed by alcohol precipitation of total RNAs including micro-RNA using isopropanol and 70% ethanol. The palate which was obtained by centrifugation of precipitating RNA was dissolved in RNAase free DEPC-treated water, and the concentration was quantified by using Thermo Scientific Nanodrop system and aliquots were made after equilibrating the concentration of RNA and stored at -80^{o} C for further use (Aronica and Gorter, 2007; Mishra et al., 2013).

Quantification of miR-3120 and miR-214 was done by using Life Biosystems micro RNA assay kit (Scott et al., 2012). cDNA of miRNA-3120 and miR-214 were synthesized and amplification was done using stemloop PCR in which stemploop primers of miR-3120 and miR-214 from Life Biosystems were used to observe expression pattern of both miRNAs. U6sn RNA was used as an endogenous control. Expression of both miRNAs was quantified by using ImageJ software through densitometric analysis of pictures taken from 4% agarose gels(Scott et al., 2012).

Quantification of Pten was done through two-step semi-quantitative PCR, using Applied Biosystems cDNA synthesis kit and using primers for Pten. GAPDH was used as an endogenous control. Primers for Pten and GAPDH were 5'CAATGTTCAGTGGCGGAACTT3' forward, 5' GGCAATGGCTGAGGGAACT3' reverse and 5'ACCACAGTCCATGCCATCAC3' forward, 5'CACCACCCTGTTGGCTGTAGCC3' reverse. Expression of the gene was quantified through densitometry analysis of pictures of 1% agarose gels using ImageJ software (Rahal and Simmen, 2010).

Statistical analysis:-

The results were expressed as mean, ± SEM. Statistical analysis was performed by using one-way analysis of variance (ANOVA) with post hoc statistical tests.

Results:-

Electrographic seizure activity and the effect of curcumin in iron-induced epileptic animals:-

Experimental animals which were given intracortical iron injection, developed epileptiform electrographic activity on their electrocorticograms (ECoG) (Fig.1). Distinct chronic epileptiform activity on ECoG began to appear around day 7 onwards (Fig-1A, a-d). The epileptiform activity was spontaneous and recurrent and consisted of isolated spikes, polyspikes, spike-waves complexes, behavioral seizures activity concomitant with ECoG paroxysms progressed with time and consisted of more facial automatisms, head nods following pauses in behavior, steadfast posture, tonic flexing concurrent with biting and chewing hindlimb extremity. The epileptiform activity was quantified by multiple unit action potentials (MUA). The MUA recordings clearly showed the progressive development and build-up of the epileptic ECoG activity (Fig. 1 B).

Curcumin treatment of epileptic animals for various durations suppressed the epileptiform activity on ECoG (Fig 1. e-h). Statistical comparison of the corresponding MUA counts from controls and curcumin-treated animals (Fig-1B) clearly showed the quantitative extent of the decrements of the epileptic activity after curcumin treatment ($F_{3,36}$ = 159, $p<0.01$)

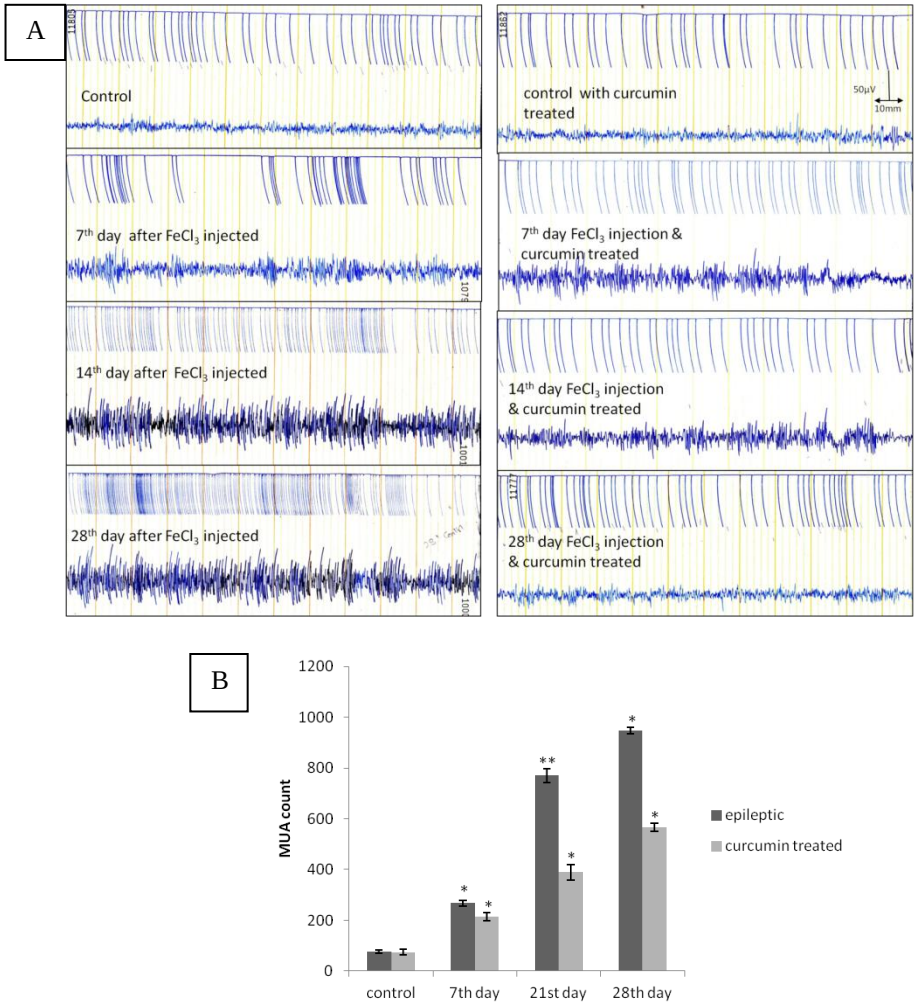

Fig. 1 – (A) Representative sample of polygraph recordings (ECoG and Multiple-unit activity) from somatosensory region of cortex of epileptic and curcumin treated epileptic rat showing epileptogenesis (a) control (b-d), day7,21 28 post-iron injection (e-h) effect of curcumin treatment on epileptogenesis (e)control, (f-h) day7,21 and 28th of curcumin treatment (B) effect of curcumin treatment for 7,21 and 28day on MUA in epileptic rats each bar represents mean SEM of 4 rat. Statistical comparision is with the respective control ($F(3,36)=159$), $p<0.01$). *p< 0.05, **p< 0.01, ***p< 0.001 (ANOVA)

Expression of miR-3120 and miR-214 in iron- induced epilepsy:-

Levels of miR-3120 and miR-214 were determined in the cortex of iron-induced epileptic rats at days 7, 14, 21, 28 after induction of epilepsy. Figure-2 shows that the levels of both miR-3120 and miR-214, compared with the controls, decreased with the progression of electrographic seizure activity (Fig.2A & B) ($F_{3,36}$ = 17, $p< 0.01$; $F_{3,36}$ = 66, $p< 0.01$), indicating that the expression of these miRNAs falls during the development of iron-induced epileptiform activity.

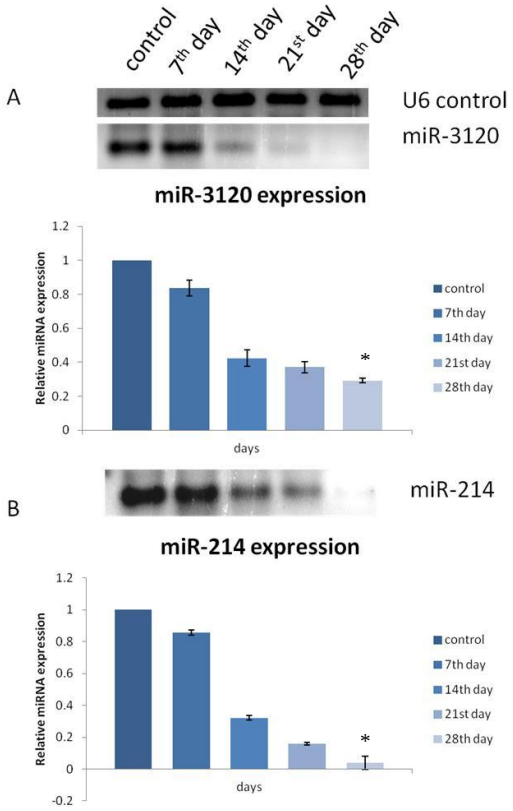

Fig. 2 – Relative miR-3120 (A) and miR-214 (B) expression levels in rat cortex of iron induced epilepsy measured by semi qRT-PCR Each bar represents the mean ± SD of SEM levels of miRNAs. Statistical comparision of values at day 7, 14, 21, and 28 of Epileptogenesis are with the controls (n=4, $p<0.05$ (ANOVA)) &(n=4, $p< 0.05$(ANOVA)). Electrophoresis bands (agarose gel) of RT-PCR products corresponding to mRNA expression are presented above the bars. Decrease in miRNAs expression with the Epileptogenesis is evident.

Effect of curcumin on the expression of miR-3120 and miR-214 in iron-induced epilepsy:-

Treatment of iron-induced epileptic rats with curcumin partially countered the epileptogenesis-associated decline in miR-3120 levels in their cortex as significantly higher levels of miRNAs were detected at day 28 after curcumin treatment (Fig.3). Seven days treatment did not significantly elevate the level of expression, 28 days treatment, however, resulted in significant elevation of the expression of both miR-3120 and miR-214 ($F_{2, 8}$ =18, $p< 0.01$; $F_{2,8}$ $p< 0.05$) (Fig. 4). Thus, curcumin treatment partially prevented the epileptogenesis associated decline in the levels of both miRNAs (Fig. 4).

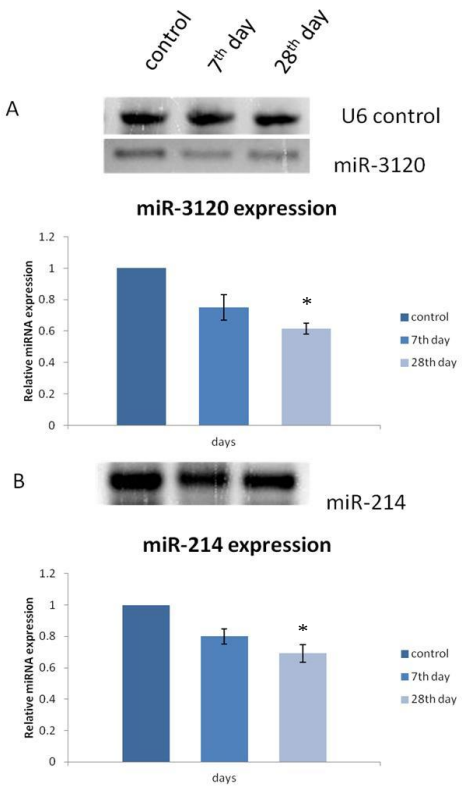

Fig 3. Effect of curcumin on relative miR-3120 (A) and miR-214 (B) expression in rat cortex of iron-induced epileptic rats measures by semi qPCR. Each bar represents the mean ± SD of SEM levels of miRNAs. Statistical comparison of values at day 7 and 28th of epleptogenesis are with the control. Electrophoresis bands (agarose gel) of RT-PCR products corresponding to mRNA expression are presented above the bars n=3, p< 0.05(ANOVA)) &. n=3, p< 0.05 (ANOVA)). Increase in levels of expression (comparison of values at day 7 day 28 with those of Fig-4 after curcumin treatment are evident.

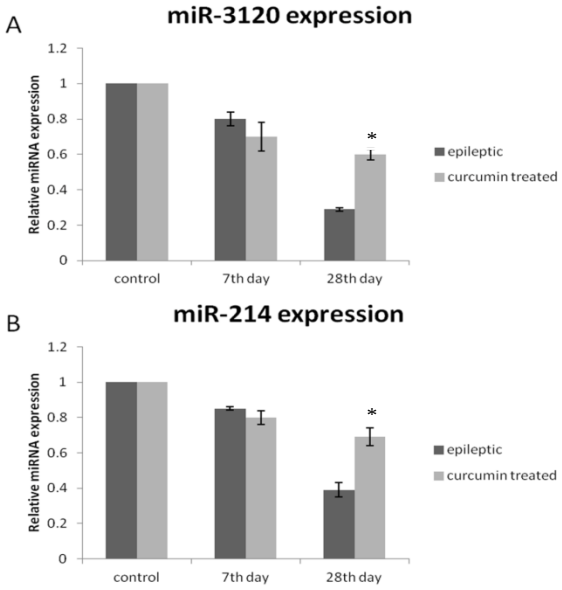

Fig 4.Effect of curcumin treatment for 7days and 28 days on miR-3120 (A) miR-214 (B) expression in the cortex of iron induced epileptic rats. Each bar represents the mean± SD of n=4 rats. Statistical comparisons of each treatment group with their respective epileptic controls cases. Increase in expression levels after curcumin treatment (28 day) is evident. (n=3, p< 0.05(ANOVA))

Expression of Pten gene in iron-induced epilepsy:-

Expression levels of Pten gene mRNA were measured at days 7 and 28 after induction of epilepsy. Pten gene expression levels increased with the progression of electrographic seizure activity ($F_{2,8}$ =44, p< 0.01) (Fig. 5).

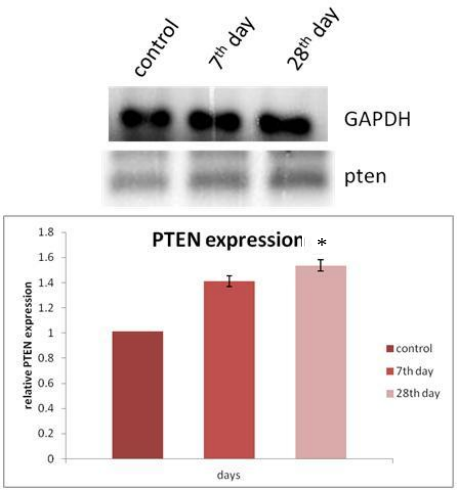

Fig 5. Relative Pten gene expression in rat cortex of iron-induced epileptic rats measured by semi qPCR. Each bar represents the mean ± SEM levels of Pten gene mRNA. Statistical comparison of values at day 7 and 28 of epileptogenesis are with the control. Electrophoresis bands (agarose gel) of RT-PCR products corresponding to mRNA expression are presented above the bars(n=3, p< 0.01 (ANOVA)) Increase in Pten gene expression with epileptogenesis , is evidnet

Effect of curcumin on Pten gene expression in iron-induced epilepsy:-

Curcumin treatment significantly decreased the expression of Pten gene (mRNA) in iron-induced epileptic rats. Seven days treatment resulted in insignificant (Fig. 6) decrease. However, 28 days treatment produced a significant decline in the expression (p< 0.01) and restored the level of expression near to the control level. Thus, curcumin treatment prevented the epileptogenesis-associated rise in Pten gene expression (Fig. 7)

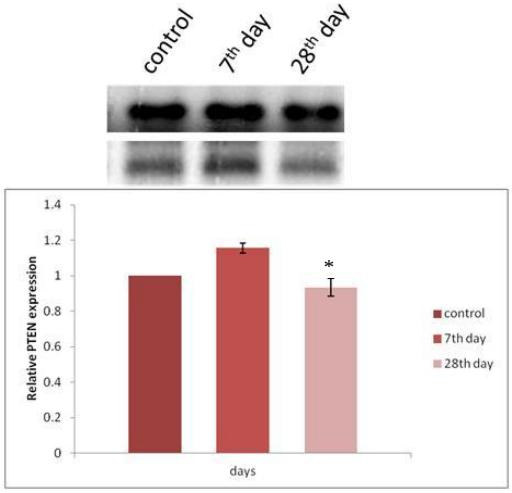

Fig 6. Effect of curcumin on relative PTEN gene expression in rat cortex of iron-induced epileptic rats measures by semi qPCR. Each bar represents the mean ± SEM of levels of Pten gene mRNA. Statistical comparison of values at day 7 and 28th of epleptogenesis are with the control. Electrophoresis bands (agarose gel) of RT-PCR products corresponding to mRNA expression are presented above the bars n=3, p< 0.01(ANOVA)) Decrease in levels of expression (comparison of values at day 7 day 28 with those of Fig-7 are evident.

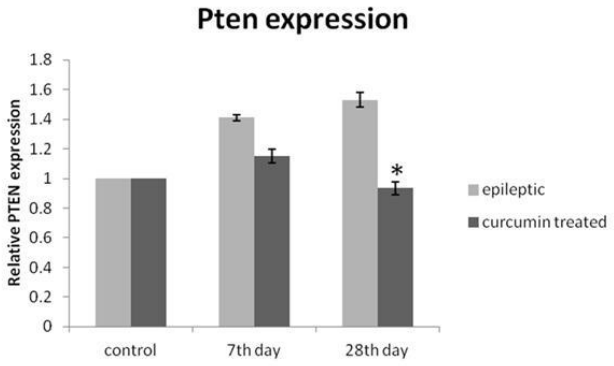

Fig 7.Effect of curcumin treatment for 7days and 28 days on Pten gene expression (levels of Pten gene mRNA) in the cortex of iron induced epileptic rats. Each bar represents the mean± SD of n=4 rats. Statistical comparisons of each treatment group with their respective epileptic controls cases. Decrease in expression levels after curcumin treatment (28 day) are evident.(n=3, $p < 0.05$(ANOVA))

Discussion:-

Many miRNAs have been indicated to be involved in the pathogenesis of epilepsy (Li et al., 2014). For example, miR-124, miR-134, miR-196 appear to be involved in glioma- related seizures, miR-146a in temporal lobe epilepsy (TLE) and in human TLE, miR-34a and miR-210 in pilocarpine-induced status epilepticus , miR-134 in status epilepticus in epileptic rats (Dogini et al., 2015; Kretschmann et al., 2015; Chen et al., 2016). miRNAs 23 a/b and let-7e also appears to be involved in TLE (Song et al., 2011). miR-219 is involved in kainic-acid induced seizures, as well as in TLE (Zheng et al., 2016). miR-214 was also found to be down-regulated in epilepsy patients.

The data obtained in the present study showed that the levels of miR-3120 and miR-214 decreased in the cortex of iron-induced epileptic rats suggesting that these miRNAs may also be involved in the pathogenesis of epilepsies. Silencing of miRNA (by antagomir) such as miR-219 has been found to induce seizure like EEG in normal mice (Zheng et al., 2016). Therefore, decreased levels of miR-3120 and miR-214 in the cortex of iron-induced epileptic rats are indicative of the involvement of these miRNAs in iron-induced epileptogenesis. Silencing of miR-219 also resulted in increased NMDA receptor levels indicating involvement of glutamatergic mechanisms in epileptogenesis. In iron-induced epileptogenesis, upregulation of NMDA receptors, down-regulation of glutamate transporters and elevation of extracellular glutamate levels occur in iron-induced epileptogenesis (Engström et al., 2001; Mishra et al., 2013). Thus, downregulation of miR-3120 and miR-214 may be involved in the induction of glutamatergic mechanism.

The present data further showed that Pten gene expression was augmented in iron –induced epileptogenesis. Disruption of Pten gene is known to causes seizures (Backman et al., 2001). Thus the observation of increased Pten expression during epileptogenesis seems rather unexpected. Increased expression of Pten, however, could be a consequence of the depression of miR-3120 and miR-214 expressions, since these two miRNAs are known to target Pten gene (Scott et al., 2012).

Curcumin was reported to be an antiepileptic agent (Jyoti et al., 2009). In the present experiments also, curcumin was found to be an antiepileptic, as curcumin treatment of epileptic rats clearly suppressed the electrographic seizure activity. The present data further showed that curcumin stimulated the expression of miR-214 and miR-3120, and reduced the expression of Pten gene in iron-induced epileptogenesis showing that the antiepileptic action of curcumin is mediated by elevation of miR-3120, miR-214 and down-regulation of Pten gene. Curcumin's antiseizure action is also supported by the finding that it inhibits mTOR signalling (Meng et al., 2013). Thus curcumin's antiepileptic action involved elevation of miR-3120 and miR-214 and may involve inhibition of mTOR signalling.

In summary, the present results demonstrate that iron-induced epileptogenesis is mediated by suppression of miR-3120 and miR-214 expression, and curcumin's seizure-suppressive effect involves elevation of miR-3120 and miR-214 expressions by curcumin. Curcumin treatment also countered epileptogenesis-associated alteration in Pten gene expression.

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