Dopamine, Glutamate cross-talk, its impact on cellular homeostasis and seizure recurrence in epilepsy

Thesis submitted to the University of Hyderabad

For the degree of

Doctor of Philosophy

By

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DECLARATION

The research work presented in the thesis entitled "**Dopamine, Glutamate Cross-talk, its Impact on cellular homeostasis and seizure recurrence in Epilepsy**" has been carried out by me in the Department of Biotechnology and Bioinformatics, School of Life Sciences, University of Hyderabad, Hyderabad, Telangana under the guidance of Prof. P. Prakash Babu. I hereby declare that this work is original and has not been submitted in part or full for any other degree or diploma of any other University or Institution.

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CERTIFICATE

This is to certify that the thesis entitled **"Dopamine, Glutamate Cross-talk, its Impact on cellular homeostasis and seizure recurrence in Epilepsy,"** submitted by Mr. Madamanchi Kishore bearing registration number 15LTPH11 in partial fulfillment of the requirements for the award of Doctor of Philosophy in the Department of Biotechnology and Bioinformatics, School of Life Sciences is a bonafide work carried out by him under my supervision and guidance.

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Further, the student has the following publication (s) before submission of the thesis/monograph for adjudication and has produced evidence for the same in the form of the acceptance letter or the reprint in the relevant area of his research: (Note: at least one publication in referred journal is required the research)

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2. Madhamanchi, K., Madhamanchi, P., Jayalakshmi, S. et al. Dopamine and Glutamate Crosstalk Worsen the Seizure Outcome in TLE-HS Patients. Mol Neurobiol (2023). https://doi.org/10.1007/s12035-023-03361-4

3. Kishore M, Pradeep M, Narne P, Jayalakshmi S, Panigrahi M, Patil A, Babu PP. Regulation of Keap1-Nrf2 axis in temporal lobe epilepsy-hippocampal sclerosis patients may limit the seizure outcomes. Neurol Sci. 2023 Jul 11. doi: 10.1007/s10072-023-06936-0. Epub ahead of print. PMID: 37432566.

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- 2019 – SNCI, International conference on frontiers in neuroscience and neurochemistry: Dynamic challenges and approaches. Jamia Hamdard, Delhi.10th - 12th October 2019
- 2019 – SNCI, Significance of antioxidant response in FCD patients. JSS College of Pharmacy, Mysore. 14th – 16th March
- 27th Annual Conference of Indian Academy of Neurology (IANCON 2019) - PP86/449: www.ncbi.nlm.nih.gov/pmc/articles/PMC6849404/
- 2017 – SNCI, Banaras Hindu University, Varanasi, U.P, India, 20th – 22nd SEP 2017
- 2016 – SNCI, Centre for Cellular and Molecular Biology, Hyderabad 9th - 11th December.

Further, the student has passed the following courses towards fulfillment of coursework (recommended by doctoral committee) on the basis of the following courses passed during his Ph.D. degree was awarded.

Name	Course No. & Credits	Pass/Fail
1. Analytical techniques	BT 801: 4	Pass
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CHAPTER 1

Background:

Epilepsy is a chronic seizure disorder due to abnormal electrical activity in the brain. An imbalance between excitatory and inhibitory neuro signals creates an electrical storm in the brain, leading to excitotoxicity and seizures [1]. Generally, neurons communicate with other neurons, muscles, or glands and promote actions, thoughts, or feelings. Many neurons (500 times more than usual) fire faster and simultaneously during seizures per second. The rapid increase in electrical activity promotes disturbances in sensation, emotions, behavior, and involuntary moments and even cause loss of awareness [2].

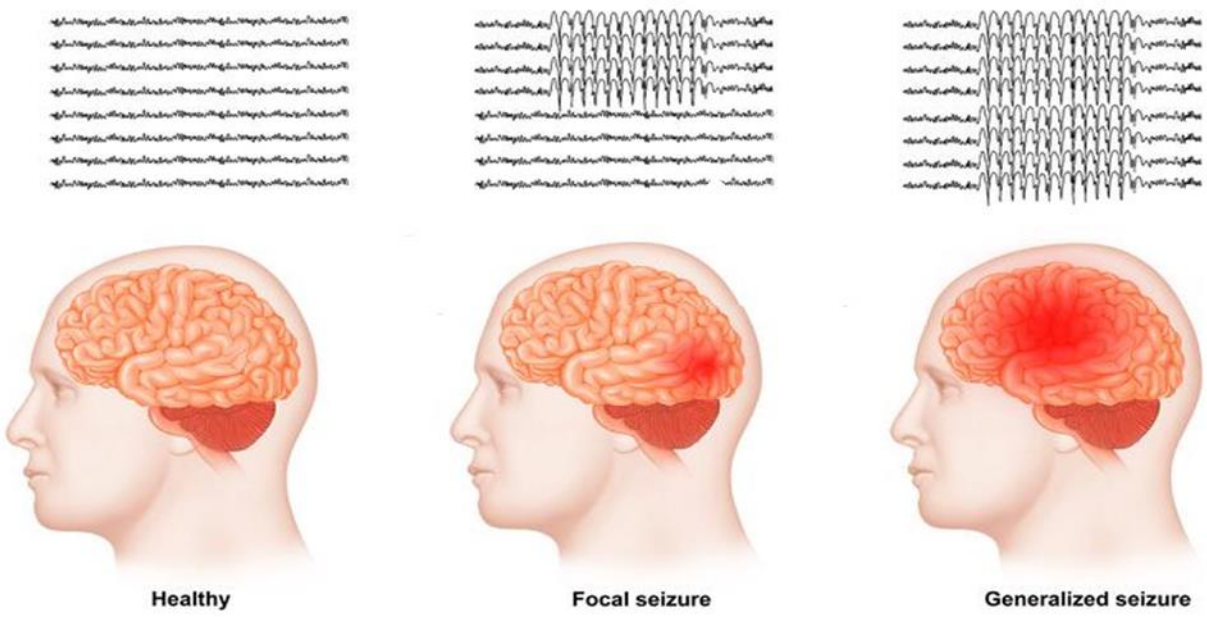

Figure 1. EEG recordings of the ordinary and epileptic brains.
Source: dreamstime.com Image218984562

Etiology of epilepsy:

Precipitation of seizures promotes epilepsy. The *incidence of two or more seizures at least 24 hours apart without an identifiable cause is considered epilepsy* [3]. Epilepsy can develop due to brain tumors, abnormal angiogenesis in the brain, stroke, traumatic brain injury, meningitis, HIV,

viral encephalitis, brain inflammation, alcoholism, dehydration, and sleep deprivation [4]. In addition, genetic factors and developmental abnormalities are also linked to epilepsy. However, half of epilepsy patients do not know the exact reasons. Any condition that disturbs the regular pattern of neuronal activity can lead to seizures. Seizures are of many types and they vary in symptoms and severity. If the seizures last more than 5 minutes, they are considered a medical emergency. Most of them last between 30 seconds to two minutes [5].

Epidemiology of Epilepsy:

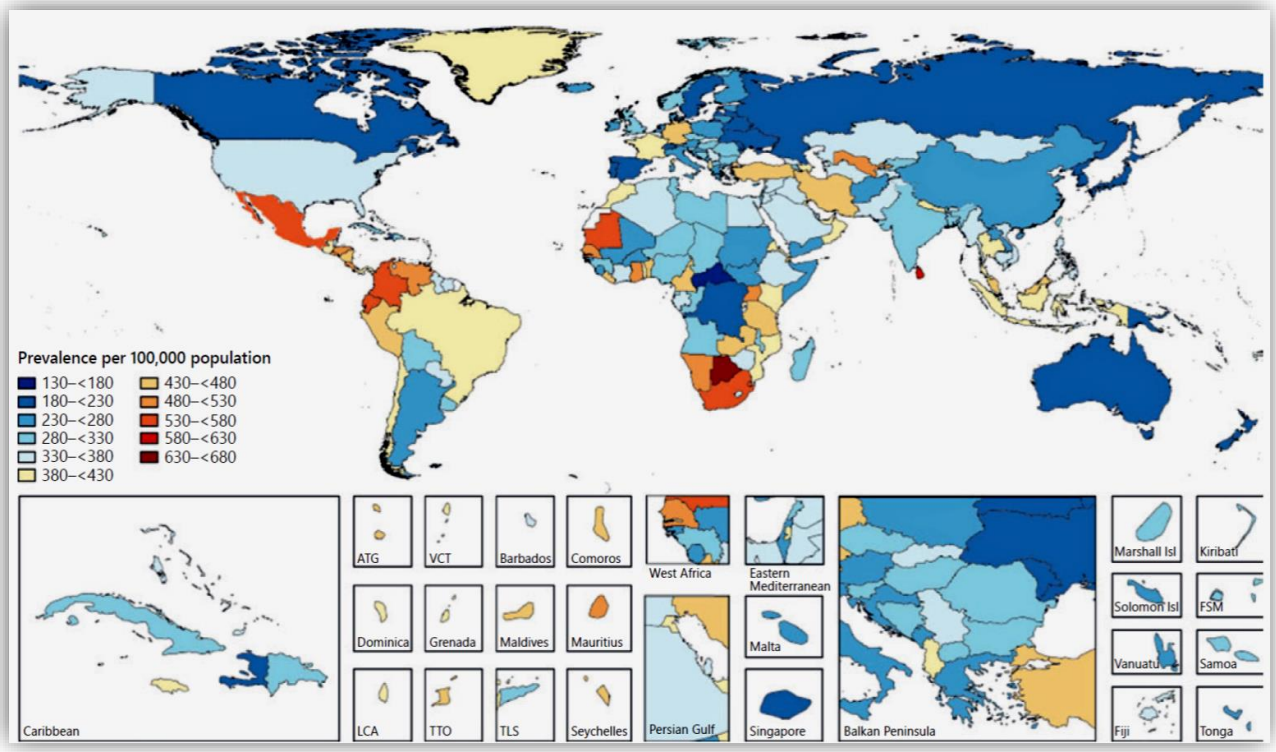

Figure: 2. Age-standardized prevalence of idiopathic epilepsy by country, 2016.[6, 7]
Source: Global Burden of Disease 2016.

Pathophysiology:

In general, electrical conductivity in the brain is non-synchronous, and we cannot observe the firing of many neurons simultaneously. But the synchronous firing phenomenon can be observed

during seizures. Neuronal activity in the brain is controlled by various intracellular and extracellular factors, like gene expression, the number and distribution of ion channels, receptors within a neuronal cell, the concentration of ions, synaptic plasticity, and stability or availability of neurotransmitters [8]. During epilepsy, excitatory neurosignals increase in affected region of patient's brain, known as "seizure focus." It may occur due to the upregulation of the excitatory signal or downregulation of the inhibitory signal [9]. This condition could occur due to developmental disorder/brain injury that disturbs the blood-brain barrier [10] and genetic factors [11].

Focal cortical dysplasia:

Type	Characteristic feature
FCDI	a. Defect in cortical lamination due to disturbances in radial migration and maturation of cortical neurons b. Disturbance in the 6-layered tangential composition of cortex comprising of Immature neurons. c. It comprises architectural abnormalities discussed in sections 'a' and 'b.'
FCDII	a. FCD with dysmorphic neuronal population b. FCD with balloon cells and dysmorphic neurons
FCDIII	a. Disturbances in cortical architecture of temporal lobe and hippocampal sclerosis b. Defects in the cortical layers adjacent to glial, glioneuronal tumors c. Defects in the cortical layers next to vascular malformation d. Defects in the cortical layer adjacent to areas affected by trauma, ischemic event, encephalitis occurs during childhood.

Table 1. Blumcke et al. proposed a new classification system to represent pathology in focal cortical dysplasia patients (2011).

It occurs due to disturbances in the brain cell organization or the development of abnormality related to brain structure. Neurons or brain cells are organized into layers of cells to form the cortex. During development, the disorganization of these cell layers disrupts brain function and increases seizure risk. Based on the type of cellular morphology and cortex affected, focal cortical dysplasia (FCD) is categorized into different types as below.

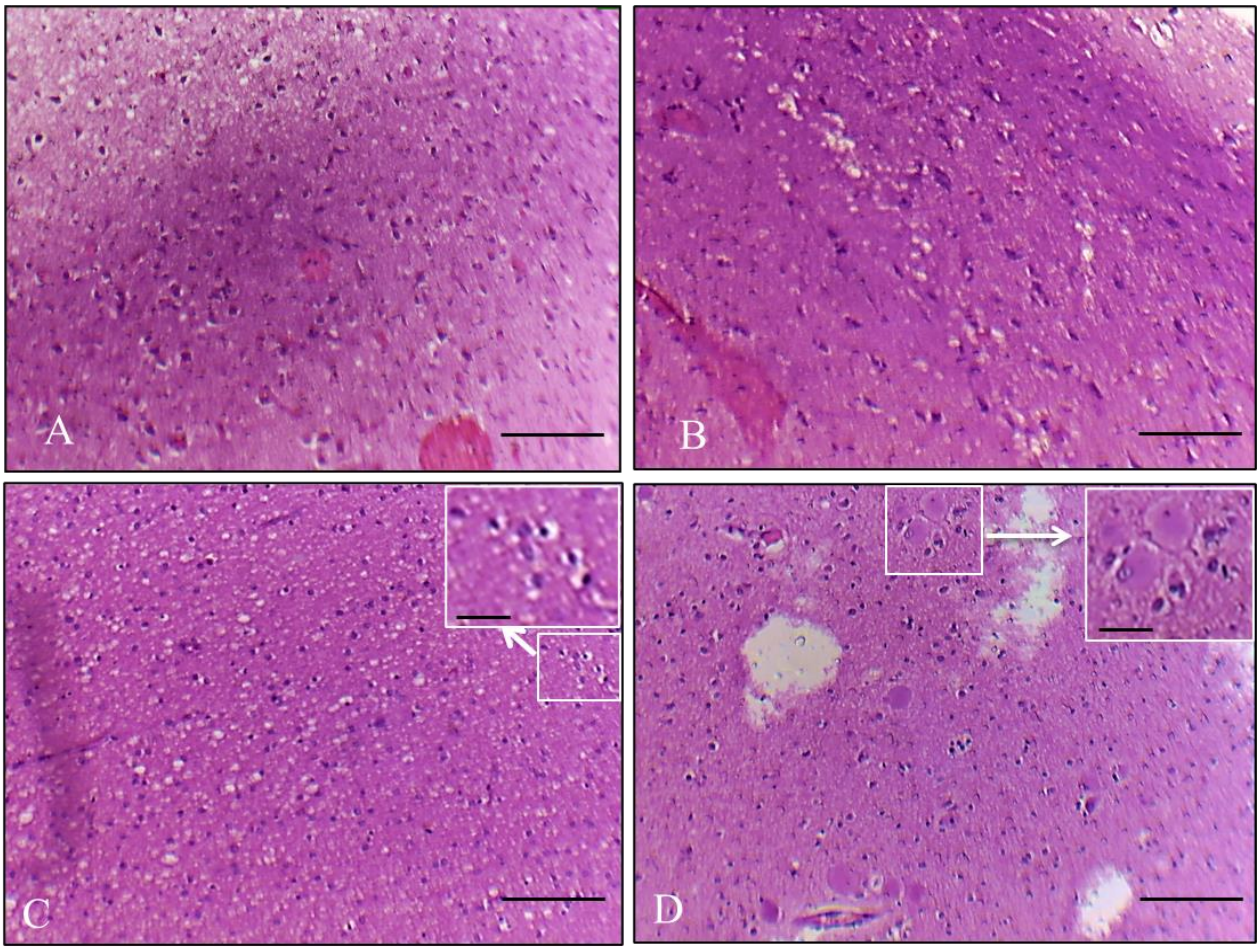

Figure 3. Histopathology of FCD types: Histopathology of FCD type I and type II: Image was taken using Olympus microscope: Hematoxylin & Eosin staining showed abnormal cortical architecture in FCDI (a) dyslamination and disorganization in the cortex, (b) columnar disorganization in the cortex, (c) FCD IIa showing dysmorphic neurons, (d) FCD IIb dysmorphic neurons and balloon cells having large cytoplasm with single or multi nuclei. The scale bar for figures 1a, 1b, 1c, and 1d is 100 µm, and for the high magnification inserts (1c, 1d in white box) 30 µm.

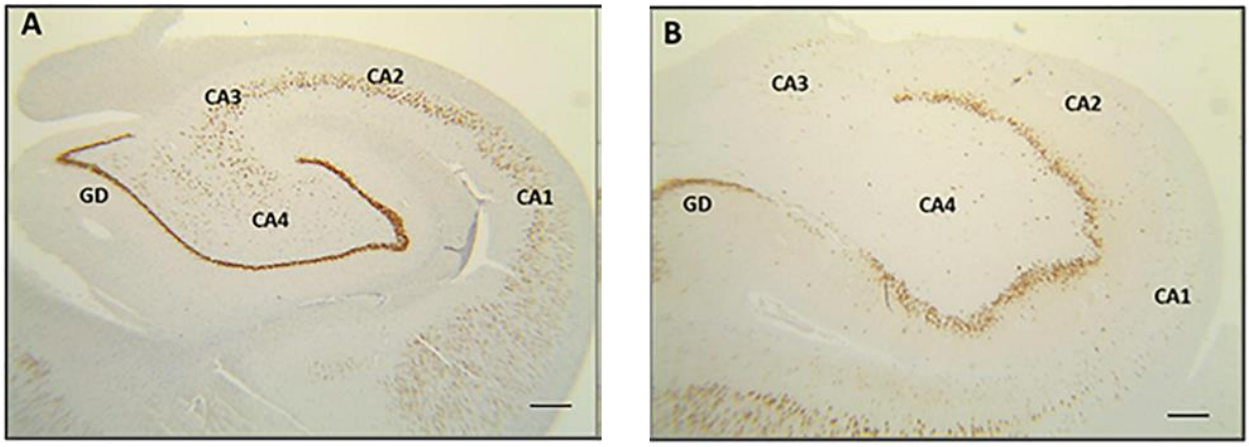

Figure 4. Normal hippocampal neuronal density obtained from a healthy individual (g) Reduced neuronal density in the hippocampal region of a patient affected with TLE-HS. (Sections were stained with NeuN, and objective magnification is 2x). Source: Yujiro (Toyoshima et.al. 2012 Feb; 62(1):53-60).

Diagnosis:

World health organization (WHO) suggest that epilepsy is not considered a single disease entity [12]. Epilepsy types have different underlying causes, so the prognoses of epilepsy type require various short-term and long-term supervision. Diagnosis of epilepsy syndrome requires information about onset age, seizure manifestation, precipitating factors, central nervous system signs, and severity.

Imaging and monitoring: To diagnose epilepsy and kind of seizures, a number of tests are used. Electroencephalogram (EEG) helps identify abnormal brain waves and the efficacy of antiepileptic drugs. In addition to EEG, video monitoring of patients is also used to rule out non-epileptic seizures. Magnetic resonance imaging (MRI), computed tomography (CT), is used to determine abnormalities in brain functions. Single-photon emission computed tomography (SPECT) and a modified SPECT called ictal SPECT were recommended to determine the exact location of seizure foci in the brain by using a tracer to understand the blood flow during seizures.

In addition, positron emission tomography (PET) was used to determine the brain metabolism [13].

Medical history: Any seizure incidents in the past, duration, and symptoms details can help determine the type of seizures and form of epilepsy; it is one of the best methods to understand the severity of illness. In addition, blood tests; to understand any metabolic or genetic disorders that can trigger seizures. Behavior, intellectual ability, and motor ability are also used to know how epilepsy affects patients.

Treatment: Most of the patients respond well to a medication when used in a prescribed manner. Nearly 80% of patients were free from generalized seizures after medication. Sodium valproate, lamotrigine, Levetiracetam, Topiramate, and Zonisamide [14] treat epilepsy. Myoclonic jerks were observed occasionally in some patients, and few exhibited pseudo resistance to treatment due to compliance or lifestyle [15], and one in six patients faced refractory epilepsy [16]. These three are associated with unfavorable responses to medication [17]. A careful diet, medicine, devices, and surgery is critical to controlling epilepsy.

The risk associated with epilepsy:

People affected with epilepsy lead an everyday life. Still, two types of life-threatening conditions were noticed, according to the National Institute of Neurological Disorders and Stroke (NINDS)

Status epilepticus: A potentially lethal condition. It can be explosive or nonconvulsive, sustained episodes of agitation, confusion, loss of consciousness, and even lead to coma. Seizures last for more than 5 minutes.

Sudden unexpected death in epilepsy (SUDEP): It occurs at any age. Some studies suggest heart and respiratory system failures due to underlying genetic abnormalities. SUDEP can be reduced by following the prescribed medication regularly.

The rationale of the present study:

The existence of an imbalance between excitation and inhibition is the symbol of epileptogenesis. Even though many studies have reported the relationship between disturbances in synaptic transmission and epileptogenesis, the exact molecular mechanism underlying the genesis of seizures in epilepsy patients, particularly with drug-resistant epilepsy (DRE), is not yet fully understood. According to the reports, 25-30 percent of the patients who underwent treatment with anti-epileptic drugs (AED) did not have complete seizure control [18]. Patients who are not showing better seizure control can be identified when they fail to respond after administering two AED [19]. These patients were again subjected to polytherapy with AEDs. If patients fail to respond to AEDs, surgical treatment is recommended to remove the seizure foci [20]. Even though patients underwent brain surgery, more than 30 percent of patients face seizure recurrence for various reasons [21]. Patients with DRE lead discomfort in social life and even increase the risk of mortality. In order to find the reasons behind the recurrence, we need to understand the seizure microenvironment in those patients. Lack of inhibitory/GABAnergic neurosignals causes a sudden surge in brain electrical activity and induces seizures [22]. Apart from GABAnergic inhibition, postsynaptic neurons regulate their excitation through the feedback inhibition mechanism by Glutamatergic and Dopaminergic receptors to prevent persistent activation and excitotoxicity [23]. Glutamate, during excitation, activates ion channels to allow Na^+ and Ca^{2+} into post-synaptic neurons and causes excitation, whereas Ca^{2+} entered into cells shows feedback negative regulation to prevent over activation of glutamate coupled ion channels. Whereas,

Dopamine D receptor can counteract it by persistent phosphorylation dependent inhibition of protein phosphatase 1γ (PP1γ). At the same time, if Dopamine (DA) levels were increased persistently, it would lead to DA receptor hetero dimer formation. All these events elevate excitotoxicity and calcium ions in post-synaptic neurons. This further leads to the formation of excess free radicals [24], and disturbs cellular homeostasis by inducing endoplasmic stress response signaling[25] or inducing cell death[26, 27].

Since epileptic patients were experiencing an imbalance between excitatory and inhibitory neurosignals, it could create excitotoxicity and disturb cellular homeostasis, we believe that understanding the molecular mechanisms of dopamine and glutamate signaling can provide information regarding the factors responsible for seizure recurrence, which helps to understand the reasons for post-surgical seizure recurrence and the administration of AED for complete seizure control after the surgery. In the current study, we have used brain tissues resected during epilepsy surgery as an ideal model system to understand the process of epileptogenesis.

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To achieve our hypothesis, we have proposed the following three objectives to study in both (FCD Type I, II, and Type III (Temporal lobe epilepsy-Hippocampal sclerosis) patient samples.

a. **Categorization of patients based on post-surgical follow-up and Thioflavin T assay data in both focal cortical dysplasia type I, II, and FCD type III (TLE-HS) patients.**
b. **To Study the impact of Dopamine and glutamate downstream signaling mechanisms.**
c. **To study the impact of excitotoxicity on dopamine receptor hetero dimer.**

The postoperative outcome was classified according to the International League Against Epilepsy's (ILAE) proposed classification [28].

Outcome classification	Definition
1	Entirely seizure-free; no auras
2	Auras only and no or <1 seizure per year
3	1 to 3 seizures per year, with or without auras
4	50% reduction of baseline seizure day, 4 seizure days per year to s; ± auras
5	<50% reduction compared to previous seizure frequency or increase up to 100% of baseline seizure days, with or without auras
6	No recovery or >100% increase compared to earlier, and ± auras

Table 2. ILAE proposed a new classification of seizure outcomes concerning epilepsy.
Source: Wieser HG, et al. Epilepsia, 42(2):282–286, 2001.

During our study, we collected epileptic brain samples from Krishna Institute of Medical Science (KIMS), Secunderabad, TS, INDIA. Based on the prior clinical observation, we categorized them as Focal Cortical dysplasia (n=26) (1a, 2a, 2b) and Hippocampal sclerosis (n=26) samples. Later with the help of post-surgical clinical observation, these patients were categorized according to ILAE seizure outcome classes. The outcome was measured regularly after the surgery.

Thioflavin T assay: Based on earlier studies, we have performed Thioflavin T assay to quantify the protein aggregates which were formed as a consequence of disturbance in endoplasmic reticulum homeostasis. We used both clinical data and ThT assay data to categorize the patient samples and used to elucidate further differences in molecular signaling mechanisms involved in the excitotoxicity of epilepsy patients.

CHAPTER 1A

Introduction:

Nearly 1-2% of the world's population suffers from epilepsy [1]. The imbalance between excitatory (glutamatergic) and inhibitory (GABAergic) neuro signals precipitates seizures [2, 3]. Chronic hyperexcitability of neuronal population (seizures) in a specific brain region results in epilepsy [4]. Ionotropic glutamate receptors like AMPAR (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor), and NMDAR (N-methyl-D-aspartate receptors) are commonly provoked during seizures [5, 6]. AMPA receptors mediate fast excitation by allowing Na^+ into the cell and inducing excitatory postsynaptic potential (EPSP) [7]. NMDAR needs the binding of agonist glycine/D-serine and AMPAR-mediated postsynaptic excitation to expel voltage-sensitive extracellular Mg^{2+} block to become active. Since NMDAR activation requires pre and postsynaptic excitation, NMDARs are called coincident receptors [8, 9]. NMDA receptors, unlike AMPARs, allow Ca^{2+} into the postsynaptic neuron [10]. Calcium ions can regulate the number of Ca^{2+}-dependent kinases and phosphatases, protein trafficking, synaptic plasticity, and long-term potentiation, and even promotes excitotoxicity and apoptosis [11–14].

Increased Ca^{2+} levels in postsynaptic neurons through NMDAR activates calpain-mediated p35 cleavage into p25 and p10 in an extracellular calcium-dependent manner [15]. It activates Cdk5, which induces the NR2B internalization [16] through pDARPP32T75 by activating PP1γ [17]. PP1γ dephosphorylates the AMPA and NMDA receptors by interacting with scaffolding proteins spinophilin and neurabin [18]. Both have F actin-binding domains with phosphorylation sites for PKA, PKC, and pCAMKIIα, which help reorganize or stabilize the actin cytoskeleton in dendritic spines [19, 20]. Spinophilin and neurabin have PDZ domains that interact with C terminal domains of AMPA/NMDA receptors [21, 22]. The calcium-dependent protein kinase pCAMKIIα modulates NMDAR current by phosphorylating at S1303 [23]. NR2B phosphorylation by PKA at

S1166 regulates dendritic spines' NMDAR function and Ca^{2+} signaling [24]. Fyn phosphorylates Y1472 at the C-terminal YEKL domain and inhibits clathrin-mediated endocytosis by interacting with postsynaptic density 95 (PSD95) at synapses and promotes LTP [25].

The pNR2B/NMDA receptor-mediated calcium inflow controls the PP1γ activity, which seems like a feedback inhibition/regulation through p25 at dendritic spines [25]. DARPP32 is a signal integration point of dopaminergic and glutamatergic signaling [26]. Dopamine inhibits PP1γ by PKA-dependent pDARPP32T34 [27, 28]. It indicates that the deregulation of the dopaminergic system contributes to neuronal synaptic activity during seizures [3, 29]. DA acts on postsynaptic neurons through two classes of DA receptors, DRD1 and DRD2, belonging to the GPCR family [30]. Despite both receptors having the same ligand, they act against each other depending on the availability of DA and regulate the activation or deactivation of kinases and phosphatases involved in AMPA/NMDA receptor synaptic localization [31]. Phasic release of DA activates DRD1, whereas tonic release of DA activates DRD2 [32–34]. DRD1 promotes the cAMP-PKAc-pDARPP32T34 axis to inhibit PP1γ and promote LTP through AMPA/NMDA receptor synaptic localization [31]. In contrast, DRD2 inhibits cAMP, thereby downregulating PKA-dependent phosphorylation of DARPP32T34 and disinhibiting PP1γ [35]. Even though D1 and D2 are antagonistic, the synergistic function of the D1-D2 heterodimer was detected in the striatum of rat brains [36]. Nucleus accumbens, caudate-putamen, globus pallidus D1, and D2 receptors form a heterodimer in medium spiny neurons [37]. The D1-D2 receptor heterodimer regulates the endoplasmic reticulum calcium reserves by Gαq, phospholipase C (PLC), and activates Ca2+/calmodulin dependent protein kinase IIα (CAMK IIα) [38, 39]. In addition, NMDARs also mobilize Ca2+, which leads to calcium overload and excitotoxic cell death [40]. The current study aims to find the importance of dopamine-DRD1 signaling on its target protein PP1γ, the AMPA

and NMDA (GluR1, NR2B) receptor subunits, and how it relates to the maintenance of epileptic seizures. We have explored the negative feedback regulatory mechanism of NMDAR-Ca^{2+} dependent activation of the p25-Cdk5-PP1γ axis on AMPA, NMDA receptor subunit phosphorylation, which directly correlates with synaptic strength. Since strong synapse plays a critical role, particularly during seizures and excitotoxicity, we believe our study with human samples can provide better insights into understanding the underlying cause of epilepsy recurrence in FCD patients.

Materials and methods:

Tissue collection and Ethical guidelines

Epileptic tissue samples were collected from Krishna Institute of Medical Sciences (KIMS), hospital, Secunderabad, India, immediately after the surgery. The studies were approved by KFRC (KIMS Foundation and Research Center) and KIMS ethical committee, Secunderabad, India, supported by the patient or close relative's consent. Institutional Ethical Committee (IEC) guidelines were followed, and all the study sets were anonymized. Based on follow-up clinical data after surgery, the patient tissue samples (n=26) were classified according to the clinical outcome (irrespective of FCD types), as suggested by the international league against epilepsy in Table 1. Control brain tissues (n=3), table 2, were collected from the National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore, India. Since control brain tissues were unsuitable for immunofluorescence study, we used them for western blot assays.

Thioflavin T fluorescence assay:

Thioflavin T (T3516, Sigma-Aldrich), a Benzothiazole salt, is widely used for protein aggregate quantification [24]. Thioflavin T binds to unfolded protein (UFP) aggregates, like β-sheets, and gives

enhanced fluorescence. 100 µg of protein was added to Milli Q water and made up the volume to 50 µl in a 96 well plate. 2 µl of freshly prepared 0.5 mM Thioflavin T stock solution was added to make the final working concentration of 20 µM. After 30 minutes of incubation, the fluorescence intensity of each sample was measured using a Tecan spectrofluorometer infinite® 200 PRO. The excitation and emission wavelengths of the microplate reader were set at 440 nm and 490 nm, respectively.

Preparation of tissue lysate and western blot assay

Whole-cell protein lysates were prepared from control (n=3) and FCD (n=26) tissue samples using a radioimmunoprecipitation assay buffer. The quantity of proteins estimated in each sample using the Bradford method (Sigma-Aldrich B6916), 6x SDS sample buffer (0.125 M Tris, pH 6.8, 4% SDS, 20% Glycerol, 0.02 M DTT, 0.02% bromophenol blue) was prepared and added to 50µg of protein. Then proteins were loaded into 10% SDS-PAGE and transferred onto nitrocellulose membrane (Bio-Rad, USA) overnight at 4^{o}C in Towbin buffer (0.048 M Tris, 0.039 M Glycine, 20% Methanol, 0.00375% SDS) at 25 V by wet transfer method. We used 5% non-fat skimmed milk powder (Sigma, cat no: M7409) in Tris buffer saline tween-20 (TBST) for the blocking process. Later the blots were incubated with primary antibody at 4^{o}C. The list of antibodies used is as follows p25/35(Cell signaling technology [CST]-2680), Spinophilin (CST-14136), Fyn (CST-4023), p-NR2B at Y1472 (Sigma-M2442), PP1γ1 (Sigma-P7609), Tyrosine hydroxylase (Novus Biologicals, 300-109), Cdk5 (Sc-6247), p-GluR1 at S845 (CST-8084), PKAc at T197 (CST- 4781), GluR1 (CST-13185S), NR2B (CST-4212), DRD1 (Novus Biologicals, NBP2-16213), DRD2 (Sc-5303), DARPP32 (Sc-135877), pDARPP32T34 (Sigma-SAB504378), pDARPP32T75 (CST-2301), CAMKIIα (CST-50049), p-CAMKIIαT286 (Sc-32289), PARP1 (CST-9542), cleaved caspase 9 (CST-9505S), cleaved caspase7 (CST-8438). Then membrane was washed with TBS

followed by TBST and incubated with secondary antibodies anti-rabbit IgG (CST-7074P2), and anti-mouse IgG (CST-G21040) conjugated to HRP with the dilution of 1:15000 for 1 hour at room temperature. Using a chemiluminescent reagent (Clarity^{TM} Western ECL substrate 1705060) and Bio-Rad (Bio-Rad, USA), the blots were developed using a molecular imager (Bio-Rad).

Double Immunofluorescence Assay

Samples were frozen and kept at -80^{o}C immediately after surgery and then fixed in formalin solution for 48 hours, then embedded in paraffin blocks. 5 µm sections were made from paraffin-fixed blocks of resected brain tissues. Sections were subjected to deparaffinization with xylene, hydrated with 100% and 95% ethanol, then boiled for 10 min in citrate buffer (10mM) pH 6.0. The slides were cooled and blocked with blocking buffer (1%BSA, 5% normal goat serum (CST-5425) dissolved in 1xPBS with 0.3% Triton X-100) for 1 hour at room temperature in a humid chamber. Sections were incubated with a primary antibody cocktail overnight (16 h) at 4^{o}C to study the neuronal expression of p25 (NBP2-34031), D1DR (NBP2-16213), D2DR (sc-5303), pCAMKIIα (Sc-32289), Cdk5 (Sc-6247), Dopamine (ab6427, from Abcam), anti-mouse NeuN (Abcam-177487), anti-rabbit NeuN (CST-24307). The sections were washed with PBS buffer for 10 min and incubated with fluorescent conjugated secondary antibodies, anti-rabbit IgG (4412S Alexa fluor® 488) 1:1000, and anti-mouse IgG (Alexa fluor® 555) 1:1000 from Cell Signaling Technology for 1 hour in dark condition at room temperature. Then sections were washed with 1xPBS buffer and mounted with Prolong® Gold anti-fade reagent with DAPI (CST-8961S). The images were taken using Carl Zeiss LSM 710 using ZEN Black 2.3 SP1 software, and the fluorescent intensity of each protein was quantified by densitometric analysis using Image J software (n= 4)

FRET analysis

Fluorescence resonance energy transfer (FRET) is a method used to estimate the interaction between two proteins based on energy transmission from donor to acceptor molecule with the help of a confocal microscope. In the current study, fluorescent conjugated secondary antibodies such as anti-rabbit IgG (4412S Alexa fluor® 488) as 'donor' and anti-mouse IgG (4409S Alexa fluor® 555) as 'acceptor' brought from Cell Signaling Technology were probed against D1 and D2 primary antibodies. In FCD sections, FRET analysis was carried out (n= 4) by measuring the distance (*r*) between acceptor and donor probes and calculating the energy transfer efficiency (*E*) in the region of interest by Carl Zeiss LSM 710 confocal microscope using ZEN black 2.3 SP1 software.

$$\text{Efficiency } (E) = 1\text{-Ida}/ ((\text{Ida} + \text{pFRET} \times \{((\Psi dd) / (\Psi aa) \times (Qd / Qa))\}$$

Ida- Donor image in the presence of acceptor, pFRET- processed FRET (algorithm for the removal of spectral bleed-through contamination between donor and acceptor), Ψaa, Ψdd-Collection of efficiency in acceptor and donor channel, Qa, Qd – Quantum efficiencies of acceptor and donor, E- the rate of transfer efficiency.

$R= R_0 [1/E - 1]1/6$ (Forster's distance R_0) is the distance at which FRET efficiency was 50% between donor and acceptor, and *r* represents the distance between donor and acceptor in the region of interest.

Data analysis:

Statistical analysis among groups performed using one-way ANOVA by the Newman-Keuls method for posthoc analysis. Data reported were as Mean and ± SEM of experiments. P-value <0.05

was considered statistically significant, determined by Sigma Plot 2000 software for the Windows version.

Results:

Accumulation of unfolded proteins:

The ER is critical for appropriately folding the proteins or enzymes and regulating many signaling pathways [26]. Thioflavin T assay (ThT) was performed to determine the fluorescent intensity of accumulated unfolded protein aggregates in (n=26) patient samples (Fig. 1). ThT assay showed a significant difference in fluorescence intensity of UFP aggregates in class 3 patients (P<0.001, 367.15±4.85) compared to class 2 (P<0.31, 280.02±3.36) and class 1 (P<0.004, 219.63±3.64).

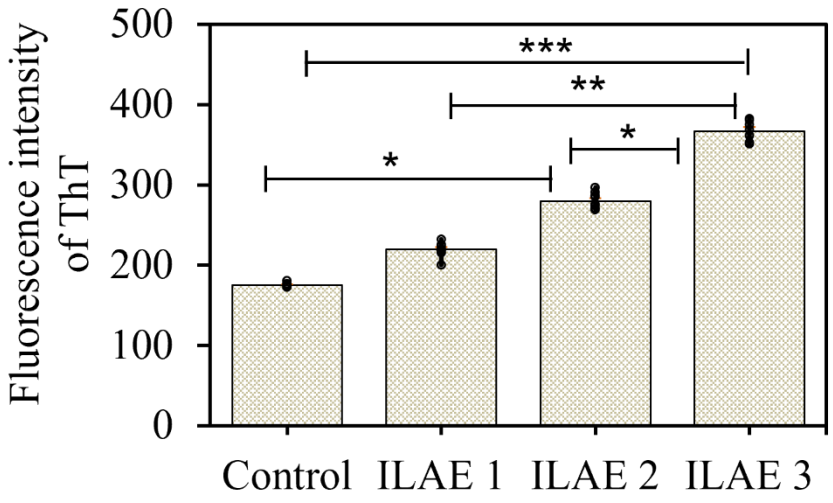

Fig. 1 Estimation of protein aggregates by ThT assay: ThT assay was performed to understand the unfolded protein aggregates in FCD patient samples (n=26). The fluorescence intensity of ThT bound to protein aggregates was measured and shown as mean ± SEM. Data represent 3 independent experiments. Figure 2 Thioflavin T assay for focal cortical dysplasia (FCD) samples was performed based on clinical parameters, class 3 ($p<0.001$) and class 2 ($p<0.03$) showed a significant increase compared to ILAE class 1.

Selection of samples for the current study:

The table. 1 represents the list of patients regularly visiting the hospital after surgery. The postsurgical assessment data was collected regularly to understand how surgery can improve the patient's seizure freeness. We use these details to classify the clinical outcome of patients as suggested by the International League against epilepsy (ILAE). We categorized them as completely seizure-free (class 1), patients experiencing focal aware seizure/auras (class 2), and patients experiencing less frequent recurrent seizures (class 3). Table. 2 represents autopsy control samples with no chronic neurological complications, collected from NIMHANS's human brain tissue repository (HBTR) facility in Bangalore, India. The tissue samples were grouped into 3 for each category based on clinical outcomes and the thioflavin T assay performed in our earlier studies [41], used for the current study.

pGluR1 and pNR2B were increased in ILAE class 2 and class 3 patients

Patient samples were subjected to western blot analyses to check the AMPA and NMDA receptor subunits critical for learning, memory formation, and excitotoxicity-induced damage. We have observed a significantly higher expression of GluR1 in ILAE classes 1, 2, and 3 (fig.2b) ($p<0.001$; 8.18±1.42, 8.67±1.34, 9.29±1.09) 1 (n=3), 2 (n=3), and 3 (n=3) respectively compared to controls (n=3) (1.14±0.02). In contrast, pGluR1 (fig.2c) was abundant in class 3 ($p<0.001$; 4.96±0.26), class 2 ($p<0.001$; 3.55±0.32) compared to class 1 ($p<0.01$; 2.17±0.66) and control (0.58±0.09). We found significantly less pGluR1 in ILAE class 1 ($p<0.03$) than in classes 2 and 3. NR2B (fig.2d) expression is significant in class 3 ($p<0.001$; 6.87±0.59) than classes 1 and 2 ($p<0.003$; 4.12±0.30, $p<0.009$; 5.18±0.79). The pNR2B (fig.2e) increased in classes 3 ($p<0.001$; 16.09±1.05) and class 2 ($p<0.01$; 9.16±3.52) compared to class 1 (4.15±0.81) and controls (3.865±1.89).

S. No	Gender	FCD type	Age of onset	Surgery age	Epilepsy duration	Location of FCD	ILAE Outcome Classification
1	Male	FCDI	12 Years	40 Years	28 Years	Frontal	Class 2
2	Male	FCDI	11 Years	13 Years	2 Years	Parietal	Class 1
3	Male	FCDI	3 Years	15 Years	12 Years	Parietal	Class 2
4	Female	FCDIIb	6 Months	4.6 Years	4 Years	Frontal	Class 1
5	Female	FCDI	16 Years	27 Years	11 Years	Frontal	Class 1
6	Male	FCDIIa	10 Years	13 Years	3 Years	Frontal	Class 3
7	Female	FCDIIb	14 Months	12 Years	10 Y 10 M	Frontal	Class 1
8	Male	FCDI	8 Years	12 Years	4 Years	Occipital	Class 2
9	Female	FCDI	6 Years	14 Years	8 Years	Frontal	Class 2
10	Female	FCDI	5 Years	18 Years	13 Years	Frontal	Class 3
11	Male	FCDI	9.5 Years	10 Years	7Months	Frontal	Class 3
12	Female	FCDIIa	2 Years	14 Years	12 Years	Frontal	Class 2
13	Male	FCDIIb	5 Years	8 Years	3 Years	Frontal	Class 1
14	Male	FCDI	34 Years	35 Years	1 Year	Frontal	Class 2
15	Male	FCDIIa	2 Years	25 Years	23 Years	Frontal	Class 1
16	Male	FCDIIb	11 Years	21 Years	10 Years	Frontal	Class 1
17	Male	FCDI	6 Years	13 Years	7 Years	Parietal	Class 3
18	Female	FCDIIb	3 Years	3 Years	2 Years	Frontal	Class 1
19	Male	FCDIIa	11 Years	14 Years	3 Years	Frontal	Class 3
20	Male	FCDIIb	15 Years	25 Years	10 Years	Frontal	Class 2
21	Male	FCDI	1 Year	11 Years	10 Years	Occipital	Class 3
22	Male	FCDI	4 Years	10 Years	6 Years	Frontal	Class 3
23	Female	FCDIIb	2 Years	4 Years	2 Years	Frontal	Class 1
24	Female	FCDIIb	2 Years	15 Years	13 Years	Frontal	Class 1
25	Male	FCDI	12 Years	25 Years	13 Years	Frontal	Class 2
26	Male	FCDI	13 Years	27 Years	14 Years	Frontal	Class 2

Table 1. Clinical data of the study population representing seizure-free outcomes according to ILAE classification. Class 1 = 38.46% (complete seizure-free, no auras); Class 2 = 34.61% (only auras and < 1 seizures per year); and Class 3 = 26.92% (<3 seizures per year and auras)

pGluR1 and pNR2B can increase Calcium-dependent p25-Cdk5 signaling

The upregulation of pGluR1 and pNR2B increase the Ca^{2+}ion movement into cells and activate proteolytic enzyme calpain, which cleaves the p35 into p25 (fig.2f). We have found increased p25 protein levels in class 3 ($p<0.001$; 5.47±0.61) and class 2 ($p<0.009$; 4.99±1.02) compared to controls (1.03±0.28) and class 1 ($p<0.04$; 3.47±0.99). Expression of Cdk5 (fig.2g) increased in class 3 ($p<0.001$; 2.86±0.36), class 2 ($p<0.001$; 2.67±0.09), and class 1 ($p<0.01$; 1.87±0.10) compared to controls. Cdk5 activates DARPP32 by phosphorylating at T75 residue (fig.2h) increased in class 1 ($p<0.3$; 3.02±0.49), class 3 ($p<0.04$; 4.03±1.07) compared to class 2 ($p<0.031.49±0.23$), and controls (1.14±0.14). Calcium ion-dependent kinase, known as calcium/calmodulin-dependent protein kinase IIα (CAMKIIα) (fig.2i), was increased in ILAE classes 1, 2, and 3 ($p<0.001$; 2.89±0.10, 2.59±0.17, 2.53±0.24) respectively compared to controls (1.330±0.190) and have no significant difference among groups. pCAMKIIα (fig.2j) expression increased in class 3 ($p<0.01$; 1.95±0.24) compared to class 2 ($p<0.05$; 1.58±0.10), class 1 (1.24±0.14) and controls (0.99±0.03). Class 2 also showed a significant increase in pCAMKII compared to class 1 and control.

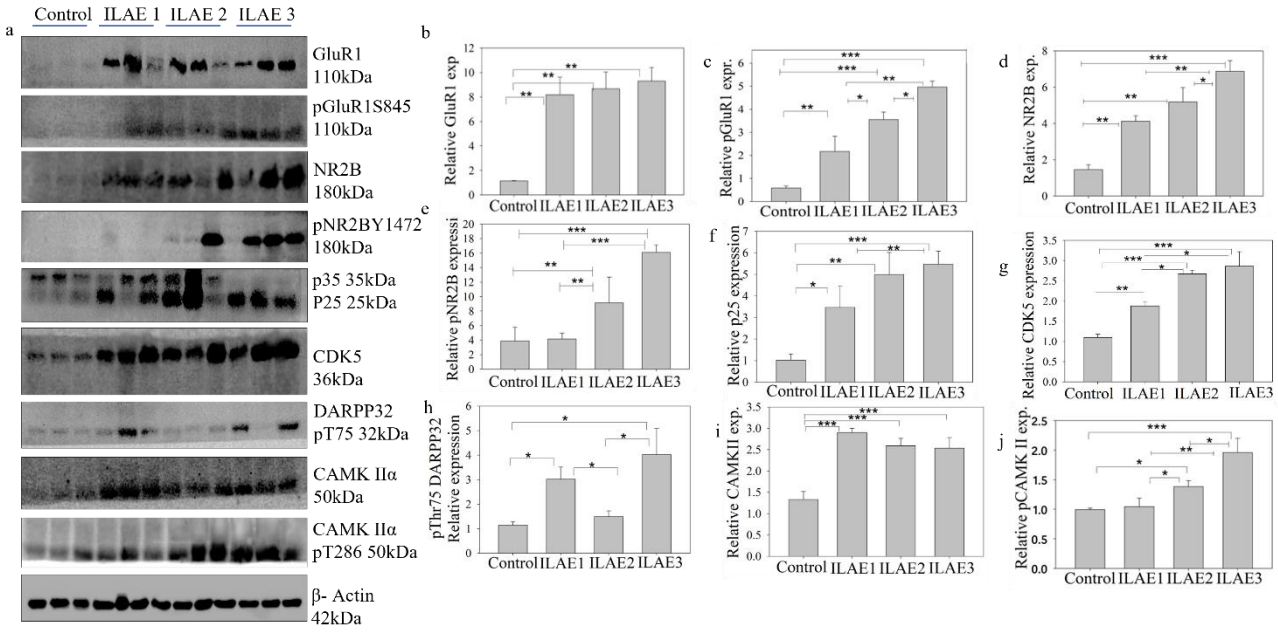

Fig.2 (a) Western blots study to check the expression of AMPA and NMDA receptor subunits and CDK5 expression in FCD patients. b-j are the corresponding bar graph representing the quantification of protein expression, GluR1 (b), pGluR1 (c), NR2B (d), pNR2B (e), p25 (f), Cdk5 (g), pDARPP32T75 (h), CAMKIIα (i), pCAMKIIαT286 (j) in both control and FCD samples. The lower panel represents an expression of beta-actin as a loading control. Data representing mean ± SEM. ***p< 0.001, **p< 0.01, *p< 0.05.

S.No.	Gender	Age	Cause Of Death	Post-Mortem Interval	Significant Neuro pathology	Disease	HIV/ HBsAg	Brain Region
1	Male	19 Years	drowning	30 Hours	Cerebral injury	No	Negative	Frontal
2	Male	25 Years	homicide	25 Hours	Absent	No	Negative	Parietal
3	Female	35 Years	Heart attack	35 Hours	Absent	Diabetes	Negative	occipital

Table 2. Control case information: The controls are obtained from NIMHANS, Bangalore. Storage and sample processing were performed by NIMHANS, the human brain tissue repository (HBTR), according to their standardized protocol to use the brain tissue for the research purpose.

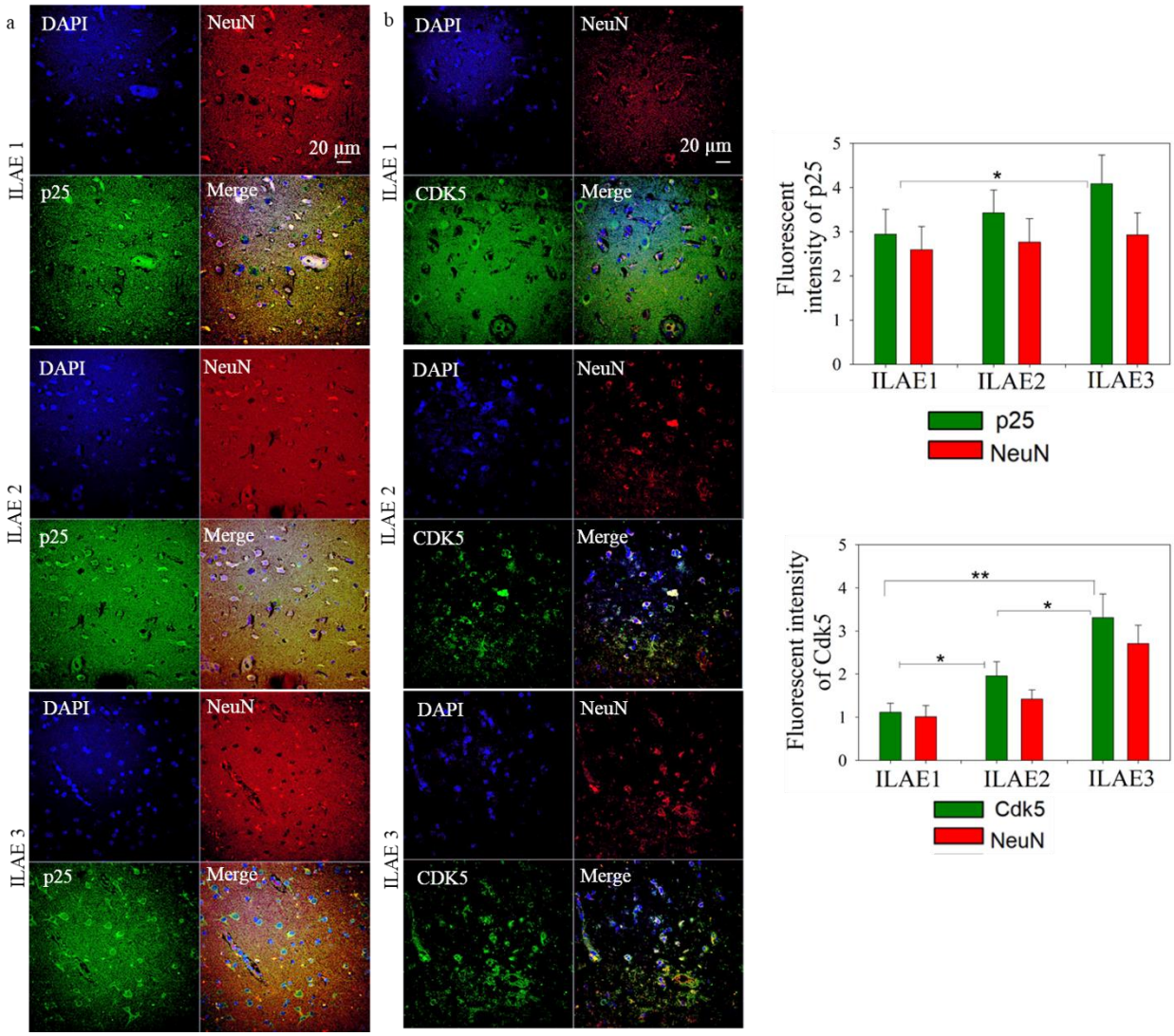

Fig: 3(a) Double immunofluorescence assays for p25 (green)colocalized with NeuN (red)(3b) Cdk5 (green) colocalized with NeuN (red) in ILAE outcome classes 1, 2, and 3. Scale bar = 20 µm

Double immunofluorescence assay for p25, Cdk5, pCAMK IIα

The p25 (fig.3a) showed a significant difference between class 3 (4.08±0.65) and class 1 (2.94±0.56), Cdk5 (fig.3b) increased in class 3 ($p<0.006$; 3.30±0.55) compared to class 1 (1.11±0.21), and class 2 ($p<0.03$; 1.96±0.33), ILAE 2 also showed a significant increase in Cdk5 than class 1. The pCAMKIIα (fig.4a) expression increased significantly in class 3 ($p<0.006$;

2.32±0.27) and class 2 (1.97±0.23) compared to class 1 (1.50±0.08). These results were correlated with western blot analysis.

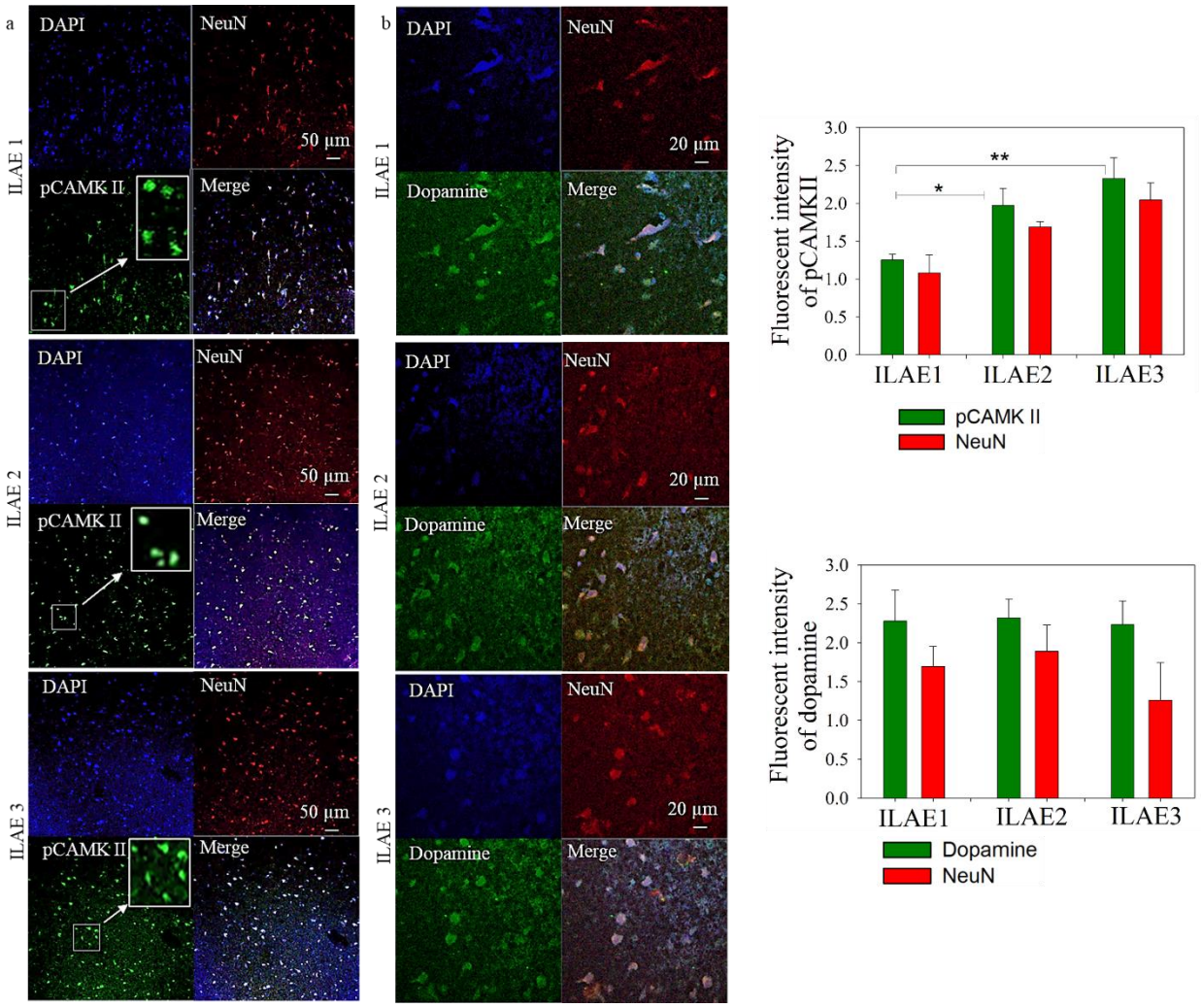

Fig. 4(a) Double immunofluorescence assay for pCAMKIIα (green) Figure (4b) Dopamine (green), and colocalized with NeuN (red). Scale bar for figure 4(a) = 50 µm, and for 4(b) = 20 µm

Dopamine synthesis increased in the epilepsy patients

Dopamine levels in FCD patient samples were analyzed by double immunofluorescence assay (fig.4b). The patients showed no difference in dopamine expression. Later we checked Tyrosine hydroxylase (fig.5d), which is required for dopamine synthesis. Tyrosine hydroxylase (TH) protein

signal intensity did not vary among the ILAE classes 1, 2, and 3 of FCD ($p<0.001$; 2.141±0.084, 1.96±0.10, 2.2±0.07), respectively, but significantly increased when compared to the controls (1.06±0.07).

DRD1 and DRD2 expression varied among ILAE classes

Since dopamine increased in FCD patient samples, we further studied the dopamine receptors expression using western blot analysis (fig 5a). DRD1 (fig.5b) showed a significant increase in class 3 ($p<0.001$; 6.48±0.91) and class 2 ($p<0.009$; 5.48±0.57) compared to controls (2.72±0.68). Class 1 (3.80±0.73) showed limited DRD1 expression than class 3 ($p<0.009$) and class 2 ($p<0.03$). D2DR (fig.5c) is significantly increased in ILAE classes 1, 2 ($p<0.001$; 4.41±0.22, $p<0.001$; 4.61±0.24) compared to controls (1.93±0.49) and ILAE class 3 $p<0.02$; 3.61±0.26). We further carried out a double immunofluorescence assay (fig. 7a) to check the relative D1 and D2 expression, which showed that DRD2 increased in ILAE class 1 ($p<0.009$, 3.89±0.15) compared to ILAE classes 3. In contrast, DRD1 expression was increased in class 2 ($p<0.008$; 3.20±0.34) and class 3 ($p<0.001$; 3.99±0.31) compared to ILAE class 1 (2.04±0.15) patient samples. The increase in DDR1 leads to the inhibition of PP1γ through cAMP-PKAc-DARPP32 Thr34 phosphorylation. DRD1 activates adenylyl cyclase 5 (AC5), which forms cAMP, an agonist for PKA activation; we found a significant increase in PKAc (fig.5e) in class 2 ($p<0.02$; 1.96±0.29) and class 3 ($p<0.03$; 1.90±0.23) samples compared to class 1 (0.95±0.33) and controls (1.02±0.01). Then PKAc target, i.e., DARPP32, was observed by western blot (fig.5f), which has no significant change in FCD patients' brain samples and controls. Furthermore, we checked the pThr34 levels of DARP32 (fig.5g), which is upregulated in class 2 ($p<0.004$; 12.22±1.52) and class 3 ($p<0.003$; 8.79±0.95), compared to ILAE class 1 (4.24±2.64) and control samples (1.83±1.18).

The role of PP1γ is limited in patients with seizure recurrence

Protein phosphatase1γ reduces excitotoxicity (fig.5h), and we found increased expression of PP1γ in ILAE class 3 ($p<0.01$, 1.76±0.16), class 2 ($p<0.03$, 1.70.29) and class 1 ($p<0.001$, 1.92±0.05) compared to controls (1.0613±0.0751), whereas class 2 did not show much difference with class 3 and class 2. Spinophilin/neurabin-2 (fig.5j), one of the PP1γ scaffolding proteins, showed a significantly decreased in ILAE classes 2 and 3 (1.23±0.21, 1.19±0.19) compared to controls ($p<0.03$; 1.52±0.11). Fyn (fig.5i) has a significant expression in class 3 ($p<0.001$; 9.29±1.94), class 2 ($p<0.008$; 7.91±1.52) compared to class 1 (2.47±1.05,) and controls (3.15±0.93).

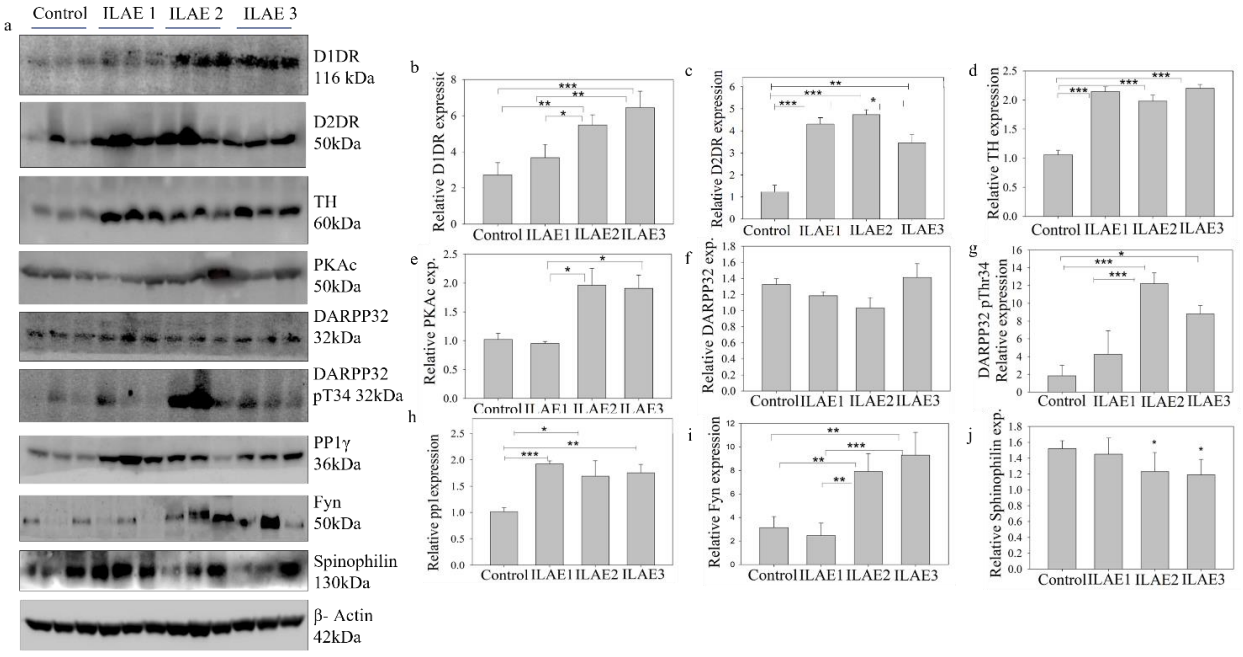

Fig: 5(a) Western blots study for DRD1 receptor and its downstream signaling molecules in FCD clinical samples. The corresponding bar graphs represent the quantification of protein expression of DRD1 (b), DRD2 (c), tyrosine hydroxylase (TH) (d), PKAc (e), DARPP32 (f), pDARPP32T34 (g), PP1γ (h), Fyn (i), Spinophilin (j) in both control and FCD samples. The lower panel represents an expression of beta-actin as a loading control. Data representing mean ± SEM. ***$p< 0.001$, **$p< 0.01$, *$p< 0.05$.

FRET analysis for dopamine receptor interaction in FCD

Since both dopamine receptors were increased in FCD conditions, we further designed an experiment to check the D1-D2 interaction through fluorescence resonance energy transfer (FRET) assay (fig. 6). By studying the FRET efficiency between D1 and D2 receptors, we concluded that they have no interaction in FCD patients. FRET efficiency value for D1D2 receptors in ILAE class 1 (a) was (-14.20±0.09), in ILAE class 2 (-0.56±0.03), and in ILAE class 3 (-60.35±15.09) (b and c) respectively. We observed negative FRET efficiency (E) between D1 and D2 receptors in ILAE classes 1, 2, and 3, which could be due to unequal distribution in terms of time and space of either D1 or D2 receptors. This could rule out the D1-D2 heteromer-Gqα mediated calcium regulation.

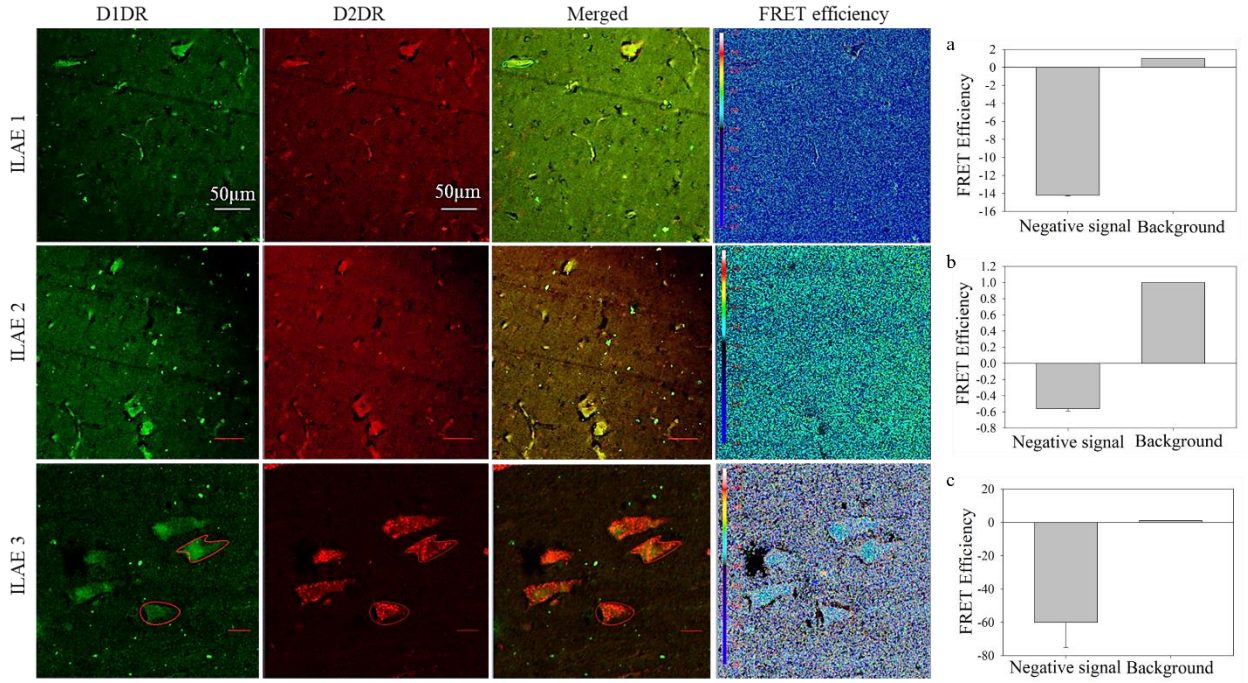

Fig. 6 Fluorescent resonance energy transfer analysis between DRD1 and DRD2 in FCD. ILAE class 1, ILAE class 2, ILAE class 3; (green color represents DRD1; red color represents DRD2; Merged represents DRD1, DRD2 combined images. The FRET efficiency represents the interaction between DRD1, DRD2, and a, b, and c represent the corresponding bar graphs about FRET efficiency.

Excitotoxicity in FCD can induce apoptosis

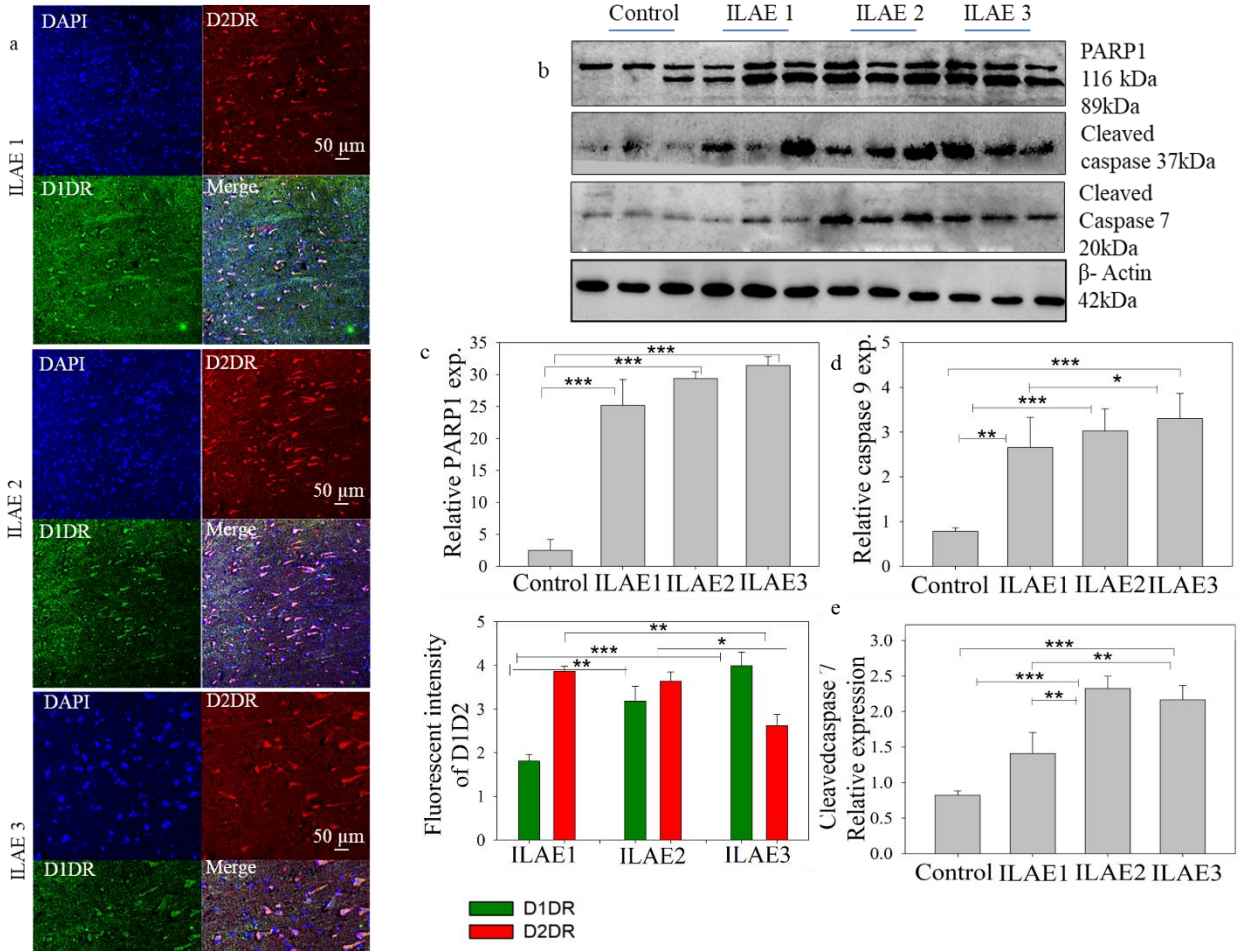

Fig. 7(a) Double immunofluorescence assay for D1DR (green) colocalized with D2DR (red), Scale bar = 20 µm. Figure (5b) Western blot assay for apoptosis markers and the corresponding bar graphs for PARP1 (c), Caspase 9 (d), and cleaved caspase 7 (e) to FCD clinical samples. Data representing as mean ± SEM. ***p< 0.001, **p< 0.01, *p< 0.05

Frequent, uncontrolled seizure-induced excitotoxicity may lead to the activation of apoptosis signal. Hence, we have performed a western blot assay to check the possibility of apoptosis signal activation (fig 7b). Interestingly we have observed the elevation of Poly (ADP-ribose) polymerase1

cleavage (fig. 7c) in class 1, 2, and 3 ($p<0.001$; 25.18±4.04, 29.39±1.05, 31.41±1.40) respectively compared to controls (2.48±1.71). PARP1 detects DNA mutations and double-strand cuts in cells. Cleaved caspase 9 (fig. 7d) also increased in class 3 ($p<0.001$; 3.30±0.56) compared to controls (0.78±0.07) and class 1 ($p<0.01$; 2.65±0.67). ILAE class 2 ($p<0.001$; 3.02±0.49) and class 1 also showed a significant difference compared to controls. Cleaved caspase 7 (fig. 7e) was more significant in class 2 ($p<0.001$; 2.32±0.17) and class 3 ($p<0.01$; 2.16±0.20) than in controls (0.82±0.06) and class 1 ($p<0.006$; 1.40±0.29) in FCD patient samples.

Discussion:

FCD is a chronic neurological disorder. Many FCD patients develop drug resistance and require surgery to control seizures. Still, many patients develop recurrent seizures and the reasons are unclear. Studies suggest that developing an epileptic cellular network around the lesion could be a major challenge in addressing seizures [42]. Hence the patient's resected tissue would be considered a typical model for understanding epileptogenesis [43]. The movement of Na^+, K^+, and Ca^{2+} is critical for neuronal firing and signal transduction [44]. AMPA and NMDA receptors on postsynaptic neurons predominantly control these ions' movement, particularly their GluR1, NR2A, and NR2B subunits [45]. Our study focused on the DA and glutamate signaling and their downstream targets, critical for postsynaptic neuron depolarization. We believe it could help to understand their relation to seizure recurrence. The FCD patients who regularly visited the hospital after surgery were observed for postsurgical seizure status to determine seizure outcomes. The clinical data showed that some patients were completely seizure-free, while some had recurrent seizures irrespective of the FCD subtypes. This data was used to categorize the patient samples according to ILAE-suggested outcomes class 1 (completely seizure-free), class 2 (no seizures, but experiencing auras), and class 3 (<3 seizure/year) [46].

Persistent activation of AMPA and NMDA receptors can lead to excitotoxicity. The postsynaptic neurons have internal regulation to bring back the resting potential upon dephosphorylation of the functional receptor subunits by PP1γ. [18, 47]. The PP1γ decreases the synaptic strength by dephosphorylating the S845 of GluR1 and Y1472 of the NR2B [25, 48, 49] can promote receptor endocytosis, long-term depression (LTD) by decoupling the synapses [10]. In our study, ILAE classes 2 and 3, patient's tissue samples showed a significant increase in pGluR1 and pNR2B of glutamate receptor subunits, which can lead to the strong synapse, long-term potentiation (LTP)/excitotoxicity by increasing ions flow into postsynaptic neurons.

The PP1γ and its adopter protein spinophilin/neurabin-2 expression increased in ILAE class 1 compared to ILAE classes 2 and 3 patient samples, which suggests negative regulation on AMPA and NMDA receptors is probably limited in patients who are experiencing recurrent seizures. DARPP32 phosphorylation acts as a signal integration point for dopamine and glutamate-dependent regulation of PP1γ [26]. Calcium ions moved through glutamate receptors activate calpain-p35/25-Cdk5 [15, 50]. The Cdk5 phosphorylates DARPP32T75 and disinhibits PP1γ [51]. Dopamine inhibits the PP1γ by pDARPP32T34 through PKAc [52]. Phosphorylation of DARPP32 at T75 was high in class 1. The pT34 significantly increased in classes 2 and 3. It suggests that DRD1 mediated PP1γ inhibition substantially increases in patients suffering from seizure recurrence. Activation of CAMKIIα is critical for both AMPA and NMDA receptor subunits for their synaptic stability [23, 53, 54]. CAMKIIα expression is similar in epilepsy patients. However, pCAMKIIα expression varied and increased in ILAE classes 2 and 3. pCAMKIIα can also improve the stability of glutamate receptors against dephosphorylation at the synapse [55]. So, we believe the upregulation of pCAMKIIα can support synaptic strength, LTP, and excitotoxicity.

TH is critical for DA synthesis. Increased TH suggests the availability of excess dopamine in FCD patients. Dopamine act through DRD1 and DRD2 receptors. These are GPCRs, which are antagonistic to each other. DRD1 requires excess DA levels, and DRD2 requires less DA for its function [33, 35]. DRD1 has a proconvulsant effect by activating PKA through cAMP, whereas DRD2 exhibits an anticonvulsant effect by reducing cAMP-dependent PKAc activation [35]. Dopamine has shown significant control over glutamate receptors in various neurodegenerative diseases [56]. DRD2 expression was limited in FCD patients belonging to ILAE class 3. At the same time, the DA hormone is also upregulated. Since the DRD2 receptor is also present in presynaptic terminals and involved in DA reuptake, it could limit excess DA effect in ILAE classes 1 and 2 compared to ILAE class 3 patients. In ILAE classes 2 and 3, DRD1 signaling is dominant because increased dopamine can bind to less affinity DRD1 and mediates PKA activation. The PKAc is abundant in classes 2, and 3 patient samples, suggesting the DRD1-PKAc axis completely dominates the DRD2-mediated anticonvulsant effect in patients with seizure recurrence. PKAc interrupts the PP1γ function by phosphorylating spinophilin at S94, S177, and neurabin1 at S461 residues [21, 57, 58]. It suggests that PP1γ is infective to dephosphorylate pGluR1 and pNR2B in ILAE class 2 and 3 patients, and it reflects through a significant increase in pGluR1 and pNR2B protein signal intensity. Probably it happens due to the excess release of dopamine, the persistence of DRD1-activated PKAc, which can dominate Cdk5-pDARPP32T75 mediated PP1γ activation [59], or by preventing the PP1γ-spinophilin/neurabin-2 interaction [60]. In our study, increased pDARPP32T34 only in ILAE classes 2 and 3, implying ILAE class 1 has a decreased DA-mediated proconvulsant effect and reflects the possibility of a complete seizure outcome than the classes 2 and 3 patients. The coexistence of dopamine receptors was observed in medium spiny neurons (MSN), globus pallidus, and nucleus accumbens [61]. These receptors form heterodimers like

D1/D2, D1/D3, D3/D2, D2/D5 [62], and homodimers like D1/D1, D2/D2 [38]. Usually, the heterodimer modulates intracellular Ca^{2+} levels [38]. We found no potential heteromer in FCD patient samples during FRET analysis using DRD1 and DRD2 fluorescent antibodies. This indicates a negative correlation between DRD1 and DRD2 receptors and has no positive interaction. Since the calcium ions entered the postsynaptic neuron through the NMDA receptor corresponding to PP1γ activation [63], the current study proved that the Cdk5-PP1γ axis is not strong enough to overcome the DRD1-mediated glutamate receptor excitotoxicity in FCD patients. Hence, the p25-Cdk5-PP1γ axis has a negligible effect on pGluR1 and pNR2B in ILAE classes 2 and 3 patients. These findings suggest the possibility of abolished PP1γ activity during status epilepticus. Another piece of evidence supporting the possibility of excitotoxicity is the upregulation of Fyn. An Src family kinase (SFK) mediates phosphorylation of NMDAR subunits at C- terminal T1472 residue in YEKL domine and prevents receptor internalization [25, 48]. These mechanisms can increase the NMDAR activity, which favors Ca^{2+} ions overload in cells and activates apoptotic signaling [63, 64]. Our study provides evidence for the upregulation of cleaved caspase 9, cleaved caspase 7, and PARP1 in ILAE classes 2 and 3. It suggests that excitotoxic insults driven by glutamate receptors can induce cell death and favor seizure recurrence by forming new irregular neural connections [65], which could also be a reason for poor seizure outcomes in FCD patients.

Conclusions:

The current study focused on understanding the dopamine and glutamate signaling cross-talk and its impact on NMDAR-mediated negative feedback regulation during excitotoxicity in epilepsy patients suffering from seizure recurrence. Since PP1γ activity is critical to reducing excitotoxicity, particularly during epileptic seizures, its function has been ousted in ILAE classes 2 and 3 patient samples. We believe it occurs due to the dopamine-mediated activation of specific serine/threonine

and tyrosine kinases such as PKAc, pCAMKIIα, Cdk5, and Fyn. This condition might favor postsurgical seizure recurrence in FCD patients with drug-resistant epilepsy. Further studies are required to find whether restoration of PP1γ function could reverse seizure susceptibility.

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CHAPTER 1B

We then moved on to studying FCD type III (TLE-HS) patient samples in comparison to FCD type I and II in order to comprehend the impact of DA and Glutamate impact on patients to understand their role in seizure recurrence and the results obtained from previous studies help us in conducting additional research in TLE-HS patient samples.

Introduction:

Temporal lobe epilepsy (TLE) with hippocampal sclerosis (HS) is considered an acquired phenomenon due to febrile illness/head trauma during early life [1, 2]. It affects the pyramidal cells and interneurons [3], ultimately causing febrile seizures (FS) [4, 5], and can lead to drug resistance epilepsy [6, 7]. As the hippocampus is deep inside the temporal lobe, 80% of TLEs have a hippocampal origin [8, 9]; hence hippocampus is regarded as a temporal extension of the cerebral cortex [10]. The development of TLE-HS conditions in patients can be influenced by other factors, including traumatic brain injury, encephalitis, meningitis, hypoxic brain injury, and stroke [11]. TLE-HS causes damage to interneurons (GABAergic), mossy cells in the hilar region, and granule cells in the dentate gyrus [11]. Dopamine (DA) and glutamate are critical for memory consolidation through the mesolimbic DA pathway. The mesolimbic pathway transports DA from the ventral tegmental area (VTA) to the nucleus accumbens, amygdala, and hippocampus. Hence, DA is critical in controlling the hippocampus and limbic system [12]. DA modulates postsynaptic neurons through D1 and D2 receptors. [13]. However, DA receptors bind with DA ligands with variable affinity. D1 receptor activation needs a phasic release of DA (Milli molar), and D2 receptor activation requires tonic (Nanomolar) release of DA hormone from presynaptic neurons [14, 15]. Also, dopamine receptors have opposing effects on cAMP synthesis in postsynaptic neurons [16] and modulate ionotropic glutamate receptors [α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPAR) and N-methyl-D-aspartate receptors (NMDAR)] function by directly or indirectly

regulating their synthesis, synaptic localization, and phosphorylation status via kinases and phosphatases [17]. D1 receptor enhances NR2A, NR2B subunits of NMDAR [18] surface expression via PKC [19] and controls synaptic plasticity by indirectly promoting CAMKIIα autophosphorylation [20] and GluR1 of AMPAR surface expression [21, 22]. Ca^{2+} enter the cell via NMDAR and stimulate AMPA receptor targeting to the membrane by pERK, CAMKIIα, and PKA-dependent phosphorylation of GluR1 subunit at S-845 in hippocampal neurons [24–28]. The pGluR1 induces long-term potentiation (LTP) by promoting excitatory postsynaptic potential (EPSP) [29]. CAMKIIα interacts with AMPA and NMDA receptors at postsynaptic density (PSD) near dendritic spines and controls their density and plasticity at synapses upon phosphorylation at T286 [34]. Fyn kinase phosphorylates Y1472 at the YEKL domain of the NR2B receptor subunit to inhibit receptor internalization and induce LTP [35]. Ca^{2+} activates Cdk5 via calpain-mediated cleavage of p35 into p25 and p10 [36]. Cdk5 activates protein phosphatase1γ (PP1γ) through pDARPP32T75 (DA and cAMP-regulated phosphoprotein 32) and by inhibiting PKA. [37]. D1R blocks Cdk5 by inhibiting PP1γ via PKAc, which results in DARPP32 phosphorylation at T34 instead of T75. Spinophilin and neurabin-1 target the PP1γ to pNR2B and regulate its synaptic location by dephosphorylation, allowing NR2B to detach from synapses for endocytosis and initiating long-term depression (LTD) [38]. D2 receptor has a high affinity to ligand (dopamine); therefore, the tonic release of DA activates the D2R pathway via $G_{i\alpha}$ and inhibits the D1R-cAMP-PKAc axis [23]. Even though D1 and D2 are antagonistic, the synergistic function of the D1-D2 heterodimer was detected in the striatum of rat brains [24]. Nucleus accumbens, caudate-putamen, globus pallidus D1, and D2 receptors form a heterodimer in medium spiny neurons [25]. The D1-D2 receptor heterodimer regulates the endoplasmic reticulum calcium reserves by Gαq, phospholipase C (PLC), and activates Ca^{2+}/calmodulin dependent protein kinase IIα (CAMK IIα)

[26, 27]. In addition, NMDARs also mobilize Ca^{2+}, which leads to calcium overload and excitotoxic death [28]. Orchestrated pre-and postsynaptic events that co-occurred (dependently/independently) will regulate normal neuronal signaling [29]. As age increases, DA and glutamate signaling also become hypofunctional due to changes in receptor synthesis, affinities, and the release of neurotransmitters [30–35]. Since epilepsy patients were found to have significant upregulation of excitatory neurotransmitters, and aberrant, dyssynchronous neuronal networks, that could lead to extraneous neuronal firing and excitotoxicity [29, 36, 37]. The current study focused on understanding the significance of NMDAR-mediated negative feedback regulation [38] that initiates long-term depression (LTD) by reducing the receptor stability at synapse through the Ca^{2+}-p25-Cdk5-PP1γ axis in TLE-HS patients. In addition, we also explored the possible DA-Glutamate crosstalk and D1-D2 interaction by hetero dimer formation, which has a significant effect on the persistence of excitotoxicity by promoting receptor trafficking and stability, using postsurgical resected brain samples of patients and postmortem control brain samples. The control brain samples used for the study have an age range between 25 to 37 years, and epilepsy patient samples have an age range between 4 to 40 years during surgery. Obtaining exact age match control for pathological studies is more challenging. Earlier reports by David C. Henshall et al. (2004) helped us to use adult controls in our study. Based on these reports, we have performed our studies to understand the influence of excitotoxicity on postsurgical seizure recurrence in epilepsy patients affected by TLE-HS.

Data analysis:

Statistical analyses among groups performed using one-way ANOVA using the Newman-Keuls method for posthoc analysis. Sigma Plot 2000 software for the Windows version was used to draw graphs. Statistical significance level was set at ***$p < 0.001$, ** $p < 0.01$, * $p < 0.05$. A student t-test

was used to compare the two groups. Data presented here as the mean ± SD; 'n' designates the number of separate experiments examined.

Results:

TLE-HS patients were categorized according to the seizure freeness and ThT assay data:

Table.2 shows the list of patients who visited the hospital after surgery. The postsurgical data of patients was collected regularly from the hospital to understand the patient's recovery. Based on these details, we followed the ILAE suggestions to classify the seizure outcome in these patients. We place the wholly recovered patients under ILAE class 1 (no seizures) and patients with recurrence of seizures under ILAE class 2 (persistent seizures). Table.1 represents the autopsy control brain samples collected from the National Institute of Mental Health and Neurosciences (NIMHANS) human brain tissue repository (HBTR) facility in Bangalore, India. ThT assay data was correlated with seizure outcome, where fluorescence intensity of protein aggregates in class 2 ($p<0.001$; 281.22±4.56) > class 1($p<0.01$; 196.07±2.7) > controls (148.8±0.91). Table.2 represents the autopsy control brain samples collected from the National Institute of Mental Health and Neurosciences (NIMHANS) human brain tissue repository (HBTR) facility in Bangalore, India. ThT assay data was correlated with seizure outcome, where fluorescence intensity of protein aggregates in class 2 ($p<0.001$; 281.22±4.56) > class 1($p<0.01$; 196.07±2.7) > controls (148.8±0.91).

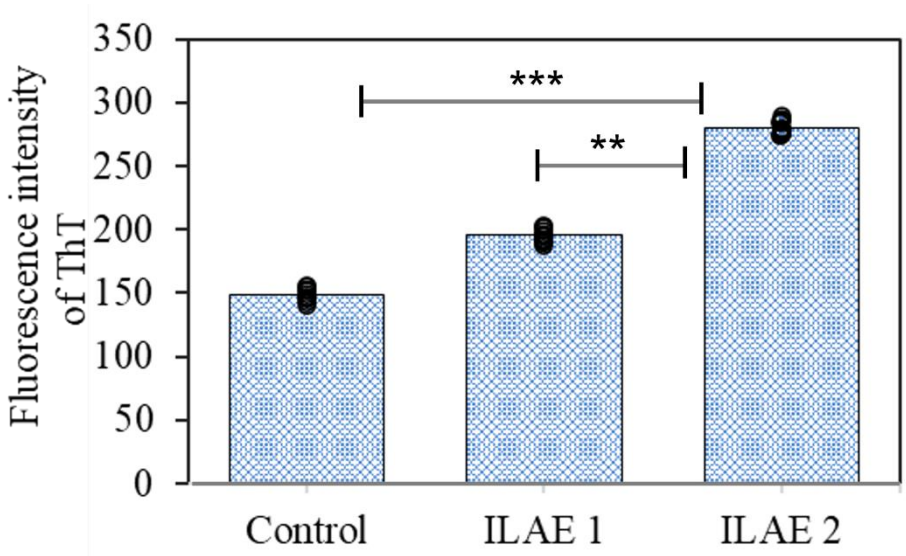

Fig.1 Represents the estimation of unfolded protein aggregates in TLE-HS patients (n=26) and controls (n=5) by Thioflavin T assay: Fluorescent intensity of aggregated proteins was measured and shown as mean ± SEM (n=3). The graph represents the ThT assay data from controls and patient samples in increasing order of protein aggregates and postsurgical clinical parameters independent of age and sex. The fluorescent intensity of controls, classes 1 and 2 patient samples indicate that UFP aggregates were significantly increased in class 2 samples compared to class 1 and controls. ***p< 0.001; **p< 0.01; *p< 0.05

S.No.	Gender	Age	Cause of Death	Post-Mortem Interval	Significant Neuro pathology	Disease	HIV/ HBsAg	Brain Region
1	M	25 years	Suicide	30 hours	Absent	No	Negative	Temporal
2	F	27 years	Drowning	28 hours	Absent	Diabetes	Negative	Temporal
3	M	37 years	Heart attack	40 hours	Absent	No	Negative	Temporal
4	M	27 years	Accident	35 hours	Cerebral injury	No	Negative	Temporal
5	F	25 years	Homicide	37 hours	Absent	No	Negative	Temporal

Table 1. Represents the control cases information and fluorescent intensity of protein aggregates: Control samples were obtained from the Human Brain Tissue Repository (HBTR), NIMHANS, Bangalore. The tissue samples were stored according to their standardized protocol at NIMHANS.

Case	Character	Gender		Onset Age	Age of surgery	Duration	Loci	Seizures/Year before surgery	Post-surgery outcome for 3 Years	ILAE Outcome Class
1	TLE-HS	M		10 Y	40 Y	30 Y	HS	09	90% decrease	Class 2
2	TLE-HS	M		9 Y	13 Y	4 Y	HS	08	seizures free	Class 1
3	TLE-HS	M		5 Y	15Y	10Y	HS	10	90% decrease	Class 2
4	TLE-HS		F	3 Y	4.6Y	1.6 Y	HS	08	seizures free	Class 1
5	TLE-HS		F	16Y	27Y	11 Y	HS	07	seizures free	Class 1
6	TLE-HS	M		10Y	13Y	3 Y	HS	09	90% decrease	Class 2
7	TLE-HS		F	4 Y	12Y	8 Y	HS	10	seizures free	Class 1
8	TLE-HS	M		7 Y	12Y	5 Y	HS	09	90% decrease	Class 2
9	TLE-HS		F	6 Y	14 Y	8 Y	HS	24	seizures free	Class 1
10	TLE-HS		F	5Y	18 Y	13 Y	HS	10	90% decrease	Class 2
11	TLE-HS	M		9 Y	10 Y	1 Y	HS	11	seizures free	Class 1
12	TLE-HS		F	2 Y	14 Y	12 Y	HS	08	90% decrease	Class 2
13	TLE-HS	M		5 Y	8Y	3 Y	HS	09	seizures free	Class 1
14	TLE-HS	M		34 Y	35 Y	1 Y	HS	10	90% decrease	Class 2
15	TLE-HS	M		2 Y	25 Y	23 Y	HS	08	seizures free	Class 1
16	TLE-HS	M		11 Y	21 Y	10 Y	HS	09	seizures free	Class 1
17	TLE-HS	M		6 Y	13 Y	7 Y	HS	09	90% decrease	Class 2
18	TLE-HS		F	1Y	3 Y	2 Y	HS	10	seizures free	Class 1
19	TLE-HS	M		11 Y	14Y	3 Y	HS	09	90% decrease	Class 2
20	TLE-HS	M		15 Y	25 Y	10 Y	HS	12	seizures free	Class 1
21	TLE-HS	M		3Y	11 Y	8 Y	HS	09	90% decrease	Class 2
22	TLE-HS	M		4 Y	10 Y	6 Y	HS	09	90% decrease	Class 2
23	TLE-HS		F	1 Y	4 Y	3 Y	HS	10	seizures free	Class 1
24	TLE-HS		F	3Y	5 Y	2 Y	HS	09	seizures free	Class 1
25	TLE-HS	M		13 Y	25 Y	12 Y	HS	10	90% decrease	Class 2
26	TLE-HS	M		15 Y	27 Y	12 Y	HS	10	90% decrease	Class 2

Table 2. Represents the TLE-HS patient's clinical data, like the type of epileptic condition, age, sex, duration of patients suffering from seizures, seizure frequency before and after surgery, and clinical outcome. Based on postsurgical seizure recurrence, patients are categorized into ILAE class 1 and ILAE class 2.

Phospho form of ionotropic glutamate receptors significantly increased in patients with seizure recurrence:

AMPR subunit GluR1 (Fig. 2b) showed no significant increase in signal intensity among controls and patients. Then we further checked pGluR1 (Fig. 2c), which showed a significant increase in ILAE class 2 ($p<0.01$; 2.69±0.35) compared to class 1 (0.97±0.23). NMDAR subunit NR2A and NR2B (Fig. 2d, 2e) upregulated in ILAE class 1 and 2 (1.41±0.20, 1.36±0.09) and (3.94±0.26, 4.00±0.08) respectively in comparison to controls (0.55±0.04), but no significant difference noticed between ILAE classes 1 & 2. pNR2B (Fig. 2f) significantly upregulated in ILAE class 2 ($p<0.009$; 1.62±0.12) compared to class 1 ($p<0.025$; 1.20±0.18). It suggests that TLE-HS patients with seizure recurrence contain a significant amount of phospho glutamate receptor subunits, which could help to maintain LTP and excitotoxicity.

Phospho forms of glutamate receptors can activate p25 dependent Cdk5-PP1γ axis:

The protein expression of p25/35 (Fig.2g) shows that p25 was significantly increased in ILAE class 2 ($p<0.001$; 6.14±0.69) compared to class 1 ($p<0.03$; 3.53±1.06) and controls (0.74±0.08). Cleavage of p35 into p25 suggests increased cellular calcium levels by NMDAR. Cellular expression of p25 was also examined against neuronal marker NeuN by double immunofluorescence assay (Fig. 3b) in both ILAE classes 1 and 2. Class 2 patients were found to have significant expression of p25 ($p<0.02$; 3.6±0.40) compared to class 1 (2.80±0.60). The p25 then activates Cdk5 (Fig. 2h), which is increased in both ILAE classes 1 ($p<0.01$; 2.14±0.15) and class 2 ($p<0.009$; 2.27±0.16). The pCdk5 S159 (Fig. 2i) was also increased in ILAE class 2 ($p<0.001$; 11.84±1.56) and class 1 ($p<0.01$; 9.93±2.68) compared to controls (1.08±0.205).

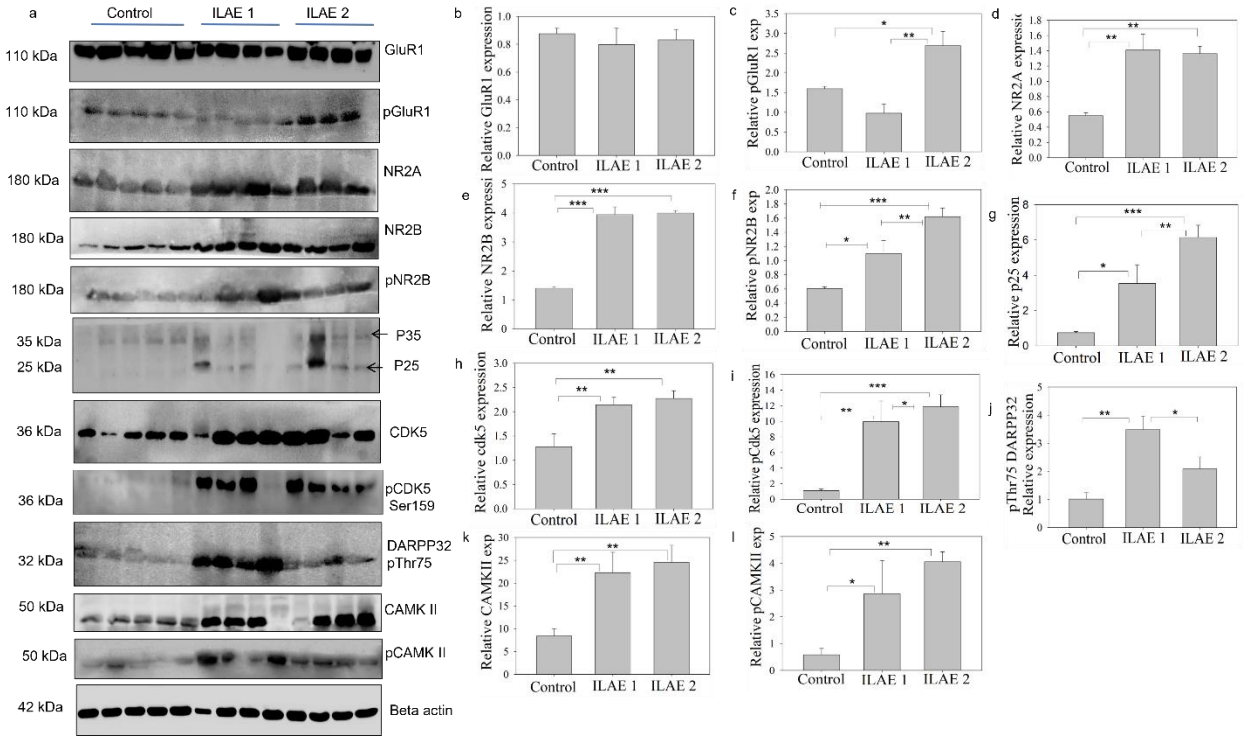

Fig.2 (a) Immunoblot analysis of (b) GluR1 and (c) pGluR1, (d)NR2A, (e) NR2B, (f) pNR2B, (g) p25/35, (h) CDK5, (i) pCDK5, (j) DARPP32 pThr-75, (k) CAMKIIα, and (l) pCAMKIIα Thr-286 in both control and TLE-HS brain tissues and the corresponding bar graphs representing the quantification of protein expressions. The base panel represents the beta-actin as an internal control. Data represents mean ± SD ***p< 0.001, **p< 0.01, *p< 0.05.

Double immunofluorescence assay (n=3) performed for pCdk5 (Fig. 3c) exhibited a significant increase in ILAE class2 (p<0.009; 5.80±0.27) compared to ILAE class 1 (3.40±0.23). The pDARPP32T75 (Fig. 2j) was found to be significant in ILAE class 1 (p<0.009; 3.48±0.48) compared to class 2 (p<0.02; 2.08±0.42) and controls (1.02±0.22).

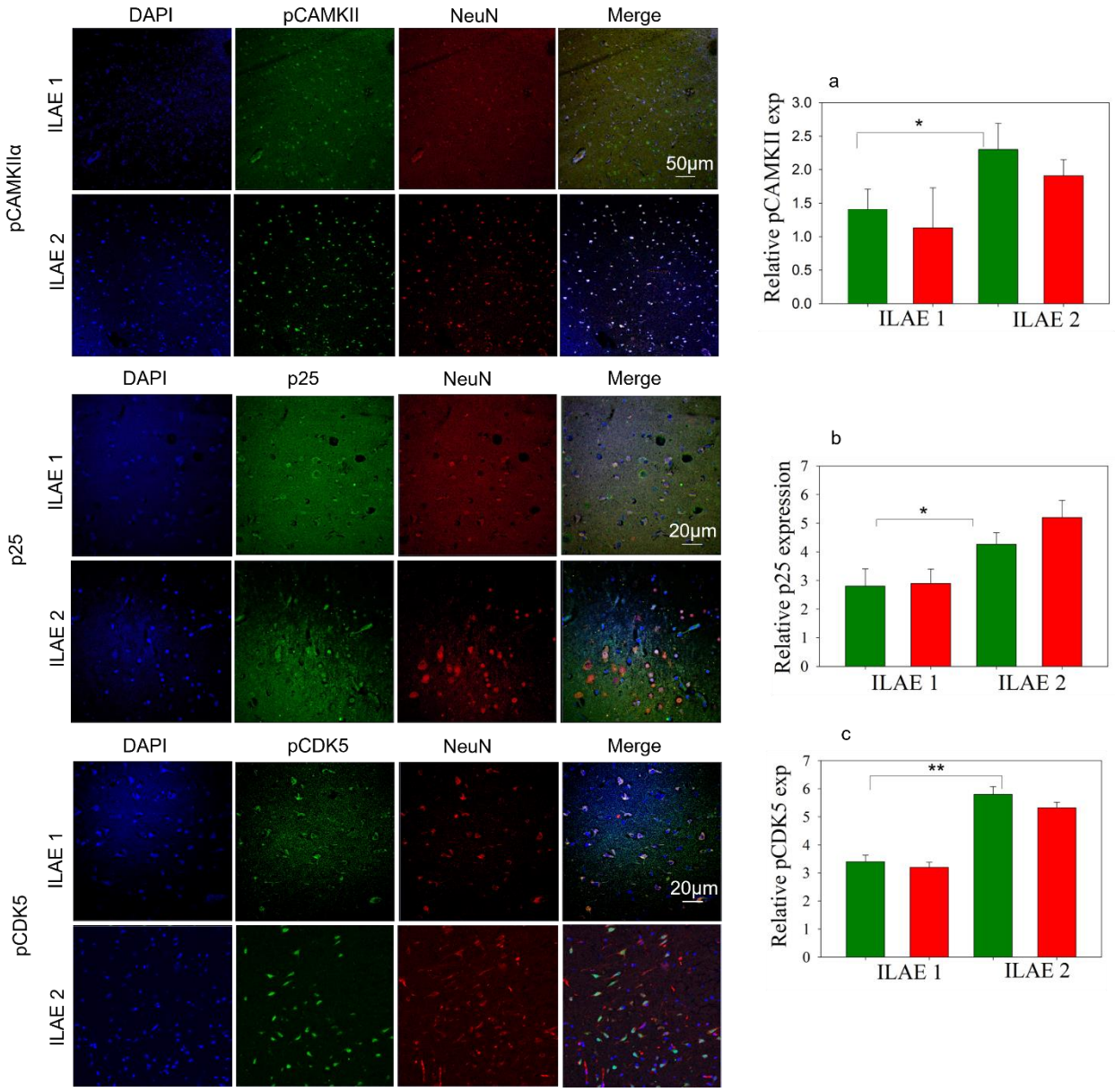

Fig.3 Double Immunofluorescence analysis for (a) pCAMK IIα (Green) NeuN (red), (b) p25 (Green), and (c) pCdk5 (Green) and NeuN (red) expression in TLE-HS clinical samples. The scale bar is 20 μm for p25, pCdk5, and pCAMK IIα. The scale bar is 50 μm

Since pDARPP32T75 disinhibits the PP1γ against cAMP-PKAc-pDARPP32T34 dependent inhibition, we further studied to observe the PP1γ (Fig. 4h) protein expression in TLE-HS patient samples. Here, we find a significant increase of PP1γ in ILAE class 2 ($p<0.009$; 1.51 ± 0.49)

compared to class 1 ($p<0.02$; 1.10±0.20) and controls (0.71±0.084). Phosphorylation of AMPA/NMDA receptor subunits and increased p25/PP1γ suggest the possibility of strong suppression of PP1γ through an alternate pathway that overturns NMDAR-mediated negative feedback regulation to control the postsynaptic excitation.

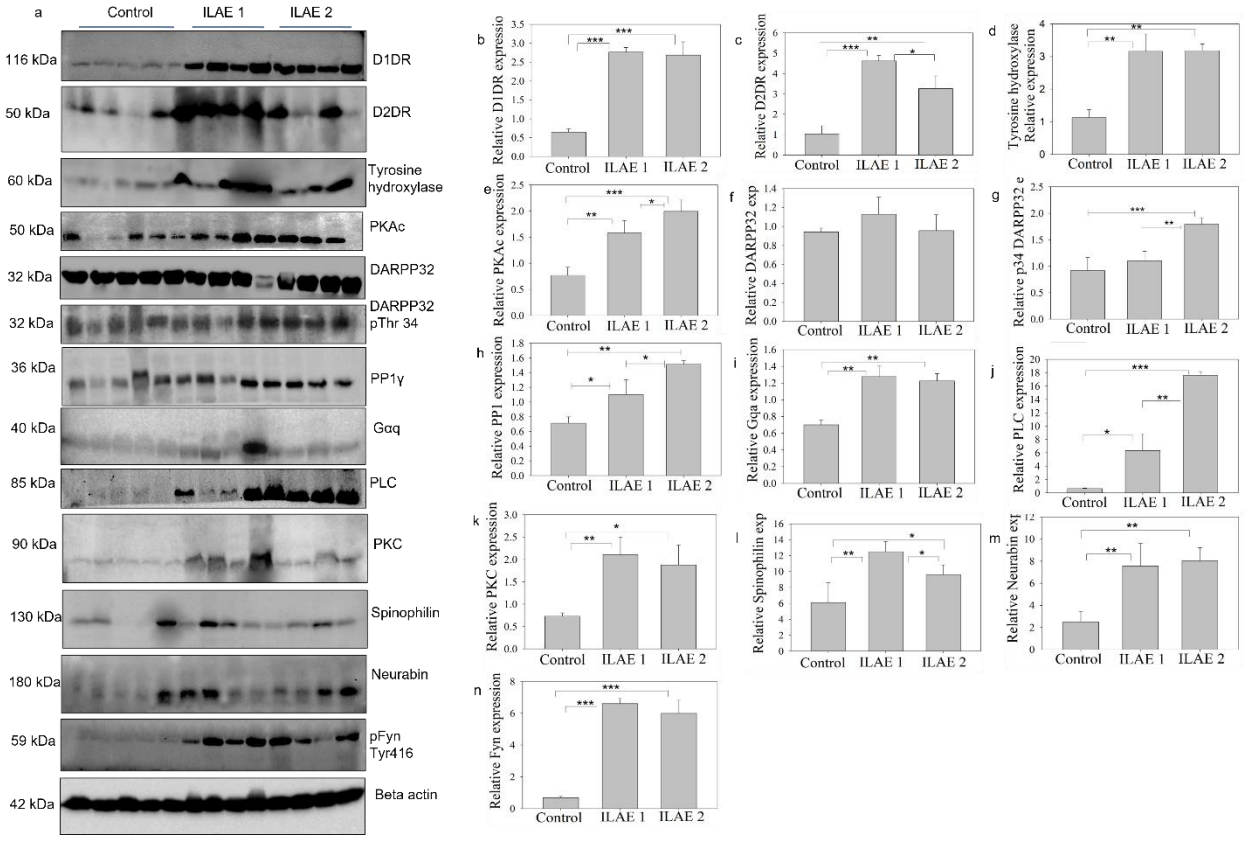

Fig.4 (a) Immunoblot analysis of (b) D1DR and (c) D2DR, (d)Tyrosine hydroxylase, (e) PKAc, (f) DARPP32, (g) DARPP32 pThr-34, (h) PP1γ, (i) Gαq, (j) PLC δ, (k) PKC, (l) spinophilin, (m) Neurabin (n) pTyr416 Fyn, in both control and TLE-HS brain tissues. The image represents Immunoblot and the corresponding bar graphs with quantification of protein expression. The base panel represents the beta-actin as an internal control. Data represents mean ± SD ***$p< 0.001$, **$p< 0.01$, *$p< 0.05$.

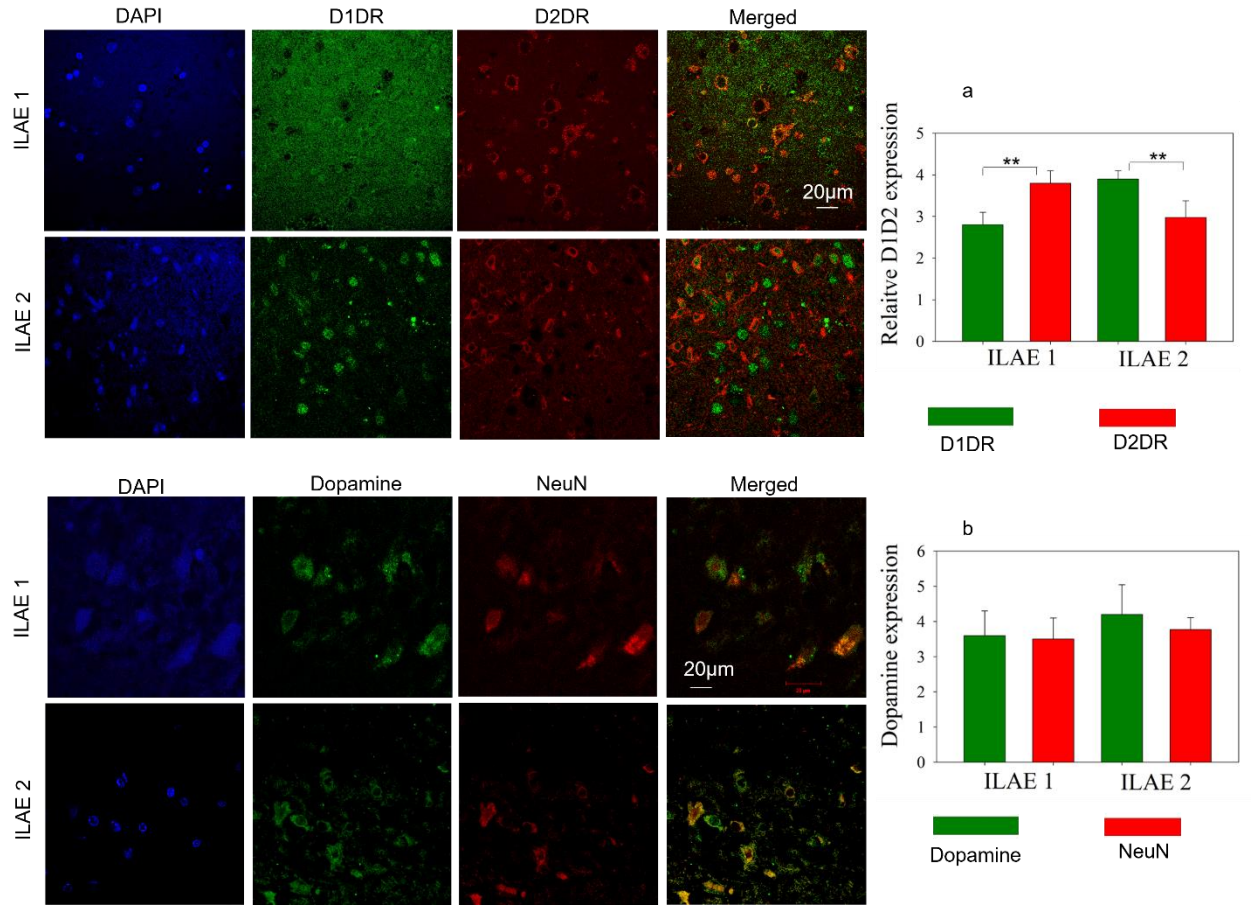

Fig.5 Double Immunofluorescence analysis for (a) D1DR (Green), D2DR (Red), (b) Dopamine (Green), and NeuN (red) expression in TLE/HS clinical samples. The scale bar is 20 µm

DA and D1, D2 receptor regulation on PP1γ:

DA signaling via D1 and D2 receptors balances neuron depolarization and maintains homeostasis. D1R (Fig. 4b) showed increased expression in TLE-HS samples belonging to ILAE class 1 ($p<0.001$; 2.76±0.12) and class 2 ($p<0.001$; 2.68±0.35). D1R is present in postsynaptic neurons, and its activity is directly associated with the depolarization of postsynaptic neurons. D2R (Fig. 4c) expression increased in ILAE class 1 ($p<0.001$; 5.61±0.28) compared to class 2 ($p<0.009$; 3.62±0.62). Since both the receptor's expression was increased in patient samples, we further examined the expression of D1 and D2 receptors by double immunofluorescent (IF) assay (Fig. 5a).

The TLE-HS patients have a remarkable difference in D1 and D2 receptor expression. D2 expression is higher ($p<0.009$; 3.80±0.30) compared to D1 (2.80±0.31) in ILAE class 1, and D1 ($p<0.005$; 3.91±0.21) is significantly greater than D2 (2.98±0.40) in ILAE class 2. Since DA availability can determine DA receptor activity, we tested the DA expression (Fig. 5b) in patient samples. According to the IF assay, DA expression had no significant difference. We also checked the Tyrosine hydroxylase (Fig. 4d), which is critical for DA synthesis and was observed through western blot analysis. We found that TH increased in both ILAE 1 ($p<009$; 3.15±0.53) and class 2 ($p<0.008$; 3.18±0.20) compared to control samples. It indicates the upregulation of the DA hormone. As we know the upregulation of DA activates the low-affinity D1 receptor, we further checked D1R downstream signaling in TLE-HS samples.

D1R increases cellular cAMP levels through adenylyl cyclase (AC5) and activates PKAc (Fig. 4e); ILAE class 2 ($p<0.001$; 2.49±0.21) patients showed a substantial increase in PKAc compared to class 1 (1.78±0.22). PKAc activates DARPP32 by phosphorylating at T34 residue. Then we observed DARPP32 (Fig. 4f) no significant difference among the ILAE groups, but its phospho form DARPP32T34 (Fig. 4g) signal intensity showed a substantial increase in ILAE class 2 samples ($p<0.009$; 1.79±0.09) compared to class 1 ($p<0.03$; 1.30±0.20). Our data suggest that the upregulation of pDARPP32T34 through D1R-PKAc can inactivate PP1γ and prevent the calcium ion-p25-Cdk5 dependent negative feedback regulation on AMPA and NMDA receptor subunits.

TLE-HS patients showed D1-D2 receptor heterodimer-mediated calcium signaling, which may involve GluR1 and NR2B receptor activation:

D1-D2 receptor heteromer manages cellular calcium levels by activating the Gαq (G protein) (Fig. 4i), which showed an increase in ILAE class 1 ($p<0.004$;1.27±0.12) and class 2 ($p<0.005$; 1.22±0.08) Gαq activates PLCδ, which cleaves the membrane phospholipid phosphatidylinositol

and formulates diacylglycerol (DAG) and inositol triphosphate (IP3). PLCδ (Fig. 4j) expression showed a significant increase in ILAE class 2 ($p<0.001$; 17.49±1.29) compared to class 2 (6.63±0.96) and controls. The IP3 generated by PLCδ creates a membrane channel to the endoplasmic reticulum, letting calcium ions escape into the cytosol. The increase of Ca^{2+} cellular levels activates Ca^{2+}/calmodulin dependent protein kinaseIIα (CAMKIIα) (Fig. 2k), which was increased in ILAE class 1 ($p<0.009$; 22.29±4.46) and class 2 ($p<0.006$; 24.52±3.75). The pCAMKIIα (Fig. 2l) substantially increased in ILAE class 2 ($p<0.007$; 4.05±0.37) compared to class 1 ($p<0.04$; 2.86±1.24) patient samples. The double immunofluorescent assay of pCAMKIIα (Fig. 3a) displayed a similar pattern of expression, where ILAE class 2 ($p<0.01$; 2.300±0.39) has a considerable expression compared to class 1 (1.41±0.30). Activation of PKC, a calcium-dependent kinase, manages the receptor membrane localization by phosphorylating S1303 and S1323 of NR2B [46]. We have observed that PKC (Fig. 4k) showed a significant increase in ILAE class 1 ($p<0.009$; 2.10±0.39) compared to class 2 ($p<0.03$; 1.87±0.45).

The above data indicates D1-D2 receptor heterodimer formation. Hence, we have examined D1-D2 receptor heterodimer formation using the confocal-based FRET method in TLE/HS patient samples (Fig. 6). FRET data revealed D1 receptor and D2 receptor-positive interaction (after removing the background) with FRET efficiency of 0.787±0.001 (n=4 regions of interest) at a relative distance of 70 Å or 0.7 nm. A negative signal was deduced when probes were not close (>100 Å). We have selected four regions of interest specific to D1-D2 heteromer co-localization. This data confirms the positive interaction between the D1-D2 receptors since the distance between two interacting proteins is <10nm and FRET efficiency is also found to be positive (between 0 - 1) could support the possibility of Ca^{2+} overload in the cells.

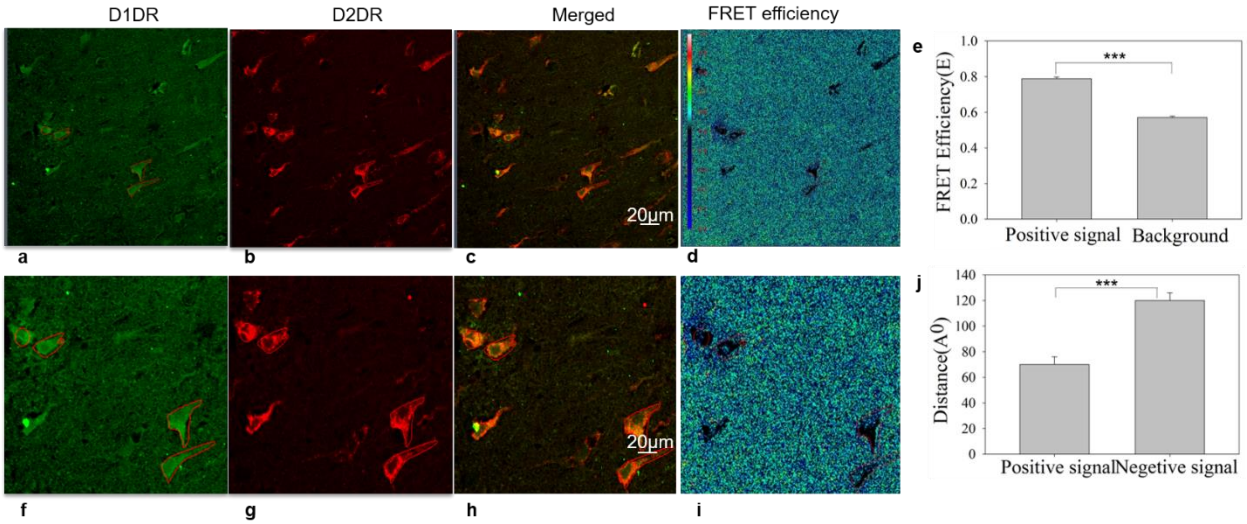

Fig.6 Fluorescent resonance energy transfer analysis for D1/D2 heterodimer in TLE/HS indicates the interaction between D1DR and D2DR. The images show the double immunofluorescence staining of D1 (green) and D2 (red) and their interaction in TLE-HS sections.

Spinophilin, Fyn also regulates the PP1γ effect on AMPA and NMDA receptor stability:

Spinophilin and Neurabin control the cytoskeleton stability at the dendritic spines and act as adaptor molecules to PP1γ during the dephosphorylation of ionotropic glutamate receptors. In TLE-HS samples, we observed a significant increase in spinophilin (Fig. 4l) in ILAE class 1 ($p<0.008$; 13.58±0.60) compared to class 2 ($p<0.03$; 10.32±2.55). Neurabin (Fig. 4m) was increased in both ILAE class 1 ($p<0.01$; 7.56±2.03) and class 2 ($p<0.009$; 8.034±1.18). We found significant expression of Fyn (Fig. 4n) in class 1 ($p<0.001$; 6.60±0.31) and class 2 ($p<0.001$; 5.99±0.81) of TLE-HS patients. Our data suggest that limited spinophilin and upregulation of Fyn could significantly affect PP1γ/spinophilin interaction and dephosphorylation events in ILAE class 2 patients. Detailed explanations can be found in the discussion section below.

Discussion:

TLE-HS is one of the common forms of focal epilepsies. It is difficult to treat with medication alone and requires surgical resection of epileptic foci to control the seizures. Even after surgery, some patients experience seizure recurrence for unknown reasons. This study aimed to determine the underlying differences between seizure-free TLE-HS patients and those with a seizure recurrence. For the present study, we have considered patient samples who regularly visited the hospital after surgery. Based on the postsurgical clinical data, we classified the patient samples as seizure-free ILAE class 1 and seizure recurrence patients ILAE class 2. The studies on the rodent model of TLE indicate the involvement of DA in epileptogenesis [39]. Several animal studies showed that D1 and D2 receptors have pro- and anti-epileptic effects on limbic epileptogenesis. DA can modulate the ionotropic glutamate receptors in different disease conditions [40]. Since postsynaptic neuronal excitation controlled by ionotropic glutamate receptors regulates Na^+, K^+, and Ca^{2+} movement [41], we believe it is essential to understand the DA and glutamate signaling and their crosstalk in epilepsy patients.

Age-related difference in DA and glutamate receptors expression and function:

Philip Seeman et al. studies suggested that during childhood D1 and D2 receptor density rise and fall together, but after 20 years, D1 declines by 3.2%, and D2 declines by 2.2% per decade [42] In the caudate nucleus and putamen, the D1/D2 ratio was independent of age and did not affect the binding affinities in males and females [43]. Francesco Amenta et al., studies on rats ages 3, 12, and 24 months reported that age-dependent reduction or change in D1 and D2 receptors expression is not homogeneous and showed the region-specific increase/decrease in receptor density even in adult rats [30]. In the case glutamate receptors system, as age increases, NMDA receptors become more hypofunctional [35]. In humans, NMDARs density in the frontal cortex showed a significant

(36%) decline between 20 to 100 years old [44]. During healthy aging, there is general agreement that the total pool for neuronal glutamate signaling decreases [45]. mRNA levels of NMDAR were reduced in patients with Alzheimer's disease [46]. Hyperactivation of AMPA/NMDA receptors was observed in the pathophysiology of hypoxic-ischemia, trauma, and epileptic condition. AMPAR dynamics were tightly regulated throughout childhood to adult age. Dysregulation of AMPARs was observed in many neurological and neurodegenerative diseases. Understanding the glutamate receptors network is still unclear due to differential phosphorylation and subunit composition patterns differ from younger to old and normal to diseased brains [47]

In our study D1, D2 receptor and AMPA, NMDA receptor subunits expression pattern showed a significant difference between controls, class 1 and class 2 patients, including children and adults. Class 2 consists of patients with seizure recurrence who showed upregulation of glutamate receptors, proconvulsant D1 receptor, and downregulation of anti-epileptic D2 receptor. This could be due to the increased seizure network supporting excitotoxicity in patients, particularly patients affected by recurrent seizures [36, 37]. These findings support our current study about the involvement of dopamine and glutamate signaling in seizure persistence and recurrence. During seizures, AMPA and NMDA receptors allow Na^+, K^+, and Ca^{2+} and support the indefinite firing of the neuron [48]. In TLE-HS patient samples, ILAE class 2 patients showing recurrence seizures were found to have higher pGluR1 and pNR2B levels, which could form a stable and long-lasting synapse. That can lead to excitotoxicity, but the neuron has its regulatory mechanism in the form of Protein phosphatase 1γ (PP1γ) to revert this condition by removing stable phospho tags from pGluR1 and pNR2B, making them weaker at the synapse, which can be leading to receptor endocytosis [49, 50].

PP1γ is increased significantly in ILAE class 2 compared to class 1 and controls but increased pNR2B and pGluR1 signal intensity suggests that PP1γ is not active against ionotropic glutamate receptors signaling. PP1γ is managed by both DA and glutamate signaling. DARPP32 functions as a signal integration point [51]. DA impedes PP1γ through PKAc-dependent pDARPP32T34 [52], whereas glutamate activates PP1γ through calcium-ions-dependent calpain activation and cleavage of p35 into p25. The p25 activates Cdk5, which phosphorylates DARPP32T75 and promotes the dephosphorylation of pDARPP32T34 [53]. In ILAE class 2, samples D1R, PKA, and pDARPP32T34 were upregulated compared to ILAE class 1. In addition, pDARPP32T75 significantly increased in class 1. Since the results suggest the differential regulation of PP1γ in ILAE classes 1 and 2 patient samples, we believe the PP1γ regulation plays a critical role depending on the seizure microenvironment in TLE-HS patients.

Double immunofluorescence assay suggested that TLE–HS patient samples have increased DA hormone. We further examined tyrosine hydroxylase (TH) enzyme expression in both ILAE class 1 and class 2 patients and observed a substantial upregulation of the TH. Since DA upregulation corresponding to D1R signaling, which is antagonistic to D2R, also supports the excitotoxicity, we further verified the D1R downstream kinase PKAc and found that ILAE class 2 patients had a substantial increase compared to ILAE class 1 and controls. It suggests that DA signaling is somehow controlled in completely seizure-free patients. When we checked the DA receptors expression, we found that D2R expression is upregulated in class 1 patients, which could prevent D1R-mediated PKAc activation. However, in patients with recurrent seizures, the D1R was upregulated, and D2R was limited. D1R could cause upregulation of PKAc, eventually inhibiting DARPP32's T75 phosphorylation and PP1γ [54].

Even though D1 and D2 receptors have an antagonistic effect on adenylyl cyclase activation, they show synergistic control over Ca^{2+} reserves of the endoplasmic reticulum. Globus pallidus, nucleus accumbens, and medium spiny neurons (MSN) DA receptors can form heterodimers [26, 55]. Based on the FRET assay, we observed D1-D2 receptor heterodimer formation. D1-D2 heterodimer activates Gαq, phospholipase C (PLC), IP3, and DAG-protein kinase C (PKC) [56]. This condition indicates that neuronal cells are critically affected by calcium ion dysregulation [57] during epileptic seizures. This condition promotes cell death by disturbing mitochondria permeability [58]. In TLE-HS patients, we found the formation of D1-D2 heterodimer and upregulation of its downstream targets such as Gαq, PLC, and PKC, so we believe it could disturb the Ca^{2+} levels in the cell and promotes cell death in TLE-HS patients.

The Ca^{2+} ions moving through NMDAR can initiate negative feedback regulation on receptors through the Ca^{2+}-calpain-p25-Cdk5-pDARPP32T75-PP1γ axis [59]. It incapacitates GluR1 and NR2B stability, reducing synaptic strength and promoting LTD [60]. However, this is not occurring in ILAE class 2 patients of TLE-HS; instead, pGluR1 and pNR2B were increased remarkably, which could promote excitotoxicity. Hence, we believe that the persistence of this condition might support seizure recurrence in class 2 patients. Other possibilities that can regulate the PP1γ function on glutamate receptors are the upregulation of cellular kinases activated directly or indirectly through DA and glutamate signaling, which are PKAc, pCAMKIIα, Fyn, and PKC, etc. PKAc can stabilize the GluR1 by phosphorylating S845 and PKC/CAMKIIα at S831 [61]. Fyn is an Src family kinase (SFK); it controls NR2B stability by phosphorylating at Y1472 residue at the C-terminal YEKL domain [62]. Another prospect is that spinophilin and neurabin are scaffolding proteins needed for PP1γ and its substrate interaction near dendritic spines [63]. In ILAE class 2 patient samples, spinophilin expression is reduced compared to class 1. PKAc can prevent

PP1γ/spinophilin interaction by phosphorylating the spinophilin at S94 and S177 residues [64], suggesting that the PP1γ-spinophilin interaction could also be a limiting factor in class 2 patients.

Conclusion:

Our study reported that even though PP1γ expression is substantial in TLE-HS patient samples, it does not affect pGluR1, and pNR2B, which causes excitotoxicity during seizures. Since D1R, D1-D2 heterodimer, and NMDA receptors manage cellular calcium, a secondary signaling molecule for different proteases, kinases, and phosphatases participate in regulating AMPA, NMDA receptor localization at synapses. We did not measure the calcium levels in the samples but presumed that there might be a possibility of growth in the cellular calcium levels in TLE-HS patients. We believe this study will further help us understand different spatiotemporal receptor interactions and their downstream signaling molecules regulating the receptor density and sensitivity at synapses.

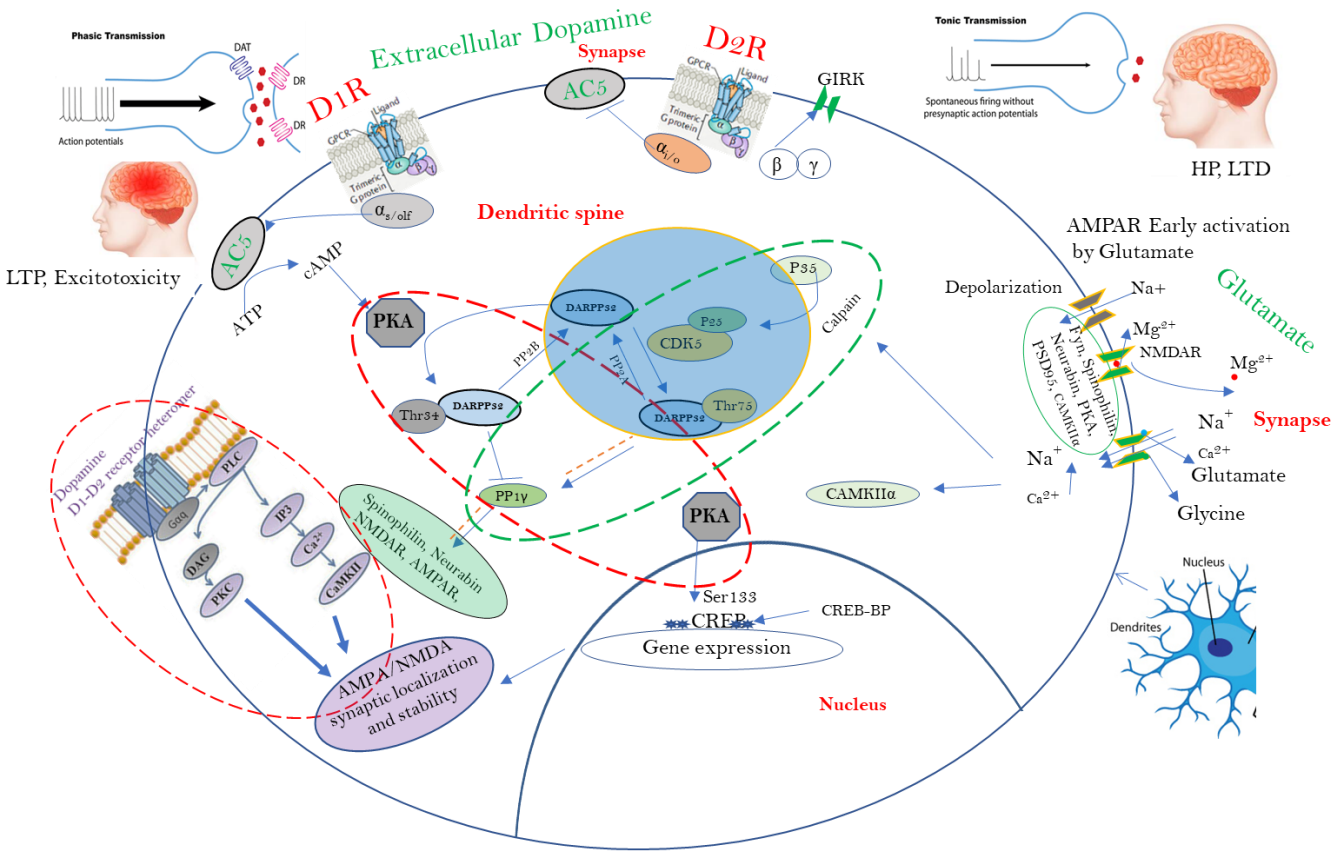

Figure 7. Representing the Dopamine, Glutamate signal crosstalk. Diagram representing the PP1γ regulation by NMDAR negative feedback inhibition signaling (green circle) and D1R signal (red circle-middle) domination over PP1γ though increased PKA, pDARPP32T34, and supports pGluR1, pNR2B in seizure recurrent patients. D1R-D2R hetero dimer activation (red circle-right) increases cellular Ca^{2+} and pCAMKIIα activation. All these events lead to calcium overload and excitotoxicity, particularly in epilepsy patients experiencing recurrent seizures.

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CHAPTER 2A

Introduction:

Cerebral cortex development is complex and involves consecutive steps to form an appropriate cortical structure [1]. Focal cortical dysplasia (FCD) is a chronic neurological disorder due to abnormal brain development in utero. It is a common cause of epilepsy in children and adults with frequent and recurrent seizures [2]. FCD has shown resistance to drug treatment [3]. Usually, symptoms are visible within the 1st year of life, however, recent studies reported that it could occur up to 60 years [4]. Reactive oxygen species (ROS) significantly damage the brain's internal environment during frequent seizures and associated comorbidities [5, 6]. Oxidative stress is responsible for the degeneration of the neuronal population in the epileptic foci [7–9]. It further leads to epilepsy initiation and progression [10]. Diets comprising antioxidants can help to reduce neuronal damage and reduce the frequency of seizures [11, 12] by providing a defense against free radicals. Cells have an endogenous robust antioxidant system to protect against the damage brought by reactive oxygen species (ROS) [13]. Enzymes such as superoxide dismutase (SOD), Catalase, peroxidase [14], NAD(P)H quinone oxidoreductase 1 (NQO1), and Heme-oxygenase-1 (HO-1), neutralize the ROS generated in the cells [15]. Nrf2 (NFE2 related factor 2, NFE2L2), a transcription factor belonging to the Cap 'N' Collar (CNC) family with conserved basic leucine zipper (bZIP) structure [16]. It activates antioxidant response by initiating the transcription of genes responsible for cellular protection against xenobiotics and oxidative stress [17, 18]. Noticeably, Nrf2 has a limited half-life and is eliminated by the ubiquitin-proteasome system in the cytoplasm [19]. Kelch-like ECH-associated protein 1(Keap1) binds to Nrf2, and negatively regulates it in the cytosol, and transfers Nrf2 to Cul3 ubiquitin for proteasomal degradation [19, 20]. During oxidative stress, Nrf2 escapes from Keap1 regulation and is associates with antioxidant response elements (ARE) at NQO1 and HO-1 gene promoters to protect the cells against oxidative stress [15, 21–24].

Rationale:

Expression of the Nrf2 transcription factor is regulated at the gene level during transcription and at the protein level in the cytosol through post-translational protein interactions [25]. At the gene level, Nrf2 expression is regulated by histone lysine methylation, leading to gene suppression [26]. Methylation sites observed in histone molecules are arginine (R), lysine (K), and histidine (H). They can be mono (me1), di (me2), and tri (me3) methylated by methyltransferases and subject to activation or suppression of a particular gene. Lysine (K) methylation of histone 3 (H3Ks) regulates Keap1 and Nrf2 [27, 28]. Enhancer of zeste homolog 2 (Ezh2) is a methyltransferase. It can trimethylates H3K9 and H3K27 residues of the Nrf2 promoter and suppresses the gene transcription [29, 30]. In addition to the Ezh2, SetD7 also monomethylates histone 3 at the 4th lysine residue. It is considered as a transcriptional activation marker [31, 32]. The presence of H3K4Me1 at the promoter of Keap1 increases the specificity of stimulation protein-1 (Sp-1) during transcription and upregulates the Keap1 expression [33]. A specific "reader" protein recognizes the H3K4Me1 and makes the chromatin accessible for transcription [34, 35]. SetD7 has several methylation target proteins such as p53 [36], estrogen receptor alpha (ERα) [37], and TBP-association factor 10 (TAF10) [38]. SetD7 also stabilizes p53 protein by monomethylation at Lys372 residue [38, 39]. Nrf2 interacts with Keap1, at DLG (aspartate, leucine, and glycine) and ETGE (glutamate, threonine, glycine, glutamate) motifs of the Neh2 domain [39]. The interaction of Keap1-Nrf2 is disturbed by a p53 target protein p21. It competes against the Nrf2 binding site to Keap1 [40] and stabilizes the Nrf2 during oxidative stress. Autophagic adopter protein p62, also known as sequestosome-1 (SQSTM1/p62), interacts with the DC pocket of Keap1, which prevents the Nrf2 binding and its degradation [40–42]. Cysteine sensors in Keap1 are sensitive to oxidative stress. The presence of ROS modifies cysteine residues, which leads to a conformational change, and stabilizes Nrf2 [43].

The stabilized Nrf2 binds to AREs along with small Maf proteins in the nucleus [44, 45] and activates phase II detoxifying enzymes [46, 47]

This study has focused on understanding the effect of histone methyltransferases, autophagy, and apoptosis-associated proteins such as p62 and p21 on the Keap1-Nrf2 axis and Nrf2 target proteins such as NQO1 and HO-1 expression in FCD with different post-surgical outcomes according to the International League Against Epilepsy (ILAE) commission suggestions [48]. This study aims to understand the significance of AO response and its regulation in patients belonging to various outcome classes, which will help predict the chances of post-surgical seizure-free outcomes concerning the antioxidant response and suggest proper medication for the FCD patients.

To proceed with the above hypothesis, we have proposed three objectives

a). To study the expression of keap1-Nrf2 dependent antioxidant enzyme expression in FCD, TLE-HS patient samples

b). To study the expression of histone methyl transferases that interfere with Keap1-Nrf2 expression

c). To study the expression of Keap1-Nrf2 complex destabilizers, which helps Nrf2 transcription factor stability

Materials and Methods:

Sample collection and Ethical guidelines:

The brain tissue (TLE/HS) samples (n=26) were frozen with liquid nitrogen and then stored at -80 ^{0}C, immediately after surgery, at the Krishna Institute of Medical Sciences (KIMS), a tertiary care center. Control (post-mortem) brain samples (n=4) were obtained from the National Institute of Mental Health and Neuro Sciences (NIMHANS), brain bank, Bangalore, India. The KIMS ethical committee and the KIMS Foundation and research center (KFRC) approved all the procedures, including experimental and sample collection processes. The patient or relative's prior consent was

taken for this study. Institutional ethical committee (IEC) guidelines were followed, and study sets were anonymized. Postoperatively, patients were followed up for at least three years for outcome assessment categorized as per International League against Epilepsy (ILAE) classification [39]. ILAE Commission Report. Proposal for a new classification of outcome with respect to epileptic seizures following epilepsy surgery [40]. Cases with ILAE class 1 and 2 were included for unequivocal analysis, as people with uncontrolled seizures (ILAE class 3 and above) could have extensive epileptogenic networks or pathways interfering with outcomes.

Study design: The tissue samples were subjected to Thioflavin T (ThT) assay to quantify the protein aggregates, which was reported in our earlier studies, and the data was correlated with clinical outcomes [41]. Based on this information, patient samples were categorized into ILAE outcome classes. For the current study, patients who were completely seizure free (n=13), and patients experiencing auras (n=13) were pooled into 4 samples in each group based on ThT assay for further analyses.

Preparation of tissue lysate and Immunoblotting:

100 mg of the patient's brain tissue sample was homogenized in a Dounce homogenizer. Radioimmunoprecipitation assay buffer (RIPA) was used to isolate the whole-cell proteins. The protease inhibitor cocktail (Sigma- Aldrich, cat no: P8340) and phosphatase inhibitors (Sigma-Aldrich, cat no: P5726) were added to the tissues before homogenizing, and proteins were isolated and quantified. 40 µg of protein from all tissue samples were subjected to SDS-PAGE. A wet transfer of proteins onto nitrocellulose membrane (Bio-Rad, USA) overnight was done in Towbin buffer at 25 V. The non-fat skimmed milk powder (Sigma- Aldrich, cat no: M7409) 5% in tris buffer saline tween 20 (TBST) was used to block the nonspecific binding of the antibody. Blots were then

incubated with primary antibodies Nrf2 (Sc-722), Keap-1 (Sc-15246), Ezh2 (CST-5246), H3K27Me3 (CST-9733), H3K4Me1 (CST-9723), H3K9Me3 (CST-13969), SetD7 (CST-2813), p21 (Novus bio, AF1047), p62 (Sc-28359), NQO1 (Novus bio, MAB7567), HO-1 (Novus bio, AF3776) diluted to 1:1000 in TBST buffer overnight at 4 ^{0}C after subsequent washing with TBS and TBST the blots were incubated at room temperature for 1 hour with secondary IgG antibodies anti-mouse (CST-G21040), anti-rabbit (CST-7074P2), anti-goat (Thermo fisher, A16005) conjugated with HRP with 1:15000 dilution. Later, blots were washed with TBS and TBST and developed with a chemiluminescent reagent (Clarity^{TM} western ECL substrate 1705060) using Bio-Rad, USA molecular imager.

Double-Immuno fluorescence:

5 µm thick tissue sections (n=4) were used to perform a double immunofluorescence assay. The paraffin-embedded tissue blocks were prepared using the tissue stored in a 10% formalin solution. The control tissues collected from the brain bank were not suitable for formalin fixation because they were held at -80 ^{o}C. Slides were subjected to deparaffinization with xylene and rehydrated with ethanol (100%, 95%, and 70%). Then citrate buffer 10 mM, pH 6.0, was boiled, and slides were kept in the buffer to retrieve the antigen. The blocking buffer was added to the section to prevent nonspecific binding. Buffer was provided with 5% normal goat serum (CST-5425) and 1% BSA dissolved in 1xPBS containing 0.3% Triton X-100. Then slides are left at room temperature for 1 hour in a humid chamber. Following the incubation, the blocking buffer was replaced with primary antibodies and incubated for 16 hours at 4 ^{o} C to check Keap1 and Nrf2 in TLE-HS brain sections. Later primary antibody was washed with PBS three times. To this 1:1000 dilution fluorescent conjugated anti-rabbit IgG (4412S Alexa fluor® 488) from CST and anti-goat IgG (A32816, Alexa fluor ® 555) from Thermo Fisher Scientific, secondary antibodies were added and incubated at room

temperature in the dark. After 1 hour, sections were washed with 1xPBS and mounted with a mounting agent (CST-8961S) Prolong® Gold antifade reagent with DAPI. The images were obtained using Carl Zeiss LSM 710 with Zen Blue software, and the fluorescent intensity was quantified with Image J software.

Data Analysis:

Statistical analysis among the groups was performed using Sigma Plot 2000 software for the Windows version. One-way ANOVA by the Newman-Keuls method was used for post-hoc analysis. Data from the experiments have been presented as Mean ± SEM. The p-value <0.05 considered as a statistically significant (***p< 0.001; **p< 0.01; *p< 0.05).

Results:

Nrf2 transcription factor availability is limited in FCD patient samples:

In FCD patients, Nrf2 (fig. 1c) protein did not show any significant increase in expression compared to control brain samples. However, the Nrf2 negative regulators such as Ezh2 (fig. 1d) was significantly upregulated in FCD patient samples (class1, 5.083±0.913; class 2, 3.567±0.673; class 3, 2.741±0.565) than controls (1.008±0.75), (p<0.001). Keap1 (fig.1b) was found to be significantly increased in ILAE's class 1 and class 3 (p<0.009; 4.046±0.61& p<0.001; 4.958±0.785) samples than controls and ILAE class 2 patient samples. In addition to Ezh2, SetD7 (fig. 1e), an H3K4 mono methyltransferase which acts positively on Keap1 was upregulated in class 1 (p<0.002; 5.098±0.143) and class 2 (p<0.001; 5.833±0.379) when compared to control samples and class 3 (p<0.03; 3.165±1.108). This might lead to immediate degradation of available Nrf2 in the cytosol by ubiquitination.

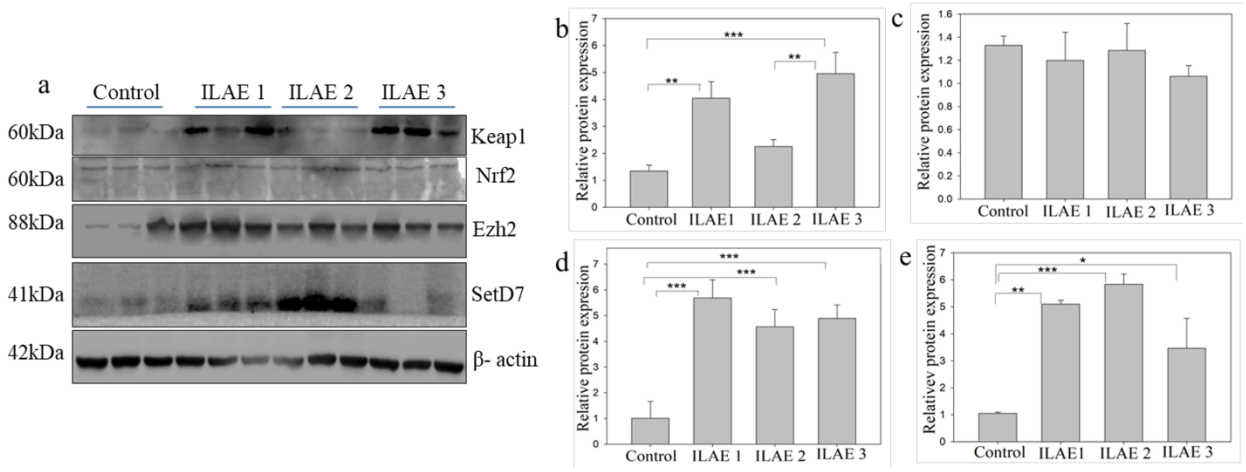

Fig. 1: Immunoblot assay of Keap1 (b), Nrf2 (c), Ezh2 (d), SetD7 (e) and internal control beta-actin performed for the control and FCD resected epilepsy human samples belonging to ILAE class, the corresponding results were represented as bar graphs. The base panel represents beta-actin. Data representing mean ± SEM, n=4. ***p< 0.001; **p< 0.01; *p< 0.05.

Histone methyltransferases (Ezh2 and SetD7) increase the Histone 3 lysine (H3K) methylated residues in FCD patients:

Histone 3 lysine methylation was found to be increased in FCD patient samples. This was correlated with increased expression of Ezh2 and SetD7. H3K9Me3 (fig. 2c) was significantly increased in FCD samples that belonged to ILAE classes 1, 2, and 3 ($p<0.001$; 32.886±3.392, 34.473±3.76, and 22.69±1.421) respectively, than controls. Similarly, H3K27Me3 (fig. 2d) showed a significant increase in class 1 ($p<0.003$; 23.24±3.074) and class 2 ($p<0.001$; 30.226±5.915) compared to class 3 and control samples. H3K4Me1 (fig. 2b) expression was more significant in ILAE classes 1, 2, and 3 ($p<0.001$; 128.693±9.674, 137.265±15.789, and 149.058±3.819), respectively, as compared to autopsy control samples.

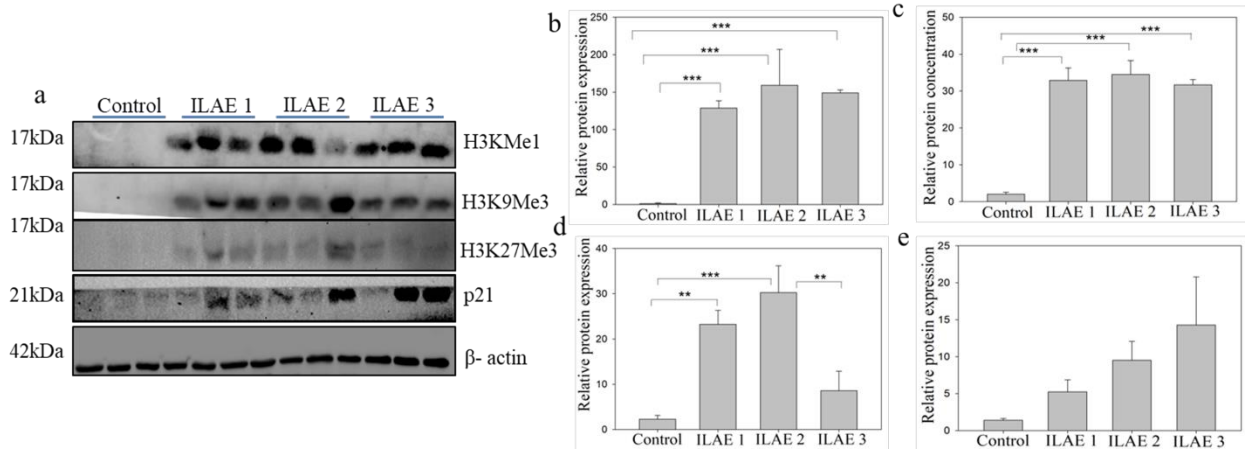

Fig. 2: Immunoblot assay of H3K4Me3 (b), H3K9Me3 (c), H3K27Me3 (d), p21 (e) and internal control beta-actin performed for the control and FCD resected epilepsy human samples belonging to ILAE class, the corresponding results were represented as bar graphs. The base panel represents beta-actin. Data representing mean ± SEM, n=4. ***p< 0.001; **p< 0.01; *p< 0.05.

Accumulation of p62 and p21 can reduce the Keap1dependent Nrf2 degradation in FCD:

Severe endoplasmic stress accumulates autophagy adaptor protein p62 and p53 target, i.e., p21, during epilepsy. In FCD, p21 (fig. 2e) protein expression was not statistically significant among the ILAE classes and controls, whereas p62 (fig. 3b), another Keap1-Nrf2 complex destabilizer, showed considerable upregulation in ILAE class 1 ($p<0.008$; 13.324±2.13), class 2 ($p<0.008$; 11.549±3.762), and class 3 ($p<0.001$; 17.827±0.779) as compared to the controls.

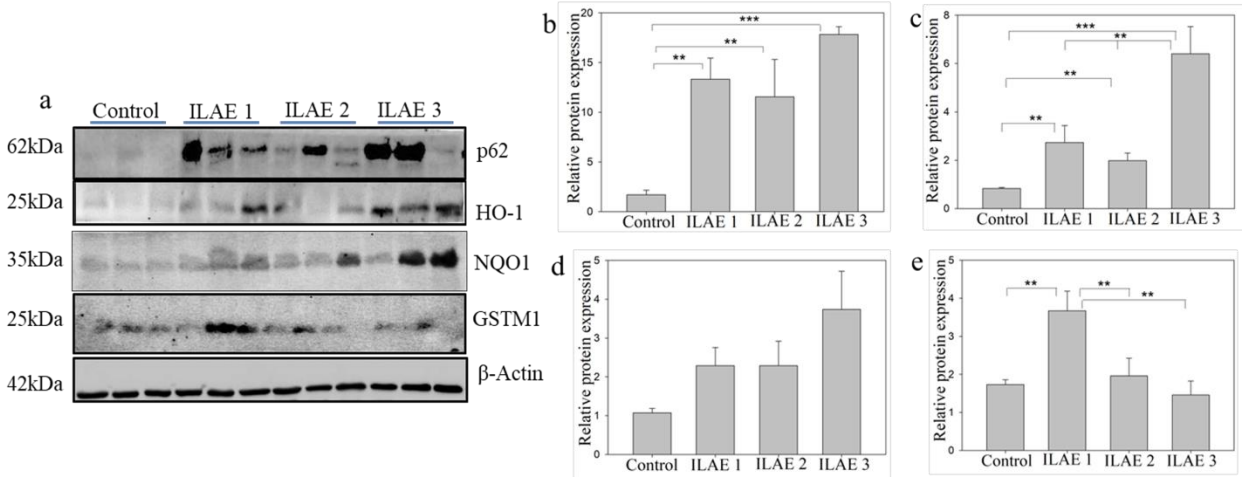

Fig. 3: Immunoblot assay of p62 (b), HO-1 (c), NQO1 (d), GSTM1 (e), and internal control beta-actin performed for the control and FCD resected epilepsy human samples belonging to ILAE class, the corresponding results were represented as bar graphs. The base panel represents beta-actin. Data represented as mean ± SEM, n=4. ***p< 0.001; **p< 0.01; *p< 0.05.

Nrf2 stabilized by p62 and p21 can increase the phase II antioxidant enzymes expression:

Protein expression of phase II antioxidant enzyme HO-1(fig.3b) was significantly increased in ILAE class 1, 2, and 3 ($p<0.005$; 2.720±0.7016), ($p<0.006$; 1.980±0.3070), ($p<0.001$; 6.390±1.1280) respectively as compared to controls (0.829±0.040). On the contrary, NQO1 (fig.3d) did not show any statistically significant increase in expression in FCD and control samples. GSTM1 (fig. 4e) showed increased protein expression in ILAE class 1 compared to classes 2 and 3 of FCD also autopsied control brain samples.

Discussion:

The resected epileptic brain samples collected during surgery are the ideal model system to study the process of epileptogenesis [49]. Post-surgical follow-up data suggested that some of these patients had seizure recurrence. Earlier studies have reported that FCD patients face severe endoplasmic reticulum (ER) stress during seizures [50]. The excitotoxicity causes the accumulation of misfolded/unfolded proteins, sometimes leading to autophagy impairment [51]. In addition, excitotoxic insults generate ROS, which is harmful to the brain cells [5, 6]. Some studies suggested that ROS can cause the initiation and progression of epileptic conditions [10]. When we performed the H_2O_2 neutralization efficiency assay for these epileptic and control brain samples, we have observed a better antioxidant (AO) response in ILAE class 1 patients, and it is close to the control samples. But ILAE class 2 and class 3 samples do not have a better AO response also significantly

differ from controls and class 1 patient samples. So, we explored the molecular changes associated with the AO response signaling mechanism to understand the reason for not having a better AO response in spite of excitotoxicity-induced oxidative stress during epilepsy. The keap1-Nrf2 axis plays a central role in detoxifying ROS [17, 18]. The current study found that FCD samples expressed Keap1 abundantly in ILAE classes 3 and 1 compared to class 2 and controls. Since Keap1 acts as a negative regulator of Nrf2, its expression limits the AO response despite the presence of Nrf2. But in our study, we haven't found any significant upregulation of Nrf2 transcription factor in FCD patient samples, and also, the Nrf2 target antioxidant enzymes such as NQO1 and HO-1vary among the groups. The reasons for not having a better antioxidant response despite seizure-induced excitotoxic insults are unclear. Therefore, we explored the role of epigenetic control over the Keap1-Nrf2 axis through histone methylation [24,25,26]. Studies in lung cancer patients reported that downregulation of Ezh2 could lead to increased expression of Nrf2 and its targets such as NQO1, HO-1 [27], which can detoxify the ROS.

Ezh2 (Enhancer of zeste homolog 2) is a histone methyltransferase that trimethylates lysine residues of histones, H3K9 and H3K27. The H3K9 and H3K27 are critical at the Nrf2 promoter [29, 30]. We found a significant increase in the trimethyl H3K9 and H3K27 proteins in the FCD patients, suggesting the strong suppression of Nrf2 transcription initiation at the promoter. Another histone methyltransferase is SetD7 [31, 32], which monomethylates H3K4 at the Keap1 promoter. In addition to H3K9 and H3K27, which negatively regulate the Nrf2, we also found H3K4Me1 upregulation, promoting the Keap1 expression. Since Keap1 inhibits Nrf2, upregulation of both Ezh2 and SetD7 indicates a robust negative regulation of Nrf2 in FCD patients by modification of the histone lysine residues. It suggests that the regulation of oxidative stress response driven by the Keap1-Nrf2 axis is not a random process. Instead, these FCD patients were probably predisposed to the Nrf2

transcription factor expression, stability, and antioxidant response. To understand it more precisely in-depth studies are required.

Reduced antioxidant response can damage brain cells and possibly promote seizure occurrence because the antioxidant response is essential for combating oxidative stress, especially during seizures [52]. The increased ROS severely affects the autophagy response [53], leading to the accumulation of autophagy adaptor protein p62 [37,38] and also promoting apoptosis by activating CHOP/GADD53 [54] and the p21 accumulation [55]. FCD patients showed strong negative regulation over Keap1-Nrf2-dependent antioxidant response through histone methylation. In the current study, the ILAE classes 1 and 2 patients had increased HO-1 and NQO1 expression compared to ILAE classes 3. The expression of NQO1 and HO-1 depended on stability and availability of Nrf2. Studies reported that several factors disturb the Keap1-Nrf2 interaction [56]. These factors include miRNAs, PKC, p62, p21, NF-κB, Brca1, and signaling pathways like Notch1, PI3-Akt, etc. [56].

The p62 and p21 proteins interact with the Keap1 at DC pocket and Nrf2 at DLG motif, respectively, to stabilize Nrf2 in cytosol against Keap1 dependent ubiquitination [40, 57]. The NLS of Nrf2 helps its nuclear translocation, where it binds to small maf proteins and activates antioxidant enzymes [58]. Since ILAE class 3 patients had increased p62, and ILAE class 2 and class 1 had p21 protein accumulation, it may increase the Nrf2 stability, thereby the expression of HO-1, NQO1 proteins, despite negative regulation of Keap1 and histone methyltransferases. But the patient samples did not show any significant presence of HO-1, NQO1 in class 3 patients, on other hand ILAE class 1 showed increased HO-1, NQO1 expression compared to class 2.

Conclusion:

Nrf2 transcription factor is critical for antioxidant response and functions by activating the genes involved in free-radical/reactive oxygen species scavenging activity. The patient cohort in our study showed no significant increase in Nrf2 expression despite oxidative stress. Our data suggest that these patients are predisposed to Nrf2 dependent antioxidant response due to Nrf2 negative regulation at the gene level through histone lysine methylation and at protein level through Keap1 dependent proteasomal degradation. It also suggests that modulation of antioxidant enzymes mediated by Keap1-Nrf2 interaction destabilizing proteins such as p21 and autophagy adaptor protein p62 have no unique advantage in FCD patients.

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CHAPTER 2B

Introduction:

Temporal lobe epilepsy (TLE) with hippocampal sclerosis (HS) is one of the most common causes of epilepsy, with nearly one-third of the patient population remaining drug-resistant. Patients experiencing seizures develop a new and random neuronal network, which induces significant changes in brain electrical activity [1]. This significantly impacts the quality of life and cognitive changes, thereby necessitating surgical resection of epileptic foci [2]. Standard temporal lobe resection surgery remains the mainstay treatment in drug-refractory cases, with 60-70% remaining seizure-free over long term [3]. Existing anti-seizure medications predominantly target seizure propagating mechanisms and act by suppressing seizures. The reasons for persistence of seizures in a significant number of patients after surgical treatment are yet unclear. An irregular neuronal network arising from recurrent seizures further promotes random neuronal firing and excitotoxicity, which is often attributed to dopamine and glutamate signaling [4]. The resultant effect includes disturbance in cellular homeostasis, lipid peroxidation, and reactive oxygen species (ROS) accumulation which leads to oxidative stress (OS) [5, 6]. Also, ROS upregulation aggravates neuroglial damage, thereby reducing the seizure threshold [7–9]. It is considered one of the significant underlying causes of epilepsy progression [10]. Endogenous antioxidant defense mechanisms counteract excessive ROS-induced cellular damage [11]. This system includes phase I antioxidant enzymes like catalase, peroxidase, superoxide dismutase (SOD), Glutathione S transferase M1 (GSTM1), NAD(P)H quinone oxidoreductase (NQO1), and heme oxygenase-1 (HO-1) that can effectively detoxify excessive ROS formed in the tissues [12–16].

Nuclear factor erythroid 2-related factor2 (Nrf2 encoded by NFE2L2), a Cap'n Collar basic leucine zipper transcription factor, regulates transcription of phase II antioxidant enzymes such as NAD(P)H quinone oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO-1) [13, 17]. Kelch-like ECH-

associated protein 1(Keap1) negatively regulates Nrf2 by forming a complex with Cul3 (cullin 3, an E3 ubiquitin ligase) and subjecting Nrf2 to proteasomal degradation, thereby maintaining low steady state levels of Nrf2 [18, 19]. In connection with regulation of cellular antioxidant response, Nrf2 upregulates NQO1, and HO-1 [9]. Also, upregulation of HO-1 and NQO1 has been shown to confer protection on neurons from an increasing OS [14–16, 20, 21].

During oxidative stress (OS), electrophiles react with Cysteine (Cys) sensors within Keap1 and alter its conformation [22]. Therein, Nrf2 escapes Keap1 regulation and translocates into the nucleus, where it binds to ARE (antioxidant response elements) by forming a dimer with small Maf proteins and elicits an antioxidant response [23, 24]. Keap1 and Nrf2 expression and interactions are regulated at the transcriptional and post-translational levels by several factors [25]. At gene level Keap1 and Nrf2 expression is regulated by histone methylation (mono (me1), di (me2), or tri (me3)) at arginine (Arg)/lysine (Lys)/histidine (His), which is critical in the compaction of chromatin and resultant gene repression [26]. Depending on the Lys residue methylated and extent of methylation, the corresponding gene can be activated or suppressed. Lysine residues of histone 3 (H3Ks) play an important role in modifying histones by methylation that has a transcriptional consequence. Studies indicated that H3K27me3 negatively regulates Nrf2 expression and downregulates HO-1 and NQO1 gene expression [27, 28]. EZH2 (Enhancer of zeste homolog 2), the catalytic subunit of multimeric complex viz., polycomb repressive complex 2 (PRC2), is a histone Lys methyl transferase that induces transcriptional repression by trimethylating H3K27 and also influencing H3K9 methylation at Nrf2 gene promoter to repress its transcription [27, 28]. Another histone methyl transferase known as SetD7 (SET7/9; SET domain-containing 7 histone lysine methyltransferase), specifically mono-methylates H3K4, facilitating the binding of stimulating protein-1 (Sp-1) at Keap1 gene promoter and upregulates Keap1 protein expression [29]. Also, post-translational modifications modulate

Keap1-Nrf2 interaction during oxidative stress, allowing Nrf2 translocation into the nucleus and binding to ARE elements of HO-1 and NQO1 [30, 31]. Besides histone methylation, SetD7 also methylates Lys372 of p53, thereby increasing protein stability, which upregulates target gene p21 [32, 33]. The C-terminal region of p21 can interact with DLG (ASP-21, Leu-25, and Gly-31) and ETGE (76LDEETGEFL84) motifs of Nrf2 [34] and compete with Keap1-Nrf2 complex formation [35], thereby facilitating the Nrf2 stabilization. Another potent Nrf2 stabilizers are the autophagic adopter protein, sequestosome-1 (SQSTM1/p62) [36]. It binds to the DC (Double glycine repeat domain and C-terminal region) pocket of Keap1 like Nrf2 and facilitates Nrf2 to escape from ubiquitin-dependent degradation [37, 38].

We tried to understand the molecular foundation of antioxidant response consisting of the aforementioned components in the current study due to the vital role of the Keap1-Nrf2 axis for antioxidant response against increased oxidative stress in an epileptogenic setting.

Data Analysis:

The statistical analysis was performed using a student t-test between groups by the Newman-Keuls method for post hoc analysis. Data were expressed as mean$\pm$SEM. A p-value of <0.05 was considered statistically significant and was determined by Sigma Plot 2000 software for the Windows version.

Results:

Nrf2 protein expression was decreased in TLE-HS patients with seizure recurrence:

Nrf2 plays a central role in regulating the expression of phase II detoxifying enzymes to neutralize ROS. In our study, it was observed that Nrf2 (fig. 1c) protein expression was higher in ILAE class 1 ($p<0.005$; 2.58±0.43) compared to class 2 (0.86±0.071) and controls ($p<0.02$; 1.65±0.30). Therefore, we focused on the Nrf2 negative regulator Keap1, which directs Nrf2 for ubiquitination in the cytosol. It was observed that Keap1 (fig. 1b) protein expression was significantly upregulated in TLE-HS patients belonging to ILAE class 2 ($p<0.02$; 1.57±0.13) compared to class 1 (1.43±0.24) and controls (1.05±0.06).

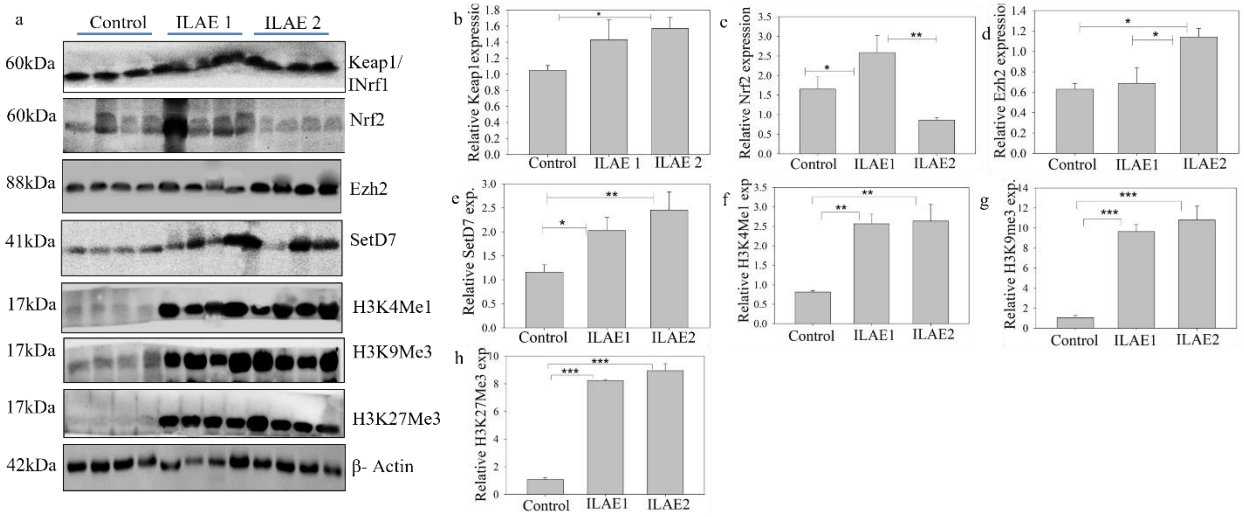

Fig. 1 a. Immunoblot analysis for Keap1 (b), Nrf2 (c), Ezh2 (d), SetD7 (e), H3K4Me1 (f), H3K9Me3 (g), H3K27Me3 (h) and β-actin was done for the controls and resected epileptic patient brain samples and protein quantification of corresponding immunoblot results represented by bar graphs. Beta-actin is used as internal control, represented by the base panel. Data represented as mean ± SEM, n=4. ***$p< 0.001$; **$p< 0.01$; *$p< 0.05$.

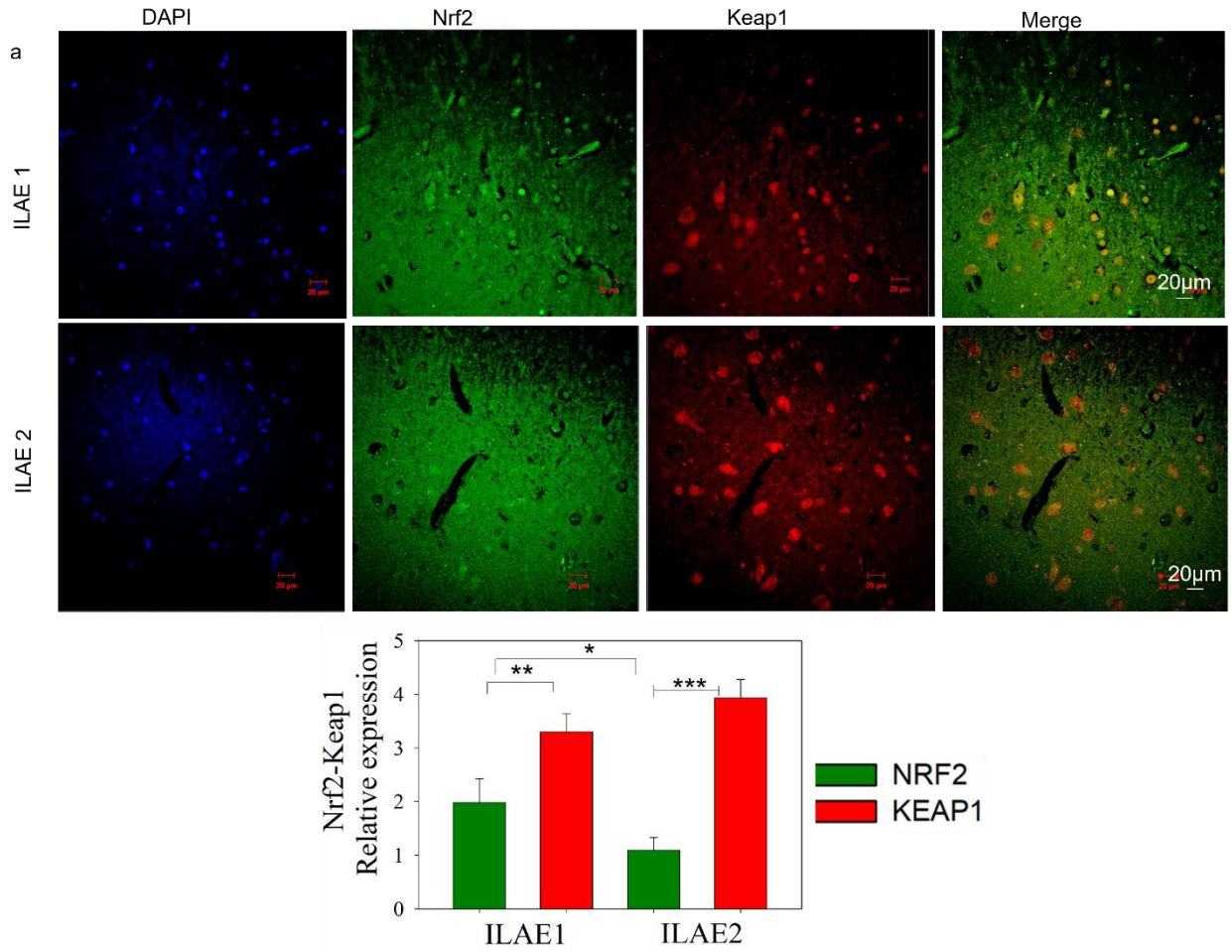

Fig. 2 Double immunofluorescence analyses to estimate the relative fluorescence intensity of Keap1 (Red) and, Nrf2 (Green), DAPI (Blue) in TLE-HS clinical samples. The scale bar represents 20 µm, n=3. ***p< 0.001; **p< 0.01; *p< 0.05.

Further, a double immunofluorescence assay was performed for Keap1 and Nrf2 in ILAE class 1 and class 2 TLE-HS patient samples (fig. 2a), It was observed that the fluorescence intensity of Keap1 was higher in class 2 ($p<0.001$; 3.93±0.34) and class 1 ($p<0.001$; 3.29±0.34 when compared with Nrf2. This data correlated with Keap1 (fig. 1b) and Nrf2 (fig. 1c) immunoblot assay findings. This finding suggests that Nrf2 is significantly negatively regulated in class 2 patients compared to completely seizure-free patients.

Histone methyltransferases regulate Keap1 and Nrf2 expression:

Previous studies reported that HMTs SetD7 and EZH2 are known to methylate H3K4 and H3K27 residues in Keap1 and Nrf2 promoter regions, respectively, and control their expression by modifying the methylation status of histones [27, 28], [29]. Based on these reports, we evaluated the protein expression of SetD7 and EZH2 in TLE-HS patient samples. EZH2 (fig. 1d) expression significantly increased in ILAE class 2 patients ($p<0.02$; 1.14±0.08) as compared to class 1 (0.68±0.15) and controls (0.63±0.05). SetD7 (fig. 1e) expression was found to be increased in ILAE class 2 ($p<0.009$; 2.45±0.091) and class 1 ($p<0.03$, 1.91±0.30) compared to controls (1.19±0.21). This indicates that significant negative regulation over Nrf2 expression is imposed by histone methylation.

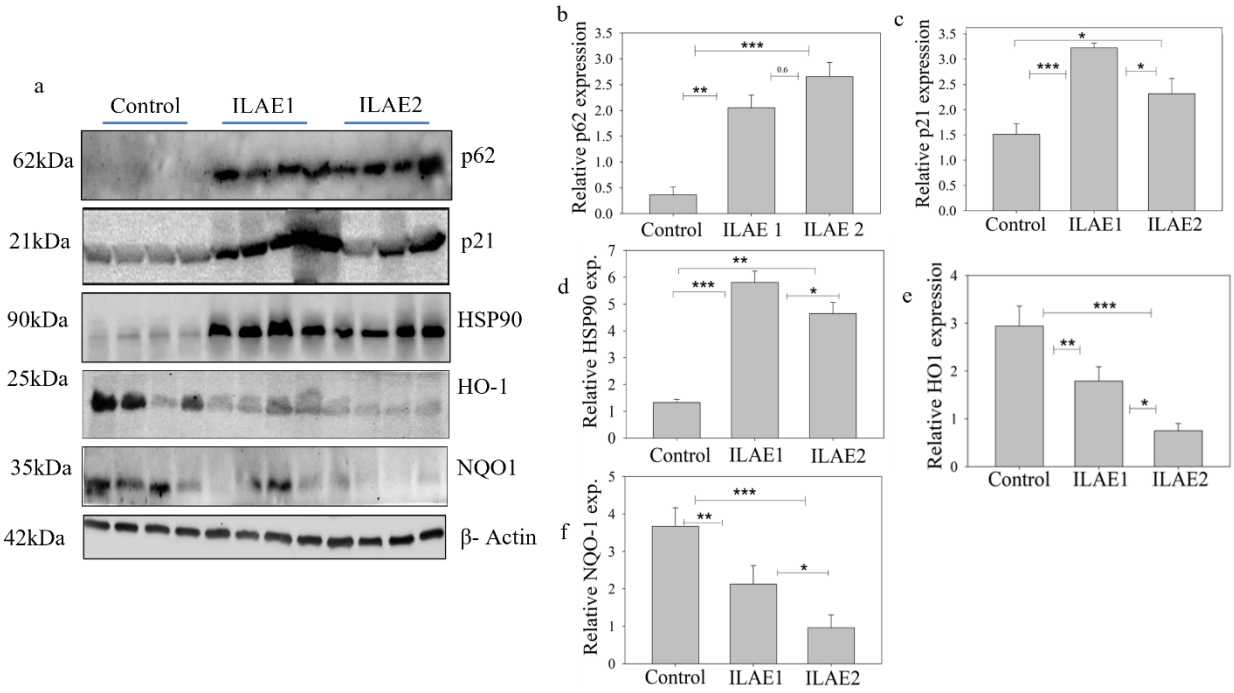

Fig. 3 a. Immunoblot analysis for p62 (b), p21 (c), HSP90 (d), HO-1 (e), NQO1 (f), and β-actin were probed for the control and resected epileptic patient brain samples and protein quantification of corresponding immunoblot results represented by bar graphs. Beta-actin is used as internal control, represented by the base panel. Data represented as mean ± SEM, n=4. ***p< 0.001; **p< 0.01; *p< 0.05.

EZH2 and SetD7 could methylate histones responsible for Nrf2 and Keap1 expression in TLE-HS patients:

EZH2 trimethylates H3K27 and influences H3K9 trimethylation [42]. H3K27me3 and H3K9me3 histone marks in promoters transcriptionally repress Nrf2 gene expression. In our study, it was observed that H3K27me3 (fig. 1h) expression significantly increased in ILAE class 1 and 2 categories ($p<0.001$; 8.22±0.08, $p<0.001$; 8.93±0.51), respectively, compared to controls (1.09±0.11). H3K9me3 (fig. 1g) band intensity also increased in ILAE class 1 and 2 ($p<0.001$; 9.64±0.70, $p<0.001$; 10.80±1.39) respectively than controls (1.06±0.22). Further, SetD7 mono-methylates H3K4 (fig. 1f). H3K4me1 positively regulates Keap1 expression, leading to additional negative control over Nrf2 availability in the cytoplasm. In TLE-HS patient samples, we found a significant increase of H3K4me1 expression in classes 1 and 2 ($p<0.005$; 2.56±0.25, $p<0.007$; 2.63±0.43), respectively, compared to autopsy control samples (0.81±0.03). Therein, upregulation of histone methylation could contribute to significant suppression of Nrf2 and could also support Keap1 expression.

p62 and p21 proteins which can bind to Keap1 and Nrf2 were upregulated in TLE-HS patients:

Looking into the interactor proteins of Nrf2, autophagy adapter protein p62, and apoptosis inhibitor protein p21 can interact directly with Keap1 and Nrf2, respectively. In TLE-HS patients, we found increased expression of p62 (fig. 3b) in ILAE class 1 ($p<0.001$; 2.05±0.24) and class 2 ($p<0.001$; 2.65±0.27) as compared to controls (0.36±0.15). Nrf2-interacting protein p21 (fig. 3c) band intensity was also significantly high in TLE-HS samples belonging to ILAE 1 ($p<0.001$, 3.28±0.20) compared to controls (1.95±0.15) and ILAE 2 ($p<0.03$, 2.32±0.12). The keap1 interacting protein HSP90 [43] (fig.3d) was found to be increased in class 1 ($p<0.03$; 5.89±0.37) as compared to class 2 (4.52±0.27)

and controls (1.26±0.15). This could suggest that p21 and p62 interactor proteins interfere with Nrf2-Keap 1 interaction, thereby preventing ubiquitin-mediated Nrf2 degradation.

Expression of phase II detoxifying enzymes is compromised in TLE-HS patients:

Phase II detoxifying enzymes are essential to clear excessive ROS produced during excitotoxicity-induced oxidative stress as in epileptic setting. Therefore, expression patterns of phase II detoxifying enzymes such as HO-1 and NQO1 were investigated. We found an increased expression of HO-1 (fig. 3e) in ILAE class 1 ($p<0.02$; 1.81±0.21) compared to ILAE class 2 (0.62±0.09) but not with respect to controls ($p<0.001$; 2.97±0.32). Similarly, NQO1 (fig. 3f) band intensity increased significantly in class 1 ($p<0.02$; 2.12±0.35) compared to ILAE class 2 patient samples (1.06±0.2). However, controls (3.67±0.49) showed a significant increase compared to class 1 ($p<0.009$) and class 2 ($p<0.001$). This indicates that phase II antioxidant response is reduced in class 2 TLE-HS patients.

Discussion:

Antioxidant activity is pivotal in protecting neurons and other cells in seizure microenvironment. A defective antioxidant response can lead to ROS-induced membrane lipid oxidation and cell death [44]. Excessive free radicals generated during excitotoxicity and frequent seizures have been reported in TLE-HS patients [45]. This could lead to a reduction of seizure threshold resulting in spontaneous, recurrent seizures. Since cell death due to excitotoxicity is apparent in TLE-HS patients [46], it is critical to understand the molecular basis governing the antioxidant response signaling mechanisms and their impact on inherent epileptogenesis in TLE-HS patients. Keap1-Nrf2 axis is an important antioxidant response mechanism involved in neutralizing the free radicals by inducing phase II detoxifying enzyme expression [11].

The current study aimed at understanding the epigenetic underpinnings of Keap1-Nrf2 signaling and the downstream antioxidant response [47, 48]. Histone methylation is one of the gene regulation mechanisms which can either repress or promote transcription, and hence gene expression depending on the methylation sites and extent of methylation on Lys residues in the respective histones. It has been reported that H3K27me3 negatively regulates Nrf2 expression and downregulates HO-1 and NQO1 gene expression [27,28]. The catalytic component of PRC2, viz., EZH2 trimethylates H3K27 and influences H3K9 methylation so as to repress Nrf2 transcription [49–51]. In line with this, earlier studies reported that EZH2, that trimethylates H3K27 promotes cell death by reducing Nrf2-dependent drug resistance in lung cancer tissues [47]. In the current study, TLE-HS patients showed a significant increase in EZH2 and SetD7 expression in ILAE class 2 compared to class 1. This indicates that there could be a decrease in Nrf2-mediated antioxidant response by promoting Keap1 expression. In line with this, we observed a significant increase in Keap 1 expression and a corresponding decrease in Nrf2 protein in class 2 patient samples. In our study, we observed that histone trimethylation status of H3K27 and H3K9 corresponded with reduced Nrf2 expression in class 2 samples compared to class 1. Alternatively, SetD7 monomethylates H3K4 and has been reported to positively regulate Keap1 expression in diabetic retinopathy [29]. A similar increase in SetD7 expression was observed in class 2 patient samples in our study.

It has been well documented that Keap1-Nrf2-ARE pathway regulates NQO1 and HO-1 expression in central nervous system [14–16, 20, 21, 49]. Nrf2 is a transcription factor that binds to ARE of phase II antioxidant enzymes genes during oxidative stress [50, 51]. In the current study, NQO1 and HO-1 protein expression was elevated in class 1 compared with class 2 patient samples. Based on significant contribution of Nrf2-Keap, it could be suggested that Keap1-Nrf2 dysregulation could possibly aggravate epileptic seizures and cognitive impairments [10].

The N-terminus of the Nrf2 protein has ETGE and DLG sequence motifs that bind explicitly with the two Kelch domains of Keap1 homodimer and is targeted by cullin3-based ubiquitin E3 ligase [52–54]. Since the half-life of Nrf2 is significantly less (20 minutes), and sequestered by Keap1 in the cytoplasm before it enters the nucleus [55], Nrf2 needs to overcome Keap1 regulation. Earlier studies reported that the Keap1-Nrf2 interaction is destabilized by p62 and p21 [34, 56]. Komatsu M, et al. suggested that p62, an autophagy adapter protein can also interact with the Kelch motif of Keap1 and increase Nrf2 half-life [37, 56]. The Kelch motif is critical for interaction with Nrf2's ETGE and DLG motif for the purpose of ubiquitination of Nrf2 in the cytosol [51]. Also, p21 stabilizes Nrf2 against Keap1 and facilitates its nuclear translocation. The KKR motif of p21 interacts with ETGE and DLG motifs in Nrf2 and compromises the Keap1-Nrf2 ubiquitination [34]. In an attempt to understand the role of p62 and p21 in relation with Nrf2-Keap 1 axis in an epileptogenic setting, we examined their corresponding expression patterns in our study population. We found a significant upregulation of p62 in TLE-HS patient samples than controls due to the accumulation of protein aggregates and chronic ER stress and resulting cell death during seizures in TLE-HS patients. With respect to p21, there was a significant increase in its expression in class 1 compared to class 2. Given the regulatory effect of p21 and p62 on the stabilization/destabilization of Keap1-Nrf2 complex, it could be suggested to play a role in mediating the phase II antioxidant enzyme-mediated detoxifying response in TLE-HS patients.

Our study indicated that TLE-HS patients did not exhibit an increase in Nrf2 expression despite seizure-induced oxidative stress. It indicates that Nrf2 is strongly and negatively regulated in TLE-HS patients prone to seizure recurrence. Commenting on the significance of the results in current study, with respect to epigenetic underpinnings of Keap1-Nrf2 axis, a significant variation in the expression of histone methylases (as discussed above) and their target antioxidant response

components was observed in our study population. Compromised functioning of Keap1-Nrf2 axis could lead to excessive ROS accumulation, cytotoxicity and cell death in an epileptic brain that could lead to reduction of seizure threshold [7–9]. All these observations point towards a relationship between the downregulation of antioxidant response that in turn has implications for development of random neural networks that propagate seizure microenvironment in patients and, eventually seizure recurrence [57].

Conclusion:

In the current study, TLE-HS patients with seizure recurrence displayed significantly reduced antioxidant enzyme expression that could be attributed to excitotoxicity. There was an upregulation of histone methyltransferases and methylated histones that, in turn, affected Nrf2 transcription. Also, Keap1, a negative regulator of Nrf2, was upregulated, particularly in ILAE class 2, compared to class 1. Therein, our study findings conclude that antioxidant response might be critical in alleviating seizure recurrence in TLE-HS patients. In view of significant tissue metabolic stress in an epileptogenic setting, inquiring into the basis of antioxidant response as in the current study would enable the understanding of molecular basis and development of possible novel therapeutic targets for mitigating epileptic seizure recurrence.

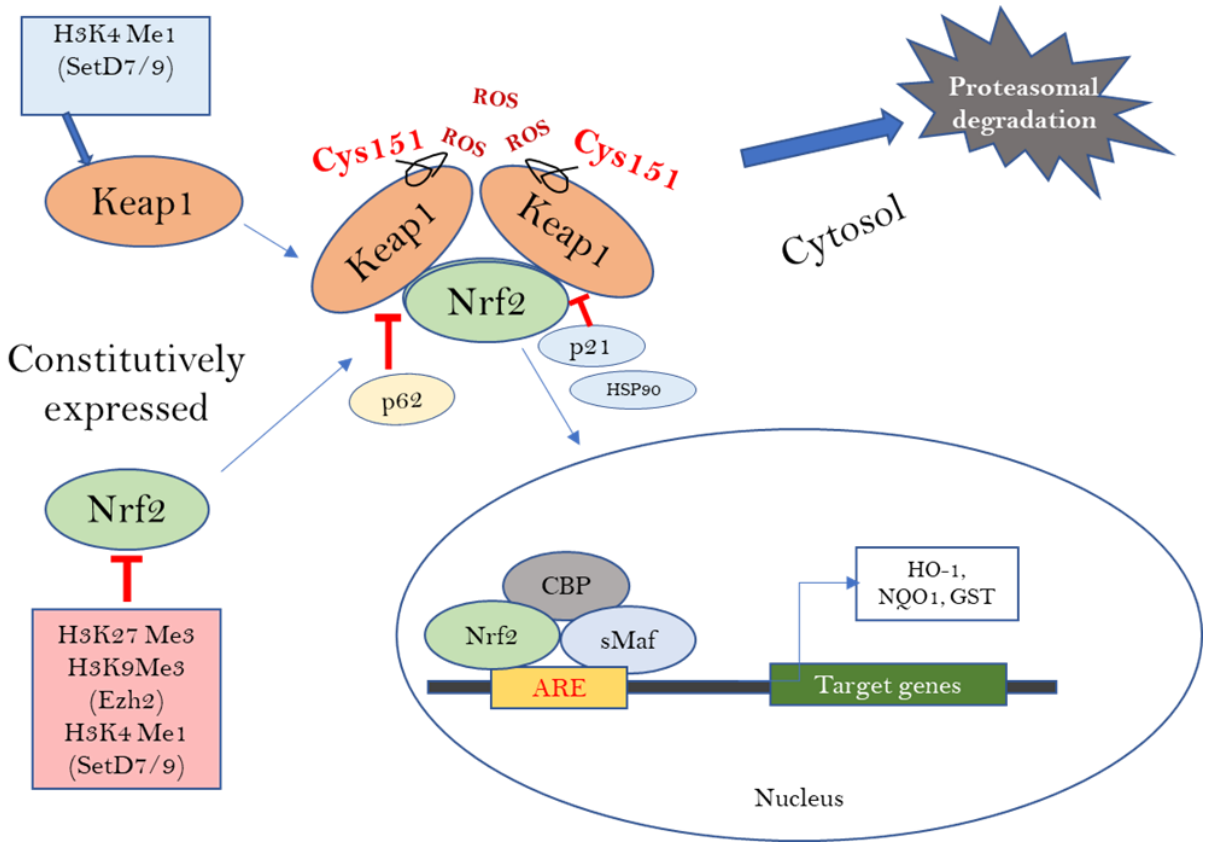

Figure representing the significance of Keap1-Nrf2 signaling mechanism in generation of phase II antioxidant response.

Keap1-Nrf2 controls antioxidant response by regulating phase II antioxidant enzymes like HO-1 (heme oxygenase-1), NQO1 (NADPH-Quinone Oxidoreductase1), and glutathione-S-transferase (GST). The Nrf2 released from negative regulation by Keap1 causes its translocation into nucleus, forming a complex with cAMP response-element binding protein (CBP) and small Maf proteins (sMaf). This complex subsequently binds antioxidant response element (ARE) and elicits an antioxidant response involving expression of phase II antioxidant enzymes. Reactive oxygen species (ROS) modify Cysteine 151 residue, p62 (sequsetosome-1), and interacts with Nrf2- binding site in Keap 1. p21 and HSP90 prevent Nrf2 interaction with Keap1. At transcriptional level, histone methyltransferases like EZH2 (Enhancer of zeste homologue2), and SetD7 (SET7/9; SET domain-containing 7 histone lysine methyltransferase) and corresponding histone targets viz., H3K27me3, H3K9me3 and H3K4me1 influence Nrf2 and Keap1 expression respectively.

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CHAPTER 3

Introduction

In both the pediatric and adult populations, focal cortical dysplasia (FCD) is a major cause of drug-resistant epilepsy [1]. FCD occurs due to the malformation of the cortex during brain development. Amongst its subtypes, type I has disruption of intracortical lamination and columnar organization. Cortical dyslamination and dysmorphic neurons characterize type IIa, while balloon cells describe type IIb [2–4]. Both FCD types I and II are associated with intractable epilepsy. Most patients with FCD show recurrent seizures even after using antiepileptic drugs [5]. One-third of the patients affected by epilepsy respond poorly to contemporary antiepileptic medicines [6]. As a chronic neurological disorder, epilepsy often contributes to neurodegeneration due to disturbance in the endoplasmic reticulum (ER) homeostasis. ER stress is triggered by various intracellular and extracellular stimuli, such as reducing disulfide bonds, ER calcium reserves, impaired protein transport to the Golgi, increased protein load, and absence of ER-associated protein degradation [7]. Nearly 30% of newly formed proteins in the ER degrade due to folding defects [8]. Any perturbations in the ER function led to the accumulation of unfolded proteins and initiate unfolded protein response (UPR) to maintain ER homeostasis [9, 10].

Rationale

ER-dependent UPR is initiated by the release of ER chaperone (BiP/GRP78) from three proximal ER stress sensors: PERK [protein kinase R-like ER kinase], IRE1 [Inositol-requiring enzyme 1], and ATF6 [activating transcription factor 6] [11]. GRP78 binds to UFP and assists in folding. The kinase domain of PERK successively phosphorylates eIF2α [12] and attenuates protein translation to ease the ER protein load. ATF4, a transcription factor unaffected by peIF2α, turns on genes required for protein folding and autophagy [13]. Autophosphorylation of IRE1α splices XBP1 [X-box binding

protein1] mRNA, leading to the generation of a potent transcriptional activator that induces ER stress-responsive genes. All three major arms of ER stress signaling pathway, i.e., PERK, IRE1, and ATF6, converge on the elements in the CCAAT enhancer-binding protein (C/EBP) homologous (CHOP) promoter region to induce the expression of pro-apoptotic molecule CHOP/GADD153 [14–16] during chronic ER stress conditions [17]. XBP1s axis also regulates Beclin1 and microtubule-associated protein1 light chain-3B (LC3B) to activate the autophagy mechanism. NADPH oxidase 2 (Nox2) enhances ROS production and oxidative stress [18, 19]. Accumulation of ROS can also inhibit the autophagy flux [19, 20]. ER stress contributes to neuronal damage during epilepsy-induced seizures [21]. Chronic ER stress can alter neuronal morphology and potentially enhances apoptotic signaling in epilepsy patients [11, 22, 23]. Further research into the relationship between ER stress and seizure severity is required to establish a link between the ER stress response and post-surgical seizure outcomes in patients.

To proceed with the above hypothesis, we have proposed three objectives

a). To study the expression of endoplasmic reticulum stress response proteins in FCD.

b). To study the expression of autophagy signaling proteins in FCD patients.

c). To study the expression of ER stress-induced translational attenuation marker and apoptosis initiator CHOP.

Methods

Sample collection and ethical guidelines

We collected the epileptic brain samples from the hospital immediately after the surgery. An experienced epilepsy pathologist classified the patients as FCD type I, IIA, and IIB at Krishna Institute of Medical Science (KIMS). Written informed consent was obtained from patients or their

relatives. The KIMS Foundation and Research Centre & KIMS Ethical Committee, Secunderabad, India, approved the study protocol, which followed the University of Hyderabad Institutional Ethical Committee. All the subjects were completely anonymized. For further studies, the epilepsy samples were collected immediately after the surgery and stored at -80 ^{o}C. The human brain tissue repository at the National Institute of Mental Health and Neuro-Science (NIMHANS), Bangalore, India, provided the autopsied control brain samples for this study. The control brain samples were not suitable for immunofluorescence assay. Hence, we used these control brains only for western blot assays.

Tissue lysate and western blot analyses

The radioimmunoprecipitation assay buffer was used to prepare the whole-cell lysate in the Dounce homogenizer at 4 ^{o}C. Protease inhibitor (Sigma-Aldrich, cat: no. P8340) and phosphatase inhibitor (Sigma-Aldrich, cat: no. P5726) were added to 100 mg of tissue sample before adding RIPA buffer. After that, tissue lysate was centrifuged at 14000 rpm for about 15 minutes at 4 ^{o}C. The supernatant was separated, and proteins were quantified using the Bradford reagent. (B6916 Sigma-Aldrich). 45 µg of protein sample suspended in a 6x sample buffer (20% glycerol, 4% Sodium dodecyl sulfate (SDS), 0.125 M Tris, pH 6.8, 0.02 M dithiothreitol, 0.02% bromophenol blue). After that, proteins were resolved by the SDS-PAGE. The gel was transferred to the nitrocellulose membrane (Protran Amersham GE) overnight at 4 ^{o}C using Towbin buffer (Tris base, Glycine, Methanol, pH 8.3) at 25 V. Tris-buffered saline (TBS) and TBS containing 0.05% Tween 20 (TBST) were used to wash the membrane. The nitrocellulose membrane was blocked with non-fat skimmed milk powder (Sigma-Aldrich: M7409) in TBST to reduce non-specific binding. With primary antibodies, these blots were incubated overnight at 4 ^{o}C. The antibodies used in our study were BiP (Cell signaling technology [CST]-3177), p-IRE1α (Novus Biological [NBP]100-2323SS), CHOP (CST-2895), XBP1 (NBP2-

20917), p-eIF2α (CST-3398), Beclin (CST-3495) P62 (CST-39749s), NOX2 (NBP2-41291), LC3B (CST-3868), ATG16L (CST-8089), ATG12+5 (MA5-27801), and ATG7 (CST-2631) diluted to 1:1000 in TBST buffer overnight at 4 °C. After subsequent washing with TBS and TBST, the blots were probed with secondary antibodies (1:15000 dilutions), anti-rabbit (CST-7074P2), and anti-mouse IgG (CST-G21040) conjugated to HRP for one hour at room temperature. The blots were developed using a chemiluminescence reagent (ClarityTM Western ECL substrate 1705060) and a Bio-Rad (Bio-Rad, USA) molecular imager.

Double immunofluorescence assay

5 µm thick paraffin-embedded tissue sections were deparaffinized, hydrated with xylene, 100%, and 95% ethanol, respectively, and washed with distilled water. Antigen was retrieved using boiled citrate buffer for 15 min (10 mM citrate buffer pH 6.0) and allowed to cool. The sections were covered with blocking buffer (5% normal goat serum, CST-5425, 1% BSA in 1x PBS supplied with 0.3% Triton X-100) and kept in a humid chamber for one hour at room temperature. The blocking buffer was removed from the tissue sections after incubation. The primary antibody cocktail was added and then incubated at 4 ºC for 16 hours to examine the neuronal expression of XBP1s (CST 12782) rabbit monoclonal antibody and NeuN (MAB377B) mouse antibody at 1:100 dilutions in antibody dilution buffer (CST-12378). The sections were washed with 1x PBS and incubated secondary antibodies that were conjugated with fluorochrome like anti-rabbit IgG with Alexa fluor® 488 (CST-4412S), anti-mouse IgG with Alexa fluor® 555 (CST-4409S) diluted in antibody dilution buffer for 1-2 hours at room temperature in the dark. Sections were washed in 1x PBS and mounted with Prolong® Gold anti-fade reagent with DAPI (CST-8961S). ZEN Blue software was used for image acquisition with Carl Zeiss LSM 710, and Image J software was used to quantify fluorescent intensities.

Data analysis

Statistical analysis among the groups performed with a one-way analysis of variance (ANOVA) using the Newman-Keuls method for post-hoc analysis. Data reported as mean ± SEM. The p-value <0.05 was considered statistically significant and was determined using Sigma Plot 2000 software for Windows.

Results

ER stress response in FCD patients

The correlation between ThT and post-surgical clinical data suggests that patients who showed complete seizure outcomes had reduced accumulation of UFP and vice versa. We have performed the western blot analysis by selecting n=3 samples from each ILAE class to understand the molecular changes associated with protein folding and protein degradation signaling mechanisms. The patient samples found no significant change or close variation while performing UFP detection assays were pooled and taken as one sample. Figure 2a represents the western blot assay for ER stress response signals against the accumulation of unfolded proteins by activating protein degradation machinery. All FCD patient samples belonging to ILAE classes 1, 2, and 3 showed a significant increase (P<0.001, 2.35±0.12, 2.59±0.24, 2.63±0.23) in the staining intensity of BiP/GRP78 (Figure 1b). The ER transmembrane receptor pIRE1α protein (Figure 1c) staining intensity showed a significant increase in class 3 (P <0.001, 2.59±0.18) than class 2 (P <0.02, 1.87±0.16) but not with class 1 (2.15±0.39). The downstream target of pIRE1α is XBP1. We have measured the XBP1 spliced (active) forms using western blot analysis (Figure 1d) and immunofluorescence assay (Figure 2). The XBP1s (s-spliced) form staining intensity was significantly increased in ILAE classes 1 and 3 (P<0.003, 1.29±0.10; p<0.003, 1.35±0.13), respectively, compared to class 2 (P<0.03, 0.90±0.09),

and immunofluorescence assay reconfirms the upregulation of XBP1s through increased fluorescence intensity in ILAE classes 1 and 3 (P <0.002, 3.15±0.25; P <0.003, 3.25±0.23), respectively, compared to class 2 (P <0.04, 1.69±0.24). The p-IRE1-XBP1 axis can further activate ER-associated protein degradation machinery and chaperones, i.e., BiP, to increase the protein folding capacity to ameliorate unfolded protein response (UPR) by ER. In our study, we observed a significant increase in peIF2α (Figure 1e) staining intensity in ILAE class 3 (P <0.002, 2.86±0.33) compared to class 1 (P <0.006, 1.26±0.25) and class 2 (P <0.008, 1.57±0.29) respectively. The above data suggest reduced protein aggregates and the absence of translational attenuation in ILAE class 1 patient samples, and the occurrence of severe ER stress due to the accumulation of unfolded proteins in ILAE class 2 and 3 samples.

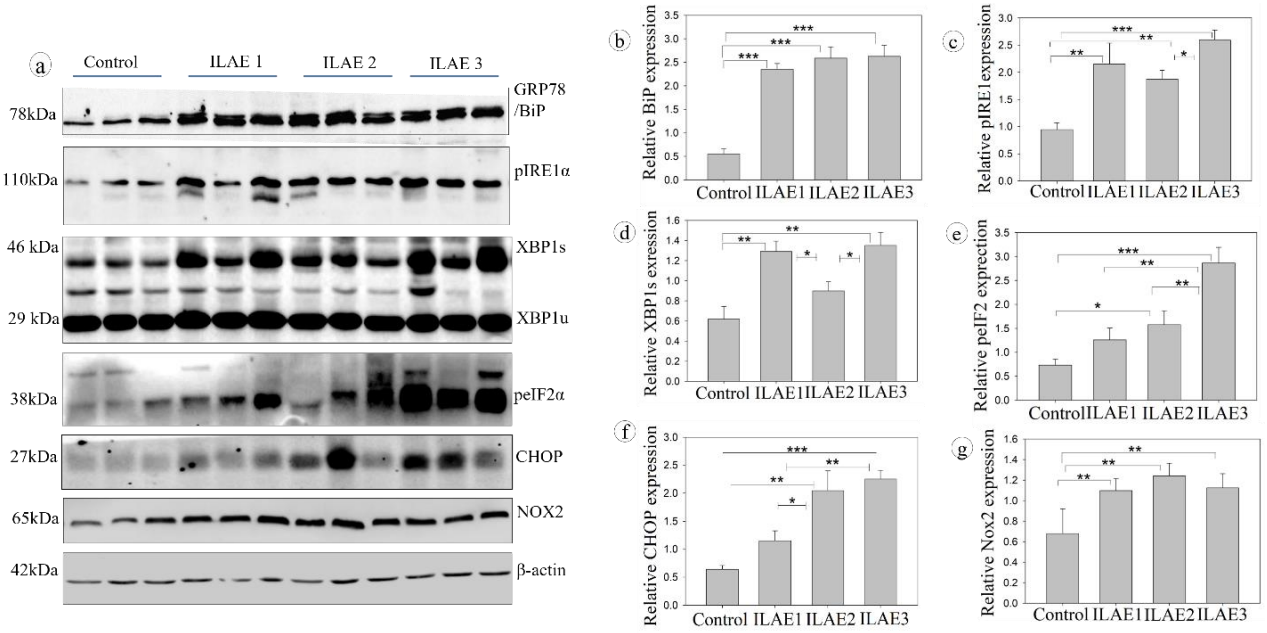

Fig. 1 Western blot analysis of ER stress response in FCD patient samples: Immunoblot assay for ER stress: Bip/GRP78, p-IRE1α, XBP1, p-eIF2 α, CHOP, NOX2, and β-actin in control and epileptic patient resected brain samples. An equal amount of protein was loaded in SDS-PAGE and transferred onto NC membranes, then blocked with nonfat milk and probed with primary antibody. The lane represents the control and FCD (n=3). The band/signal intensity was quantified (Relative protein expression) and shown as mean ± SEM. Densitometry analysis (b-g) showed a significant increase in BiP/GRP78, p-IRE1α, XBP1, p-eIF2 α, CHOP, and Nox2 in the FCD samples belonging to ILAE classes 2 & 3 than class 1 and controls. *p<0.05, **p<0.01, ***p<0.001

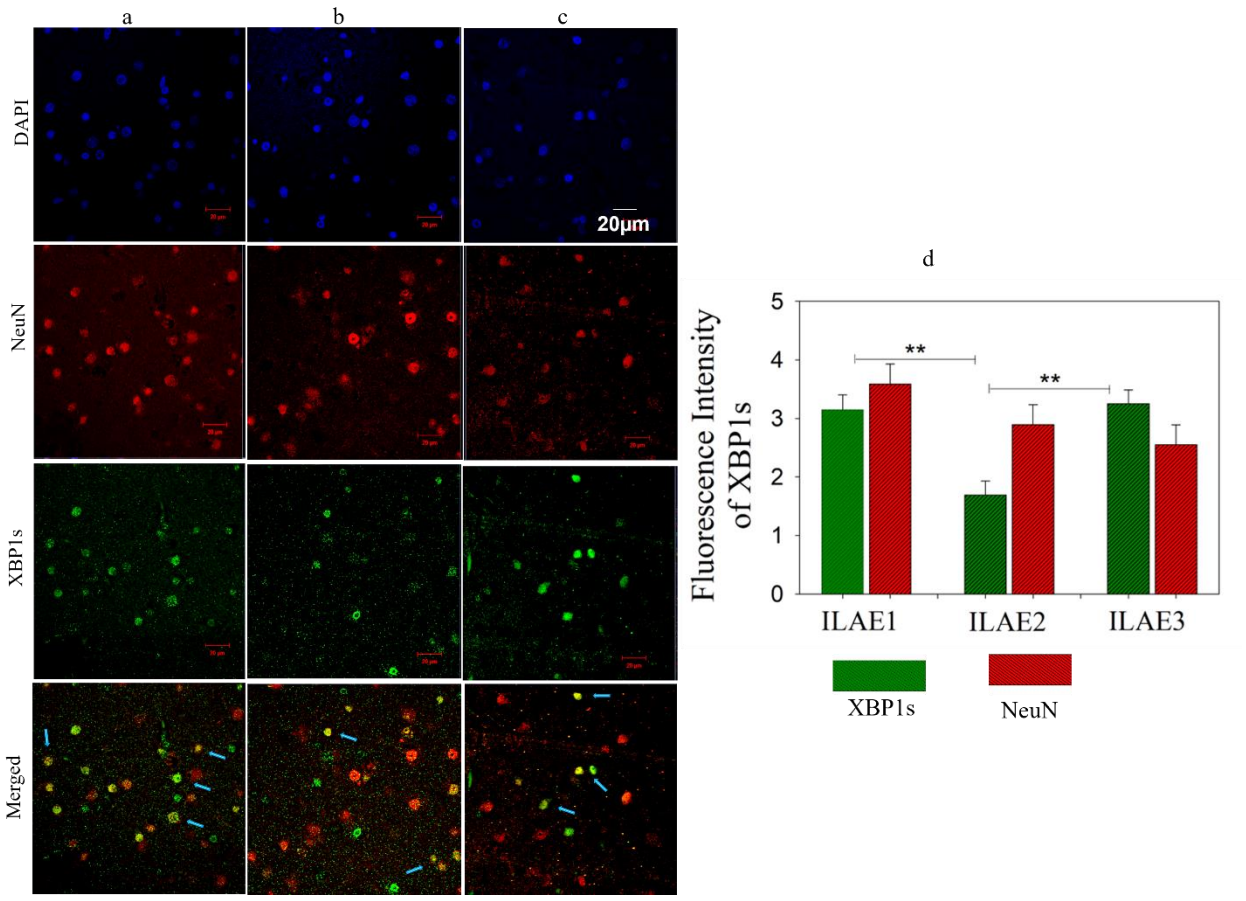

Fig. 2 Double immunofluorescence study for XBP1s expression in FCD: (Colored Image)

Double Immunofluorescence assay. Staining of XBP1s (green) and NeuN (red) 2a, 2b, and 2c represents the neuronal expression of XBP1s in ILAE outcome classes 1, 2, and 3 of FCD patient's brain sections (n=3), respectively. The blue color represents Di-amidino phenylindole (DAPI) staining used as a nuclear counterstain. The arrowheads in the merged image of NeuN+XBP1s represent the localization of XBP1s in neuronal cells. The fluorescence intensity of XBP1s was quantified and shown as mean ± SEM. Class 1 (a) > Class 2 (b) < Class 3 (c). Scale bar = 20µm. Data is representative of 3 independent experiments.

ER-induced autophagy response may not be sufficient to clear protein aggregates

The Beclin 1 (Figure 3b) staining intensity was significantly higher in ILAE class 1 (P<0.01, 1.09±0.10) class 2 (P <0.009, 1.29±0.12) and class 3 (P<0.05, 1.24±0.10). Upregulation of Beclin 1 suggests how critical the autophagy signal is for classes 1, 2, and 3 to clear the aggregated proteins.

Furthermore, the staining intensity of ATG7 (Figure 3c) increased in class 3 (P<0.001, 14.02±3.23) than the ILAE 2 (P<0.03, 7.81±2.02) and ILAE 1 (P<0.02, 7.94±1.51), ATG12-5 (Figure 3d) significantly increased in class 2 (P<0.003, 2.21±0.1) and class 3 (P<0.002, 2.56±0.39) than class 1 (P<0.05, 1.07±0.051), and ATG16L1 (Figure 3e) significantly higher in class 3 (P<0.001, 76.88±5.57) than class 2 (P<0.03, 45.92±11.59) and class 1 (P<0.02, 37.12±6.34). Sequsetosome1/p62 (Figure 3f), a critical autophagy cargo protein's staining intensity was found to be increased in ILAE classes 2 (P<0.01, 3.61±0.52) and class 3 (P<0.001, 4.84±0.27) significantly than class 1 (P<0.04, 1.76±0.55), since p62 is degraded along with autophagy proteins, its abundance infers impaired autophagy. The LC3B type II (Figure 3g) staining intensity was significantly higher in ILAE class 3 (P<0.001, 10.17±1.27) than in class 1 (P<0.005, 4.74±1.53). At the same time, class 2 (P<0.01, 7.61±0.65) showed a significant increase compared to class 1 (4.74±1.53), Since LC3B II and p62 themselves undergo autophagy degradation [24], their abundance/accumulation is a good sign of impaired autophagy and increased ER stress in ILAE class 2 and class 3 patients.

Chronic ER stress response inducing apoptosis

ER stress response initiates autophagy signaling to clear the UFP aggregates during stress conditions. In some chronic stress conditions, the cells may fail to eliminate excess proteins due to factors that inhibit or overthrow the autophagy machinery and lead to the activation of the apoptosis signal. In FCD patients, Nox2 (Figure 1g), protein staining intensity was increased in class 1 (P<0.01, 1.09±0.12), class 2 (P<0.007, 1.13±0.12), and 3 (P<0.009, 1.12±0.13), this suggests the ROS accumulation in patient samples. Nox2 increases the synthesis of ROS and also disturbs the autophagy mechanism. In addition, we have observed a significant increase in CHOP protein staining intensity through western blot assay in class 2 (P<0.009, 2.05±0.35) and class 3 (P<0.001, 2.25±0.16) compared to the ILAE class 1 (P<0.02, 1.15±0.18) patients (Figure 1f). CHOP acts as an

apoptosis initiation factor during impaired autophagy and severe ER stress. All the FCD samples belonging to ILAE classes 1, 2, and 3 were found to have a significant increase in UPR and autophagy signaling proteins and UFP aggregates compared to control brain samples.

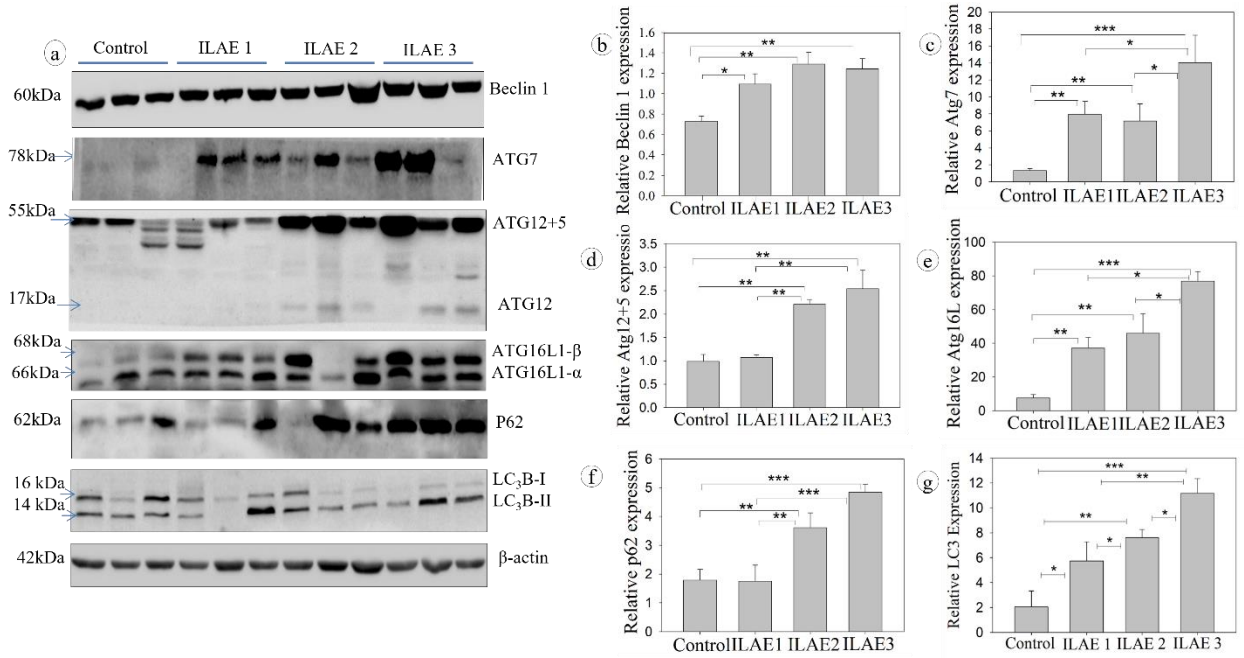

Fig. 3 Western blot analysis of autophagy response in FCD patient samples:

Immunoblot assay for autophagy: Beclin, ATG7, ATG12-5, ATG16L1, p62, LC3B, and β-actin in control and epileptic patient resected brain samples. An equal amount of protein was loaded in SDS-PAGE, transferred onto NC membranes, blocked with nonfat milk, and then probed with a primary antibody. The lanes represent the control and FCD samples. The band/signal intensity was quantified (Relative protein expression) and shown as mean ± SEM. Densitometry analysis (b-g) shows a significant increase in Beclin1, ATG7, ATG12+5, ATG16L1, P62, and LC3B in the FCD samples belonging to ILAE classes 2 & 3 than class 1 and controls (n=3). *p<0.05, **p<0.01, ***p<0.001

Discussion

One-third of patients who have epilepsy show poor responses to current anti-epileptic drugs [25] and require the surgical resection of epileptic foci. Many patients benefit from epilepsy surgery, but

still, some are vulnerable to seizure recurrence [26]. During epilepsy, excitotoxicity disturbs cellular functions such as protein folding and sorting [27] and free radicle production [28], but the severity and duration vary among individuals. These events disturb the ER homeostasis and lead to the activation of stress response signals to maintain cellular homeostasis by attenuating new protein synthesis and clearing the UFP aggregates. Since patients exhibit different cellular and molecular changes during seizures, the epileptic brain tissue resected during surgery is archetypal to understanding epileptogenesis [29]. This study aimed to find the UPR and autophagy changes that occurred in the FCD patients using resected epileptic brain samples to understand the possibility of a relation between ER stress and clinical outcomes in FCD patients.

Thioflavin T assay indicates the accumulation of UFP in FCD patients. Irrespective of age, gender, seizure foci, and severity, some patients had limited UFP, whereas few did not. Since we have observed the post-surgical follow-up data regularly to understand the clinical outcomes, we have categorized the patient cohort (n=26) according to ILAE suggested outcome classes [30]. The ILAE class 1 (seizures-free), class 2 (<1 seizure episode/year and auras), and class 3 (two to three seizure episodes/year and auras). Our findings showed the correlation between seizure outcomes and UFP aggregates. Patients with limited UFP levels suggest possible re-establishment of ER homeostasis, whereas increased UFP represents the possibility of repeated acute or chronic ER stress with auras and seizure recurrence. We believe that understanding the role of ER stress significance can help us to predict the possibility of seizure recurrence.

Based on these observations, we further studied the expression pattern of UPR and autophagy signaling proteins. We began with GRP78/BiP protein expression; BiP is an ER lumen resident chaperone that helps in the proper folding of nascent/unfolded proteins [31]. Since UFP aggregates increased in patients, we have observed significant upregulation of BiP in all the patient samples,

which could help in protein folding. In epilepsy, activation of IRE1α (pSer723) is a sign of ER stress UPR response [32–34]. IRE1α is inactive as long as BiP is bound to it, presence of UFP in epilepsy patients released the BiP, which allowed the autophosphorylation and activation of IRE1α. Activated IRE1α (pSer724) splices XBP1 mRNA into XBP1s [35–37]. During the accumulation of UFP, cells can adapt to acute (last for a few minutes to hours) or chronic ER stress responses (a few days to years) to retain ER homeostasis [25]. The increased expression of XBP1s is a possible sign of chronic ER stress in ILAE classes 1 & 3, and we assume the possibility of adaptive chronic ER stress in ILAE class 1 compared to class 3. Similarly, class 2 patients probably adopted acute ER stress, which lasts for a while and helps to reduce the UFP aggregates. Therefore, adaptation to acute ER stress response could be one of the reasons for reduced XBP1s expression in class 2 patient samples. The acute-repetitive and chronic non-adaptive ER stress responses can worsen seizures in epilepsy patients by reducing the seizure threshold [38–40]. Studies on human and mouse models showed significant upregulation of peIF2α (Ser51) during status epilepticus and hippocampal sclerosis [41, 42], which represents the attenuation of global protein synthesis due to the inability of cells to limit the UFP in the ER [43]. The expression pattern of GRP78/BiP, pIRE1α, XBP1s, peIF2α, and ThT assay results suggest that ILAE class 1 patients readily get rid of UFP aggregates compared to ILAE classes 2 and 3.

ER stress response can control both autophagy and apoptosis signals depending on the severity of ER stress [44–48]. In our study, we observed a significant increase in the expression of Beclin1 protein in all the FCD patient samples. Growing evidence also supports the relationship between impaired autophagy and epilepsy [49, 50]. Hence, we further elucidate the difference in autophagy signaling among the ILAE outcome classes. Autophagy response proteins (ATGs) are recruited to the ER and autophagy membrane upon autophagy induction [51]. Since ATG 7 increases autophagy

and reduces ER stress [52], class 1 samples showed ATG7 upregulation but not class 2, which suggests impaired autophagy. Atg16L is a part of the core autophagy protein associated with the Atg12-5 complex [53], which was increased in ILAE classes 2 & 3 patients. P62/SQSTM1 is an autophagy adaptor protein that degrades in the autophagosome [54]. In our patient cohort, samples belonging to ILAE classes 2 and 3 showed the accumulation of p62 protein and LC3B, suggesting the possibility of impaired autophagy because of chronic ER stress [24]. FCD patients were found to have increased NADPH oxidase 2 expression. Nox2 increases ROS formation, which could induce chronic ER stress, alter autophagy, even causes cell death, and contribute to increased seizure susceptibility [20, 55–57]. Increased Nox2, peIF2α, UFP, p62, and LC3B suggest the possibility of dysregulated chronic ER stress that can disturb the UPR and autophagy. CHOP has a dual role, which acts as an autophagy or apoptosis inducer [58]. Several studies have reported that during severe ER stress, CHOP acts as a pro-apoptotic molecule and promotes cell death [11, 59], which can create hyperexcitable seizure-sensitive cellular circuits [50]. Our findings were in line with the above studies.

Conclusion

The epileptic patients, who are seizure-free, were found to have reduced protein aggregates. On the other hand, patients with seizure recurrence had increased UFP aggregates due to disturbed ER homeostasis. This study explained how the brain's protective mechanism deals with seizures. We believe further studies about ER stress and epilepsy using more clinical samples and animal models can help consolidate our findings on epilepsy progression and clinical outcomes.

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Manuscripts

Accepted

- Endoplasmic reticulum stress and unfolded protein accumulation correlate to seizure recurrence in focal cortical dysplasia patients. doi.org/10.1007/s12192-022-01301-0
- Dopamine and glutamate crosstalk worsen the seizure outcome in TLE-HS patients. doi.org/10.1007/s12035-023-03361-4
- Regulation of Keap1-Nrf2 axis in Temporal Lobe Epilepsy- Hippocampal Sclerosis patients may limit seizure outcomes. doi.org/10.1007/s10072-023-06936-0

Under peer-review

- Dopamine-Glutamate cross-talk limits the feedback inhibition of ionotropic Glutamate receptors and seizure outcome in Focal Cortical Dysplasia.
- Regulation of Keap1-Nrf2 signaling by histone methyltransferases: Significance of antioxidant response in FCD patients

PUBLICATIONS

Cell Stress and Chaperones
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ORIGINAL ARTICLE

Endoplasmic reticulum stress and unfolded protein accumulation correlate to seizure recurrence in focal cortical dysplasia patients

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Abstract
Epileptic seizures occur due to an imbalance between excitatory and inhibitory neurosignals. The excitotoxic insults promote the accumulation of reactive oxygen species (ROS), unfolded proteins (UFP) aggregation, and sometimes even cell death. The epileptic brain samples in our study showed significant changes in the quantity of UFP accumulation. This part explored the efficiency of ER stress and autophagy responses at neutralizing the UFP using resected epileptic brain tissue samples. Meanwhile, we regularly observed these patients' post-surgical clinical data to find the recurrence of seizures. According to International League against Epilepsy (ILAE) suggestions, we classified the patients ($n=26$) as class 1 (completely seizure-free), class 2 (less frequent seizures or auras), and class 3 (auras with < 3 seizures per year). The classification helped us understand the reason for variations in the UFP accumulation in patient samples. We have observed the protein levels of ER chaperone, glucose-regulated protein 78 kDa (GRP78/BiP), inositol-requiring enzyme 1α (IRE1α), X box-binding protein 1 s (XBP1s), eukaryotic translation initiation factor 2α (peIF2α), C/EBP homologous protein (CHOP), NADPH oxidase (NOX2), and autophagy proteins like BECLIN1, ATG 7, 12, 5, 16, p62, and LC3. Our results suggested that ER stress response limitation may contribute to seizure recurrence in epilepsy patients, particularly in classes 2 and 3. In addition, we have observed significant upregulation of ER stress-dependent apoptosis initiation factor CHOP in these patients. These results indicate that understanding the ER stress response pattern infers the possibility of post-surgical outcomes in focal cortical dysplasia (FCD) patients.

Keywords Focal cortical dysplasia (FCD) · Endoplasmic reticulum stress · Unfolded protein response (UPR) · Oxidative stress · Autophagy · Clinical outcome

Introduction

In both the pediatric and adult populations, focal cortical dysplasia (FCD) is a major cause of drug-resistant epilepsy (Kabat and Król 2012). FCD occurs due to the malformation of the cortex during brain development. Amongst its subtypes, type I has disruption of intracortical lamination and columnar organization. Cortical dyslamination and dysmorphic neurons characterize type IIa, while balloon cells describe type IIb (Palmini et al. 2004; Krsek et al. 2008; Kim and Choi 2019). Both FCD types I and II are associated with intractable epilepsy. Most patients with FCD show recurrent seizures even after using antiepileptic drugs (Kwan et al. 2010). One-third of the patients affected by epilepsy respond poorly to contemporary antiepileptic medicines (Liu et al. 2019). As a chronic neurological disorder, epilepsy often contributes to neurodegeneration due to disturbance in the endoplasmic reticulum (ER) homeostasis. ER stress is triggered by various intracellular and extracellular stimuli, such as reducing disulfide bonds, ER calcium reserves, impairment of protein transport to the Golgi, increased protein load, and absence of ER-associated protein degradation (Kadowaki and Nishitoh 2013). Nearly 30% of newly formed proteins in the ER degrade due to folding defects (Schubert

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Dopamine and Glutamate Crosstalk Worsen the Seizure Outcome in TLE-HS Patients

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Abstract

Temporal lobe epilepsy (TLE), accompanied by hippocampal sclerosis (HS), is the most common form of drug-resistant epilepsy (DRE). Nearly 20% of the patients showed seizure recurrence even after surgery, and the reasons are yet to be understood. Dysregulation of neurotransmitters is evident during seizures, which can induce excitotoxicity. The present study focused on understanding the molecular changes associated with Dopamine (DA) and glutamate signaling and their possible impact on the persistence of excitotoxicity and seizure recurrence in patients with drug-resistant TLE-HS who underwent surgery. According to the International League against Epilepsy (ILAE) suggested classification for seizure outcomes, the patients ($n=26$) were classified as class 1 (no seizures) and class 2 (persistent seizures) using the latest post-surgery follow-up data to understand the prevalent molecular changes in seizure-free and seizure-recurrence patient groups. Our study uses thioflavin T assay, western blot analysis, immunofluorescence assays, and fluorescence resonance energy transfer (FRET) assays. We have observed a substantial increase in the DA and glutamate receptors that promote excitotoxicity. Patients who had seizure recurrence showed a significant increase in (pNR2B, $p<0.009$; and pGluR1, $p<0.01$), protein phosphatase1γ (PP1γ; $p<0.009$), protein kinase A (PKAc; $p<0.001$) and dopamine-cAMP regulated phospho protein32 (pDARPP32T34; $p<0.009$) which are critical for long-term potentiation (LTP), excitotoxicity compared to seizure-free patients and controls. A significant increase in D1R downstream kinases like PKA ($p<0.001$), pCAMKII ($p<0.009$), and Fyn ($p<0.001$) was observed in patient samples compared to controls. Anti-epileptic DA receptor D2R was found to be decreased in ILAE class 2 ($p<0.02$) compared to class 1. Since upregulation of DA and glutamate signaling supports LTP and excitotoxicity, we believe it could impact seizure recurrence. Further studies about the impact of DA and glutamate signaling on the distribution of PP1γ at postsynaptic density and synaptic strength could help us understand the seizure microenvironment in patients.

Keywords Temporal Lobe Epilepsy · Seizures Recurrence · Dopamine · D1-D2 Heterodimer · Ionotropic Glutamate Receptor

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ORIGINAL ARTICLE

Regulation of Keap1-Nrf2 axis in temporal lobe epilepsy—hippocampal sclerosis patients may limit the seizure outcomes

Madhamanchi Kishore[1] · Madhamanchi Pradeep[1,2] · Parimala Narne[1] · Sita Jayalakshmi[3] · Manas Panigrahi[3] · Anuja Patil[3] · Phanithi Prakash Babu[1]

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Abstract

Background Accumulation of reactive oxygen species (ROS) exacerbates neuronal loss during seizure-induced excitotoxicity. Keap1 (Kelch-like ECH-associated protein1)-nuclear factor erythroid 2–related factor 2 (Nrf2) axis is one of the known active antioxidant response mechanisms. Our study focused on finding the factors influencing Keap1-Nrf2 axis regulation in temporal lobe epilepsy (TLE) associated with hippocampal sclerosis (HS) patients.

Methods Based on post-surgical follow-up data, patient samples ($n = 26$) were categorized into class 1 (completely seizure-free) and class 2 (only focal-aware seizures/auras), as suggested by International League Against Epilepsy (ILAE). For molecular analyses, double immunofluorescence assay and Western blot analysis were employed.

Results A significant decrease in expression of Nrf2 ($p < 0.005$), HO-1; $p < 0.02$) and NADPH Quinone oxidoreductase1 (NQO1; $p < 0.02$) was observed in ILAE class 2. Keap1 ($p < 0.02$) and histone methyltransferases (HMTs) like SetD7 (SET7/9; SET domain-containing 7 histone lysine methyltransferase) ($p < 0.009$) and enhancer of zeste homolog 2 (EZH2; $p < 0.02$) and methylated histones viz., H3K4me1 ($p < 0.001$), H3K9me3 ($p < 0.001$), and H3K27me3 ($p < 0.001$) was upregulated in ILAE class 2. Nrf2-interacting proteins viz., p21 ($p < 0.001$) and heat shock protein 90 (HSP90; $p < 0.03$) increased in class 1 compared to class 2 patients.

Conclusion Upregulation of HMTs and methylated histones can limit phase II antioxidant enzyme expression. Also, HSP90 and p21 that interfere with Keap1-Nrf2 interaction could contribute to a marginal increase in HO-1 and NQO1 expression despite histone methylation and Keap1. Based on our findings, we conclude that TLE-HS patients prone to seizure recurrence were found to have dysfunctional antioxidant response, in part, owing to Keap1-Nrf2 axis.

Keywords Temporal lobe epilepsy (TLE) · Hippocampal sclerosis (HS) · Oxidative stress · Epigenetic regulation · Histone methylation · Antioxidant response

Introduction

Temporal lobe epilepsy (TLE) with hippocampal sclerosis (HS) is one of the most common causes of epilepsy, with nearly one-third of patient population remaining drug-resistant. Patients experiencing seizures develop a new and random neuronal network, which induces significant changes in brain electrical activity [1]. This significantly impacts the quality of life and cognitive changes, thereby necessitating surgical resection of epileptic foci [2]. Standard temporal lobe resection surgery remains the mainstay treatment in drug-refractory cases, with 60–70% remaining seizure-free over long term [3]. Existing anti-seizure medications predominantly target seizure propagating mechanisms and act by suppressing seizures. The reasons for the persistence of seizures in a significant number of patients after surgical treatment are yet unclear. An irregular neuronal network arising from recurrent seizures further promotes random neuronal firing and excitotoxicity, which is often attributed to dopamine and glutamate signaling [4]. The

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Dopamine, Glutamate Cross-talk, its Impact on Cellular Homeostasis and Seizure Recurrence in Epilepsy

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