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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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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 [23–27]. The pGluR1 induces long-term potentiation (LTP) by promoting excitatory postsynaptic potential (EPSP) [28]. 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 [27]. Fyn kinase phosphorylates Y1472 at the YEKL domain of the NR2B receptor subunit to inhibit receptor internalization and induce LTP [29]. Ca^{2+} activates Cdk5 via calpain-mediated cleavage of p35 into p25 and p10 [30]. Cdk5 activates protein phosphatase1γ (PP1γ) through pDARPP32T75 (DA and cAMP-regulated phosphoprotein 32) and by inhibiting PKA. [31]. 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) [32]. 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 [33]. Even though D1 and D2 are antagonistic, the synergistic function of the D1-D2 heterodimer was detected in the striatum of rat brains [23]. Nucleus accumbens, caudate-putamen, globus pallidus D1, and D2 receptors form a heterodimer in medium spiny neurons [24]. 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) [25, 26]. In addition, NMDARs also mobilize Ca^{2+}, which leads to calcium overload and excitotoxic death [27]. Orchestrated pre-and postsynaptic events that co-occurred (dependently/independently) will regulate normal neuronal signaling [28]. As age increases, DA and glutamate signaling also become hypofunctional due to changes in receptor synthesis, affinities, and the release of neurotransmitters [27, 29, 34–37]. 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 [28, 30, 31]. The current study focused on understanding the significance of NMDAR-mediated negative feedback regulation [32] 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 [24], using postsurgical resected brain samples of patients and postmortem control brain samples. The control brain samples used for our 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. Getting exact age match control for pathological studies is more challenging. Earlier studies by David C. Henshall et al. (2004) helped us to use adult controls in our study. Based on these reports, we perform our studies to understand the impact of excitotoxicity on postsurgical seizure recurrence in epilepsy patients affected by TLE-HS.

Methods and Materials

Tissue Collection and Ethical Guidelines

Postmortem control brain samples were obtained from the National Institute of Mental Health and Neuro Sciences

(NIMHANS), Bangalore, India, for the current study. The diseased samples do not have any chronic neurological disorders, particularly epilepsy. The mean age for control is 27.8 ± 1.8 years (range 25 to 37). Epilepsy (TLE-HS) patient samples were obtained from Krishna Institute of Medical Sciences (KIMS), Secunderabad, India. Since the control and patient samples have undergone acute or chronic ER stress as a consequence of death or due to seizure-induced excitotoxicity [38], we have estimated the quantity of protein aggregates using Thioflavin T assay in both controls and TLE-HS samples. Irrespective of age or sex, some patient samples showed a limited amount of protein aggregates, and some showed a significant increase, but control samples were found to have a much lesser quantity of protein aggregates compared to patient samples.

Meanwhile, the patient's postsurgical seizure experience, who was visiting the hospital, was observed regularly. With the help of postsurgical clinical data, TLE-HS patients were categorized into class 1 (completely seizure-free) and class 2 (recurrent seizures), according to the suggestions of the International League against Epilepsy (ILAE) [39]. The mean age of class 1 patients is 13.2 ± 2.4 years (range 3 to 27), and class 2 is 19.5 ± 2.8 years (range 10 to 40). The ThT assay data showed a correlation with the clinical outcome, where class 1 patients have reduced protein aggregates compared to class 2, and controls were also found to have a much lesser quantity of protein aggregates compared to patients (Tables 1 and 2). We cannot observe the age or sex correlation regarding protein aggregates in patients or control samples. Our earlier studies also support the impact of protein aggregates in epilepsy patients [40]. In addition, we also followed the earlier studies done by David C. Henshall et al. (2000, 2004), where they used controls and patient samples with a significant range of age differences to check the expression pattern of proteins like bcl-2, caspases, and death-associated protein kinase (DAP kinase). With the help of our ThT assay data and the work done by David C. Henshall et al. [30, 31] on TLE patient samples, we further proceed to check the impact of chronic seizures induced excitotoxicity on seizure outcomes in TLE-HS patients using autopsy control samples. The TLE-HS resected brain tissue samples were snap-frozen with liquid nitrogen and transferred to -80˚C immediately after surgery. Some tissues were kept in 10% formalin for paraffin fixation at Krishna Institute of Medical Sciences (KIMS). All the studies were approved by the KIMS ethical committee KFRC (KIMS foundation and research center) and are supported by the patient or close relative's consent. Institutional ethical committee (IEC) guidelines were also observed, and all study sets were entirely anonymized.

Thioflavin T Fluorescence Assay

Thioflavin T (T3516, Sigma-Aldrich) is widely used to quantify protein aggregate [41]. Thioflavin T is a Benzothiazole salt; it binds to unfolded protein (UFP) aggregates, like β-sheets, and gives enhanced fluorescence. The experiment was performed in a 96-well plate. We added 100 µg of protein to Milli Q water and made up the volume up to 50 µl. Before the experiment, we prepared 2 µl of 0.5 mM Thioflavin T stock solution and added it to the 96 well plate to make the final working concentration of 20 µM. Incubated the reaction for 30 min to develop the color, the fluorescent intensity of each sample was measured with the help of infinite® 200 PRO, Tecan spectrophotometer using the excitation and emission parameters at 440 nm and 490 nm, respectively.

Tissue Lysate and Western Analysis

The Bradford method was used to estimate the protein quantity (B6916 Sigma-Aldrich). 45 µg of protein from TLE-HS brain samples were suspended in a 6× sample buffer (20% glycerol, 4% Sodium dodecyl sulfate (SDS), 0.125 M Tris, pH 6.8, 0.02 M dithiothreitol (DTT), 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 40C using Towbin buffer (Tris base, Glycine, Methanol, pH 8.3) at 25 V. Tris-buffered saline (TBS) and TBS with 0.05% Tween 20 (TBST) were used to wash the membrane. The nitrocellulose (NC) membrane was blocked with non-fat skimmed milk powder (Sigma-Aldrich: M7409) in TBST to reduce non-specific binding. At 40C, these blots were incubated with primary antibodies overnight. The antibodies used were spinophilin (CST-14136), Neurabin-1(Santa Cruz [Sc]-37407), PP1γ1 (Sigma-P7609), Cdk5 (Sc-6247), p-GluR1 (abcam[ab]-12108), PKAc(T197) (CST- 4781), Fyn (CST-4023), GluR1 (CST-13185S), NR2B (CST-4212), NR2A (CST-4205), p-NR2B (Sigma-M2442), p25/35 (Cell signaling technology [CST]-2680), p-Cdk5 (Sc-377558), D1DR (Novus Biologicals, NBP2-16,213), D2DR (Sc-5303), DARPP32 (Sc-135877), pDARPP32T34 (Sigma-SAB504378), pDARPP32T75 (CST-2301), CAMKIIα (CST-50049), p-CAMKIIαT286 (Sc-32289), PLCδ1 (sc-393464), PKC (Sc-17804), Gαq/11 (sc-365906), in 1:1000 dilution. The blots were washed with TBS, followed by TBST after incubation. Again, blots were incubated with secondary antibody anti-rabbit IgG (CST-7074P2) and anti-mouse IgG (CST-G21040) conjugated to HRP with a dilution (1:15,000) for 1 h at room temperature. After washing with TBS and TBST, followed by incubation, the blots were developed in the Bio-Rad (Bio-Rad, USA) molecular imager with chemiluminescence (ClarityTM Western ECL substrate 1,705,060) reagent [42].

Table 1 Represents the TLE-HS patient's clinical data, with the type of epileptic condition, age, sex, duration of patients suffering from seizures, seizure frequency before and after surgery, clinical outcome, and fluorescent intensity of protein aggregates. Based on protein aggregate quantity estimated by Thioflavin T assay, patient samples were pooled (who showed similar values) and categorized as 1–4 in increasing order of protein aggregates in both ILAE class 1 (1–4 on the left side to the values in last column in table1) and ILAE class 2 (1–4 on the right side to the values in last column in table1) in the brackets

Case	Character	Gender	Onset Age	Age of surgery	Duration	Loci	Seizures/Y before surgery	Post-surgery outcome for 3 Years	ILAE Outcome Class	ThT assay Individual data points (Mean)
1	TLE-HS	M	10 Y	40 Y	30 Y	HS	09	90% decrease	Class 2	279.5 (2)
2	TLE-HS	M	9 Y	13 Y	4 Y	HS	08	Completely seizures free	Class 1	(2) 195.0
3	TLE-HS	M	5 Y	15Y	10Y	HS	10	90% decrease	Class 2	279.2 (2)
4	TLE-HS	F	3 Y	4.6Y	1.6 Y	HS	08	Completely seizures free	Class 1	(3) 199.6
5	TLE-HS	F	16Y	27Y	11 Y	HS	07	Completely seizures free	Class 1	(3) 203.0
6	TLE-HS	M	10Y	13Y	3 Y	HS	09	90% decrease	Class 2	275.3 (1)
7	TLE-HS	F	4 Y	12Y	8 Y	HS	10	Completely seizures free	Class 1	(3) 202.3
8	TLE-HS	M	7 Y	12Y	5 Y	HS	09	90% decrease	Class 2	284.2 (3)
9	TLE-HS	F	6 Y	14 Y	8 Y	HS	24	Completely seizures free	Class 1	(1) 188.2
10	TLE-HS	F	5Y	18 Y	13 Y	HS	10	90% decrease	Class 2	275.6 (1)
11	TLE-HS	M	9 Y	10 Y	1 Y	HS	11	Completely seizures free	Class 1	(2) 194.7
12	TLE-HS	F	2 Y	14 Y	12 Y	HS	08	90% decrease	Class 2	278.4 (2)
13	TLE-HS	M	5 Y	8Y	3 Y	HS	09	Completely seizures free	Class 1	(2) 195.5
14	TLE-HS	M	34 Y	35 Y	1 Y	HS	10	90% decrease	Class 2	276.1 (1)
15	TLE-HS	M	2 Y	25 Y	23 Y	HS	08	Completely seizures free	Class 1	(2) 196.4
16	TLE-HS	M	11 Y	21 Y	10 Y	HS	09	Completely seizures free	Class 1	(1) 190.3
17	TLE-HS	M	6 Y	13 Y	7 Y	HS	09	90% decrease	Class 2	283.8 (3)
18	TLE-HS	F	1Y	3 Y	2 Y	HS	10	Completely seizures free	Class 1	(1) 191.1
19	TLE-HS	M	11 Y	14Y	3 Y	HS	09	90% decrease	Class 2	274.1 (1)
20	TLE-HS	M	15 Y	25 Y	10 Y	HS	12	Completely seizures free	Class 1	(1) 192.1
21	TLE-HS	M	3Y	11 Y	8 Y	HS	09	90% decrease	Class 2	278.6 (2)
22	TLE-HS	M	4 Y	10 Y	6 Y	HS	09	90% decrease	Class 2	297.2 (4)
23	TLE-HS	F	1 Y	4 Y	3 Y	HS	10	Completely seizures free	Class 1	(1) 190.4
24	TLE-HS	F	3Y	5 Y	2 Y	HS	09	Completely seizures free	Class 1	(4) 201.4
25	TLE-HS	M	13 Y	25 Y	12 Y	HS	10	90% decrease	Class 2	295.6 (4)
26	TLE-HS	M	15 Y	27 Y	12 Y	HS	10	90% decrease	Class 2	278.3 (2)

Table 2 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

S.No	Gender	Age	Cause of Death	Postmortem Interval	Significant Neuro pathology	Disease	HIV/ HBsAg	Brain Region	ThT assay Individual data points (Mean)
1	M	25 years	Suicide	30 h	Absent	No	Negative	Temporal	146.8 (1)
2	F	27 years	Drowning	28 h	Absent	Diabetes	Negative	Temporal	147.5 (2)
3	M	37 years	Heart attack	40 h	Absent	No	Negative	Temporal	147.4 (2)
4	M	27 years	Accident	35 h	Cerebral injury	No	Negative	Temporal	152.5 (4)
5	F	25 years	Homicide	37 h	Absent	No	Negative	Temporal	149.8 (3)

Double Immunofluorescence Assay

In this study, 5 µm thick tissue sections were hydrated with 100% and 90% ethanol and then deparaffinized with xylene. Antigen retrieval was done with boiling citrate buffer (10 mM), pH 6.0. Sections were cooled immediately and blocked with blocking buffer (5% normal goat serum (CST-5425), 1% bovine serum albumin (BSA) dissolved in 1 × phosphate-buffered saline (PBS), and 0.3% Triton X-100) at room temperature for one hour in a humid chamber. 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 h to examine the neuronal expression of p-CAMKIIαT286 (Sc-32289), p-Cdk5 (Sc-377558), DA from Abcam (ab6427), p25 (NBP2-34,031), D1R (NBP2-16,213), D2R (sc-5303), mouse-generated NeuN (Abcam-177487), and rabbit-generated NeuN (CST-24307). After washing the slides for 10 min with PBS, the fluorescent conjugated secondary anti-rabbit IgG antibody (4412S Alexa fluor® 488) 1:1000 and anti-mouse IgG antibody (Alexa fluor® 555) 1:1000 dilutions from Cell Signaling Technology applied for 1 h in dark conditions at room temperature. Sections were washed in 1xPBS 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 intensity ($n=4$) [43, 44].

FRET Analysis

Fluorescence resonance energy transfer (FRET) analysis is a well-established method to estimate the interaction between two proteins. In a confocal microscope, fluorescently labeled secondary antibodies were used to test the possibility of protein interaction through energy transfer between donor and acceptor. To detect D1 and D2 primary antibodies, the tissue sections were stained with an anti-rabbit IgG secondary antibody (4412S Alexa fluor® 488) as a donor and an anti-mouse IgG secondary antibody (4409S Alexa fluor® 555) as an acceptor. FRET analysis was conducted ($n=6$) with a Carl Zeiss LSM 710 confocal microscope using ZEN blue edition 2.1 software by measuring the distance (r) between the acceptor and donor probes and calculating the energy transfer efficiency (E) in the region of interest.

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

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- rate of transfer efficiency.

R = R¬0 [1/E—1]1/6 (Forster's distance R0) denotes the distance at which FRET efficiency was 50% between donor and acceptor, and r is the distance between donor and acceptor in a region of interest.

Data Analysis

Statistical analyses among groups was 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 1 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 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).

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 (Figs. 2d and 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.

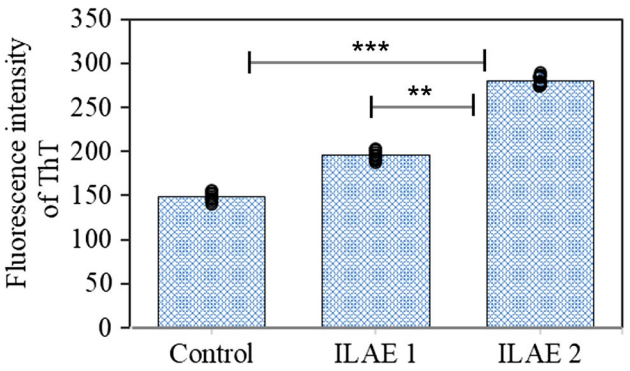

Fig. 1 Represents the Estimation of unfolded protein aggregates in TLE-HS patients ($n = 26$) and controls ($n = 5$) by Thioflavin T assay: Fluorescent intensity of aggregated proteins was measured and shown as mean ± SEM ($n = 3$). The graph represents the ThT assay data from controls and patient samples 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. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$

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). 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). 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.

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

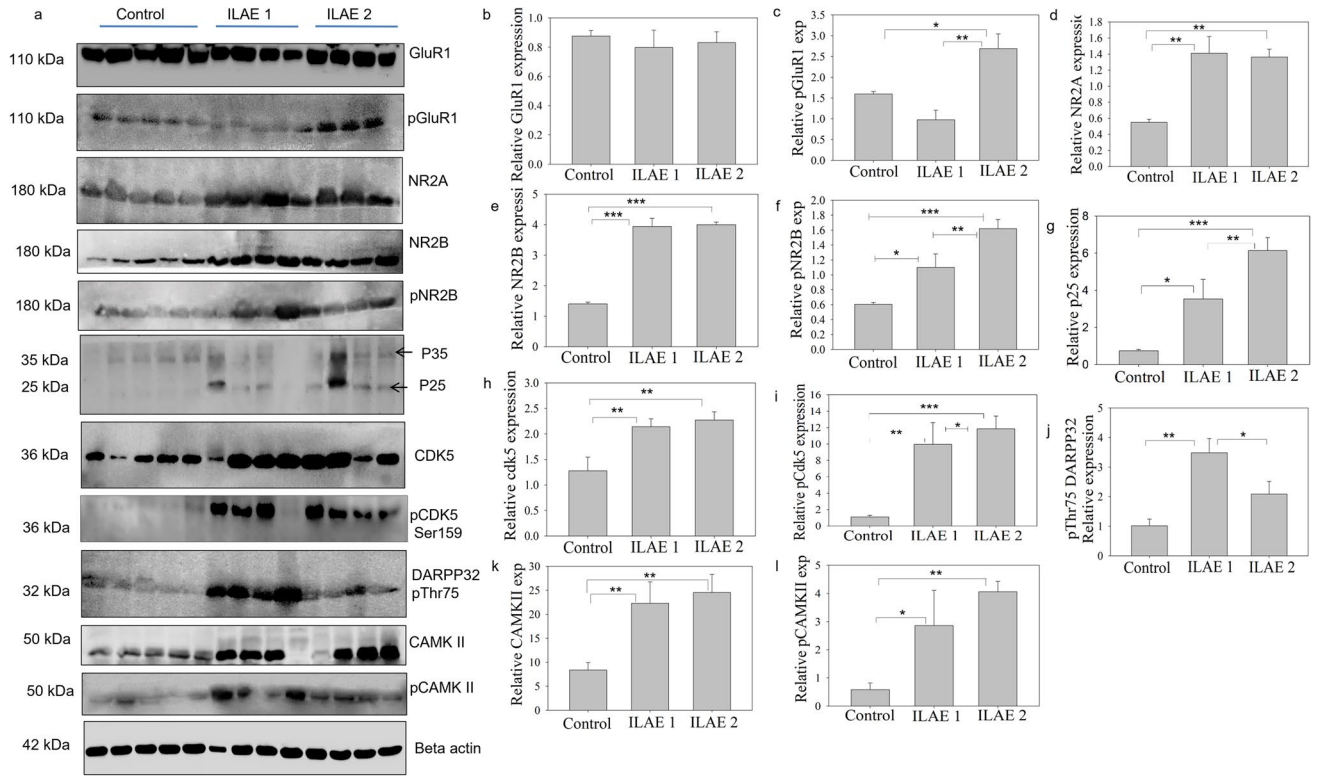

Fig. 2 **a** Immunoblot analysis of (**b**) GluR1 and (**c**) pGluR1, (**d**)NR2A, (**e**) NR2B, (**f**) pNR2B, (**g**) p25/35, (**h**) CDK5, (**i**) pCDK5, (**j**) DARPP32 pThr-75, (**k**) CAMKIIα, and (**l**) pCAMKIIα Thr-286 in both control and TLE-HS brain tissues and the corresponding bar graphs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

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 activate Ca^{2+}/calmodulin dependent protein kinaseIIα (CAMKIIα) (Fig. 2k), which was

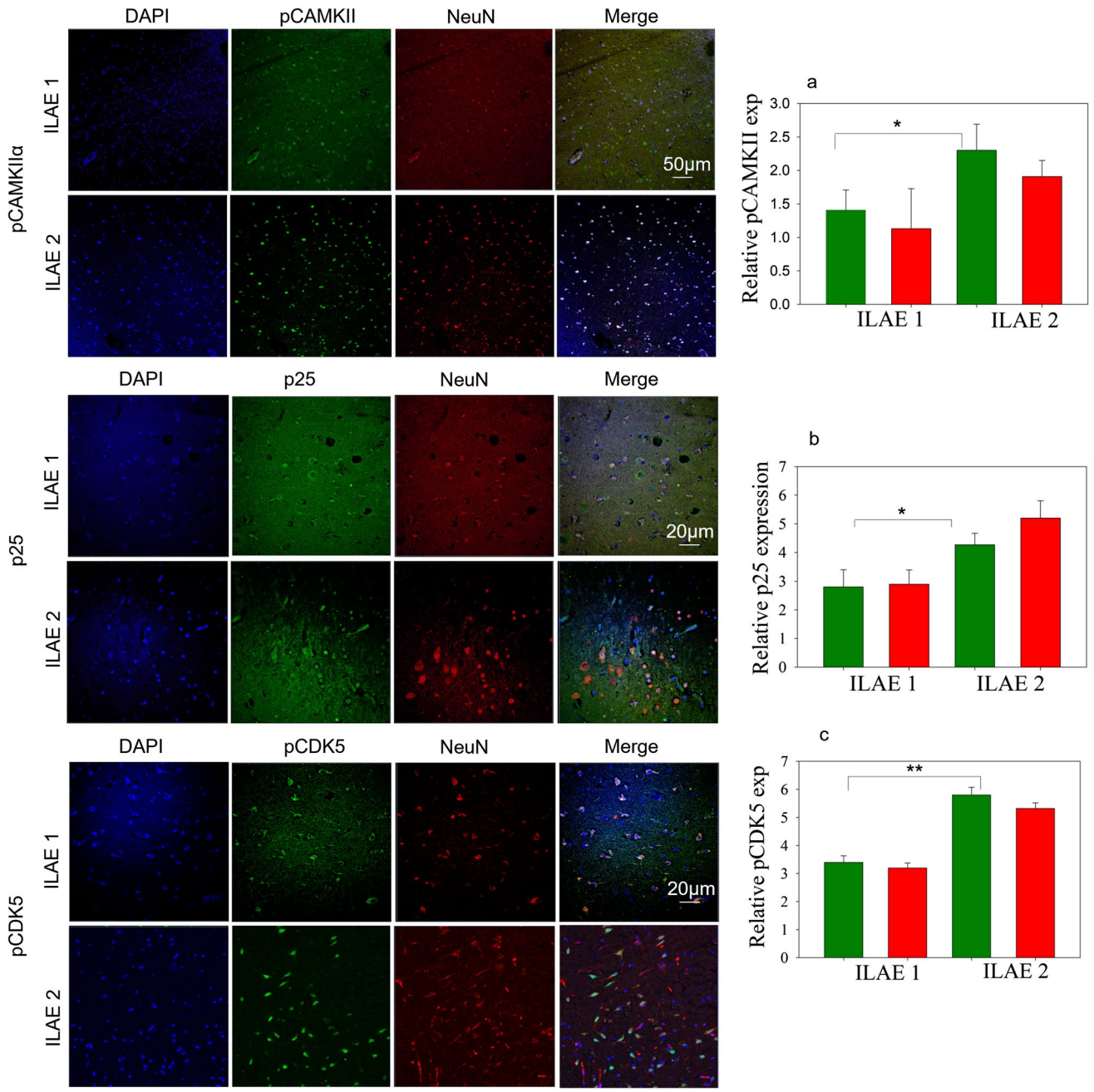

Fig. 3 Double Immunofluorescence analysis for (**a**) pCAMK IIα (Green) NeuN (red), (**b**) p25 (Green), and (**c**) pCdk5 (Green) and NeuN (red) expression in TLE-HS clinical samples. The scale bar is 20 μm for p25, pCdk5, and pCAMK IIα. The scale bar is 50 μm

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 [44]. 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

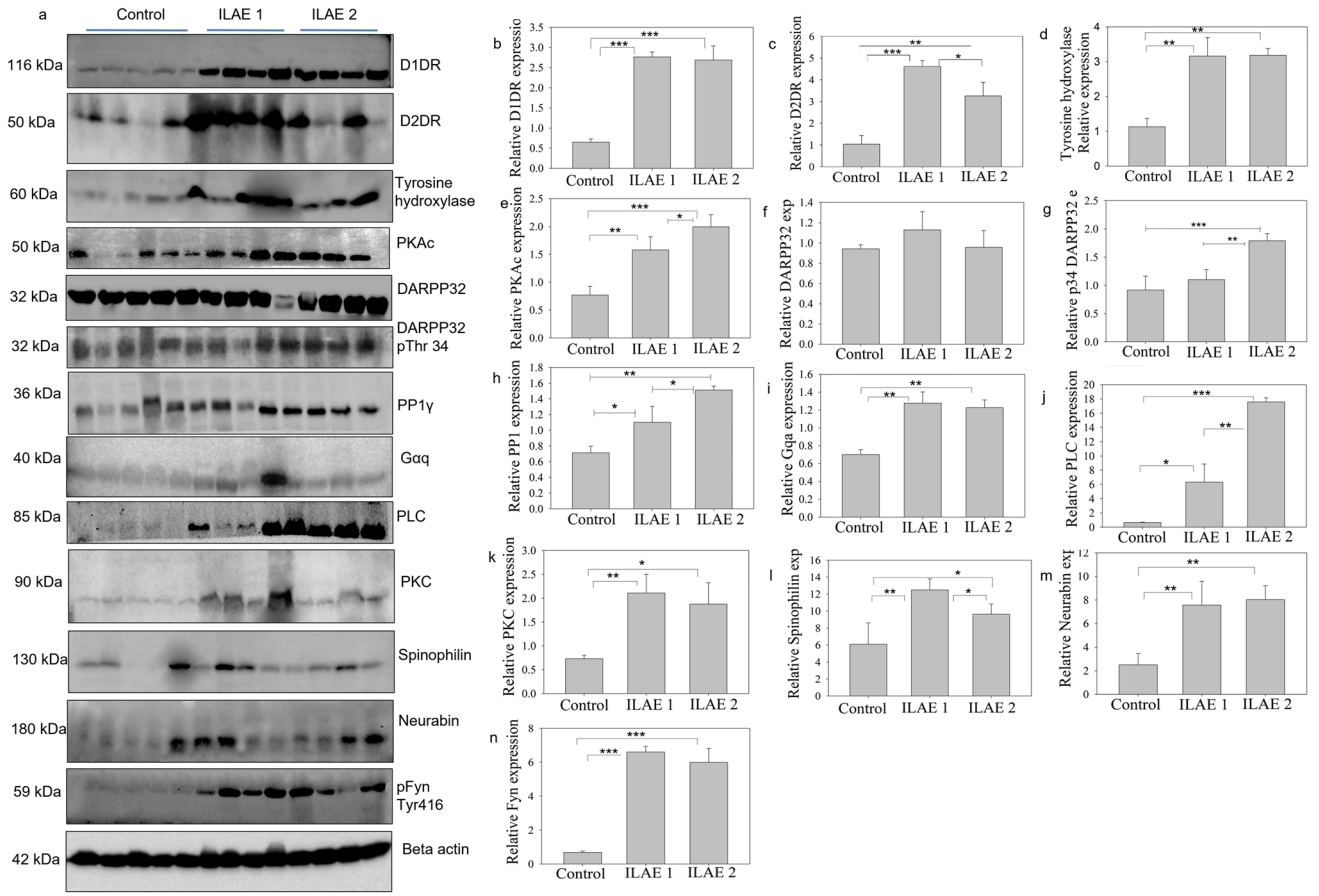

Fig. 4 **a** Immunoblot analysis of (**b**) D1DR and (**c**) D2DR, (**d**)Tyrosine hydroxylase, (**e**) PKAc, (**f**) DARPP32, (**g**) DARPP32 pThr-34, (**h**) PP1γ, (**i**) Gαq, (**j**) PLC δ, (**k**) PKC, (**l**) spinophilin, (**m**) Neurabin (**n**) pTyr416 Fyn, in both control and TLE-HS brain tissues. 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 ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

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 < 10 nm and FRET efficiency is also found to be positive (between 0—1) could support the possibility of Ca^{2+} overload in the cells.

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

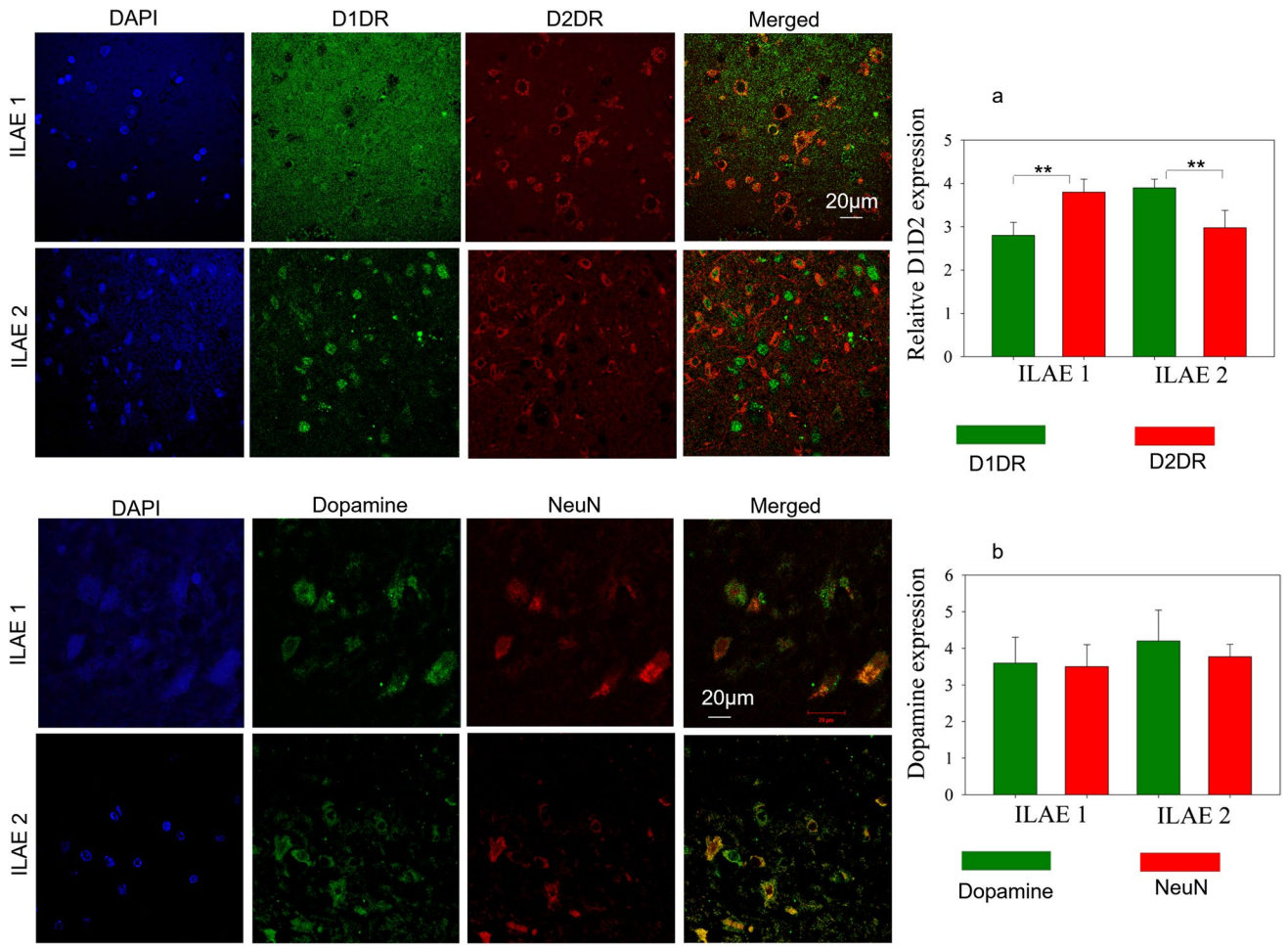

Fig. 5 Double Immunofluorescence analysis for (**a**) D1DR (Green), D2DR (Red), (**b**) Dopamine (Green), and NeuN (red) expression in TLE/HS clinical samples. The scale bar is 20 μm

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 [45]. 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 [46]. Since postsynaptic neuronal excitation controlled by ionotropic glutamate receptors regulates Na+, K+, and Ca^{2+} movement [47], 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 [48] 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 [49]. 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 [34]. In the case glutamate receptors system, as age increases, NMDA receptors become more hypofunctional [29]. In humans, NMDARs density in the frontal cortex showed a significant (36%) decline between 20 to 100 years old [50]. During healthy aging, there is general agreement that the total pool for neuronal glutamate signaling decreases [51]. mRNA levels of NMDAR were reduced in patients with Alzheimer's disease [52]. Hyperactivation of AMPA/NMDA receptors was observed in the pathophysiology of hypoxic-ischemia, trauma, and epileptic condition [27]. 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 [53]

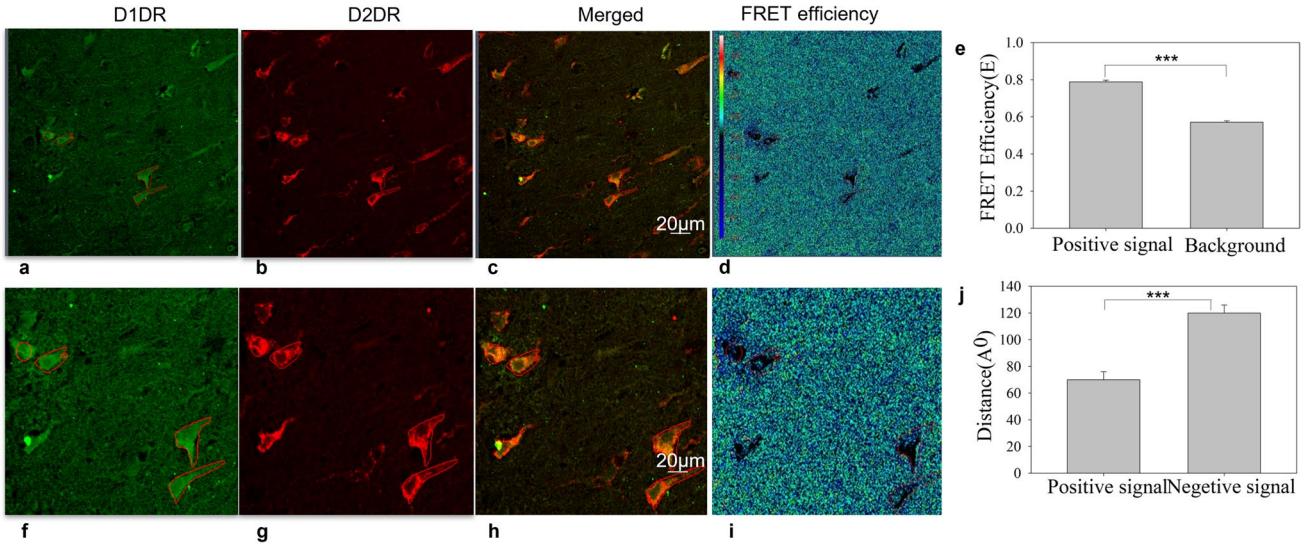

Fig. 6 Fluorescent resonance energy transfer analysis for D1/D2 heterodimer in TLE/HS indicates the interaction between D1DR and D2DR. The images show the double immunofluorescence staining of D1 (green) and D2 (red) and their interaction in TLE-HS sections

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 [30, 31]. 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 [54]. 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 [55, 56].

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 [57]. DA impedes PP1γ through PKAc-dependent pDARPP32T34 [58], 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 [59]. 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 checked 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 [60].

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 [25, 61]. 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) [62]. This condition indicates that neuronal cells are critically affected by calcium ion dysregulation [63] during epileptic seizures. This condition promotes cell death by disturbing mitochondria permeability [64]. 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 [65]. It incapacitates GluR1 and NR2B stability, reducing synaptic strength and promoting LTD [66]. 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 [67]. Fyn is an Src family kinase (SFK); it controls NR2B stability by phosphorylating at Y1472 residue at the C-terminal YEKL domain [68]. Another prospect is that spinophilin and neurabin are scaffolding proteins needed for PP1γ and its substrate interaction near dendritic spines [69]. 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 [70], 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.

Limitations

It is an observational study to correlate the expression pattern of DA and glutamate receptors and its downstream signaling proteins/kinases in the seizure-free group versus the recurrent seizures group and controls. The lack of an exact age match for children's epileptic samples could be one of the limitations. However, patients can develop an aberrant cellular network during epilepsy which induces a shift in the synchronicity of neuronal firing due to the abundance of excitatory neurotransmitters. Further research requires to confirm whether the increased DA and glutamate receptors or procrastination of PP1γ activation is behind the seizure recurrence.

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Author Contribution All the authors contributed to the study concept. The work plan was designed by [Prof. Prakash Babu Phanithi]. Data collection and data processing performed by [Kishore Madhamanchi]. Tissue sample and data collection by [Pradeep Madhamanchi]. Epilepsy surgery, storage of patient samples, and individual patient data provided by [Manas Panigrahi, Sita Jayalakshmi, and Anuja Patil. The first draft of the manuscript was prepared by [Kishore Madhamanchi] Corresponding author, and all authors commented on the previous version. All the authors read and approved the final manuscript.

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Data Availability All the data provided in the manuscript and original data supplied as a supplementary file.

Declarations

Ethics Approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the University of Hyderabad IAEC/UH/151/2017/05/PPB/P13.

Consent to Participate Written informed consent was obtained from all individual participants included in the study.

Consent to Publish For the current study, consent to publish has been received from all the patients.

Competing Interest All the authors have no relevant financial or non-financial interests to disclose.

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