RESEARCH PAPER

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Dysregulation of miR-24-3p and miR-186-5p and $GABA_A$ receptor expression in focal cortical dysplasia

Arpna Srivastava[1], Yogesh Agarwal[2], Soumil Dey[3], Ramesh Doddamani[3], Mehar Chand Sharma[4], Manjari Tripathi[1], Poodipedi Sarat Chandra[3], Sanjeev Lalwani[5], Aparna Banerjee Dixit[6]*, Jyotirmoy Banerjee[2]*

[1] Department of Neurology, AIIMS, New Delhi, India
[2] Department of Biophysics, AIIMS, New Delhi, India
[3] Department of Neurosurgery, AIIMS, New Delhi, India
[4] Department of Pathology, AIIMS, New Delhi, India
[5] Department of Forensic Medicine and Toxicology, AIIMS, New Delhi
[6] Dr B R Ambedkar Centre for Biomedical Research, University of Delhi, Delhi
** Email: jyotirmoybanerjee1@gmail.com; aparnabanerjeedixit@gmail.com*

Spontaneous synaptic activity mediated by $GABA_A$ receptor is associated with epileptogenicity in focal cortical dysplasia (FCD). miRNAs are potentially involved in the regulation of $GABA_A$ receptor subunit expression and activity. This study aimed to determine the expression of miRNAs in FCD and correlate their expression level with mRNA levels of $GABA_A$ receptor subunits. Expression of $GABA_A$ receptor subunits (α1 and α4) and miRNAs (miR-155-5p, miR-186-5p, and miR-24-3p) were evaluated using real-time PCR in resected brain samples from FCD patients. miRNA levels were also determined in the serum of FCD patients. Spontaneous $GABA_A$ receptor-mediated synaptic activity was measured using patch clamp technique. Significant increase in α1 and α4 subunit expression and miR-155-5p levels, while decrease in miR-24-3p and miR-186-5p levels, was observed in the brain samples of FCD. In the serum of FCD patients, miR-155-5p levels were increased, whereas miR-24-3p and miR-186-5p levels remained unaltered. Increased α4 subunit expression in FCD might be due to reduced levels of miR-24-3p and miR-186-5p. In addition, reduced miR-186-5p levels might be responsible for increased expression of α1 subunit. We also observed an increase in the spontaneous $GABA_A$ receptor-mediated synaptic transmission in FCD. In conclusion, dysregulation of miRNAs and $GABA_A$ receptor expression suggest that these miRNAs may contribute to altered $GABA_A$ receptor-mediated synaptic activity in FCD.

Key words: $GABA_A$ receptors, microRNAs, focal cortical dysplasia, epilepsy

INTRODUCTION

Focal cortical dysplasia (FCD) is the most common form of drug-resistant epilepsy in pediatric patients. FCD is associated with dysmature cerebral cortex which is responsible for immaturity in the neuronal network. GABA is known to be excitatory in immature neuronal networks, like during the development of the cerebral cortex. Spontaneous pacemaker GABA receptor-mediated synaptic activity has been shown in immature pyramidal neurons in the resected specimen obtained from epileptic foci of pediatric FCD patients (Cepeda et al., 2006; 2014). This increase in $GABA_A$ receptor activity has been attributed to the fact that GABA might be acting as an excitatory neurotransmitter possibly due to an immature neuronal network (Cepeda et al., 2007). We have previously shown that altered spontaneous $GABA_A$ receptor function contributes to epileptogenicity in patients with FCD (Banerjee et al., 2020). The number of α4-containing $GABA_A$ receptors is higher during development as compared to the α1-containing $GABA_A$

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receptors, which could also be possibly the case in patients with FCD (Sharma et al., 2021).

$GABA_A$ receptors switch from α4 and α5 to α1 subunit composition expression with specific biophysical properties that help rapid, transient phasic transmission. The subunit composition of neuronal $GABA_A$ receptors changes during prenatal and postnatal development (Yu et al., 2006). The reduction in α1 subunit expression potentially leads to decreased tonic inhibition and increased excitability (Semyanov et al., 2004). Increased α4 subunit expression and its redistribution to synaptic location could cause a change in the kinetics of the receptors leading to altered phasic currents. The ratio of α1/ α4 subunit expression is suspected to be changed during the development of cortical malformations in patients with FCD. Increased ratios of α4/ α1-containing $GABA_A$ receptor subunits in tuberous sclerosis complex and FCD IIB are also known to influence benzodiazepine resistance and alter GABAergic kinetics at synapses, leading to poor treatment outcomes (Talos et al., 2012). Our previous study also demonstrated that upregulated expression of α4-containing $GABA_A$ receptors, and GABAergic activity in pyramidal neurons in drug-resistant FCD is correlated with insensitivity to benzodiazepines (Sharma et al., 2021).

Receptor activity and expression are regulated by several transcriptional, posttranscriptional, and post-translational mechanisms such as phosphorylation, receptor internalization, and epigenetic mechanisms including regulation of expression by microRNAs (Chen & Roche, 2007; He et al., 2014). There are pieces of evidence that suggest that miRNA regulates the $GABA_A$ receptor subunit expression in the cerebral cortex, although details remain to be resolved. *In silico* analysis indicated potential binding sites for miRNAs on the 3'UTR of mRNAs for several $GABA_A$ receptor subunits (Barker & Hines, 2020). In experimental settings, it has been demonstrated that miR-155-5p regulates the mRNA encoding the α1 subunit, with elevated levels of miR-155-5p leading to a reduction in α1 subunit expression (D'Urso et al., 2012). Further, Zhang et al. (2019) also demonstrated the miR-155-5p up regulation was accompanied by an increase in IL-1β, IL-6, and TNF-α whereas GAT-1, GAT-3, and GABA were reduced in rats following post-ischemic seizures. We have also demonstrated the up regulation of miR-155-5p in surgically resected tissues of FCD patients (Srivastava et al., 2017). Altered $GABA_A$ receptor expression was found to be associated with FCD (Crino et al., 2001). MicroRNAs play a significant role in regulating gene expression within the nervous system, particularly during neurodevelopment (Avansini et al., 2018). Given these considerations, the role of miR-155-5p needs to be explored in FCD in the context of $GABA_A$ receptor α1 expression. The expression of miR-186-5p and miR-24-3p has been shown to downregulate $GABA_A$ receptor α4 expression. Transfection with molecular mimics of miR-186-5p, or miR-24-3p also downregulated $GABA_A$ receptor α4 expressions, whereas transfection with the corresponding inhibitors of these microRNAs normalized $GABA_A$ receptor α4 expression in cultured cortical mouse neurons. Promoter-reporter experiments supported the idea that miR-186-5p and miR-24-3p bind to the 3'UTR of $GABA_A$ receptor α4 and thereby inhibit protein production (Bekdash & Harrison, 2015). miR-186-5p and miR-24-3p are predicted to bind the $GABA_A$ receptor subunit α4 by TargetScan and miRanda (Zhao et al., 2012). To our knowledge, no information is available regarding the interplay between these miRNAs and the $GABA_A$ receptor subunit expression in FCD patients. The aim of the current study was to evaluate the expression of miRNAs (miR-155-5p, miR-186-5p, and miR-24-3p) potentially involved in the dysregulation of $GABA_A$ receptor expression in resected brain and blood samples from FCD patients. Further, we measured $GABA_A$ receptor-mediated activity in brain specimens from FCD patients.

METHODS

Sample Collection

Patients diagnosed with drug-resistant epilepsy due to FCD who underwent surgery were included in the study. Each patient was assessed preoperatively, and pathology was determined by analyzing convergent data from MRI, video electroencephalography (vEEG), fluoro-2-deoxyglucose positron emission tomography (FDG-PET), and magnetoencephalography (MEG). Pathology was further confirmed by histopathological examination by neuropathologists. Well-established epileptogenic zones resected during brain surgery were used in this study. Patients with dual pathology were excluded. For control, autopsy samples (cortex) of minimum *post-mortem* delay (within 8 h) and not having any neurological disorders were obtained. As per inclusion criteria, a total of 29 FCD and 12 autopsy samples were collected for this study, including 18 male and 11 female patients. The detailed clinical characteristics of individuals are listed in Table 1. The mean age of the patients was 20.62 ± 8.08 (ranging from 6-36 years). The mean age of autopsy control was 27.66 ± 10.53 (ranging from 16-50 years), mean age for tumor periphery control was 30.4 ± 7.45 (ranging from 15-40 years), and mean age of healthy individuals was 27.58 ± 5.69 (ranging from 18-42 years). The duration of seizures averaged at 9.96 ± 6.02 years. The seizure on-

Table 1. Clinical data of patients and controls.

Patient/Control	Age (Years)/Gender	Pathology	Seizure Frequency	Age of onset of seizure	ASDs
F1	22/M	FCD IIa	1-2 Day	1.5 yrs	LMG,CLM,LVM, TPM,
F2	24/M	FCD IIa	5-6/Month	14 yrs	VAL, PHE, CLM
F3	14/M	FCD IIb	3-4/Week	3 yrs	LVM,LCS,CLM, OXC
F4	21/M	FCD I	3-4/Day	10 yrs	VAL, CLM
F5	24/F	FCD IIa	3-4/Month	20 yrs	CLM, LVM, TPM
F6	18/M	FCD IIa	4-5/Day	6 yrs	CBZ,LVM,CLM
F7	6/F	FCD IIa	1/15 Day	0.6 yrs	VAL,CLM,LVM,
F8	14/M	FCD IIb	4-5/Day	7 yrs	CLM, VAL, LVM
F9	18/F	FCD IIa	5-6/Day	12 yrs	VAL, LVM, LCS, CLM, PHB
F10	13/M	FCD Ib	1-2/Day	8 yrs	CBZ, CLM, LVM, SV
F11	36/F	FCD IIb	20-30/Day	5 yrs	CLM,LCM, LVM, OXCBZ, SV
F12	6/M	FCD Ic	1-2/Day	0.8 yrs	CLM, LCS, OXCBZ, SV
F13	17/M	FCD IIa	1/1-2 Days	11 yrs	LVM, LCS, CLM
F14	23/M	FCD Ic	3-4/Month	5 yrs	CLM,TPM, PHB,VAL
F15	22/F	FCD Ic	3-4/Day	16 yrs	CLM, LVM, SV,
F16	13/M	FCD Ia	1-2/Day	8 yrs	CBZ, CLM, LVM, SV
A1	25/M	Pelvic Injury	NA	NA	NA
A2	18/F	Pelvic Injury and lower limb injury	NA	NA	NA
A3	16/M	Abdominal Injury	NA	NA	NA
A4	18/M	Head and abdominal injury	NA	NA	NA
A5	40/M	Respiratory failure	NA	NA	NA
A6	36/F	Heart Failure	NA	NA	NA
A7	30/M	Abdominal injury	NA	NA	NA
A8	50/M	Myocardial infarction	NA	NA	NA
A9	26/M	Asphyxia	NA	NA	NA
A10	18/M	Abdominal injury	NA	NA	NA
A11	37/M	Hanging	NA	NA	NA
A12	18/M	Pelvic limb injury	NA	NA	NA
T1	15/F	Low grade glioma	NA	NA	NA
T2	48/M	Low grade glioma	NA	NA	NA
T3	35/M	Low grade glioma	NA	NA	NA
T4	35/M	Low grade glioma	NA	NA	NA
T5	53/M	Low grade glioma	NA	NA	NA
H1	18/F	Healthy Individual	NA	NA	NA

Patient/Control	Age (Years)/Gender	Pathology	Seizure Frequency	Age of onset of seizure	ASDs
H2	30/M	Healthy Individual	NA	NA	NA
H3	24/F	Healthy Individual	NA	NA	NA
H4	29/F	Healthy Individual	NA	NA	NA
H5	25/M	Healthy Individual	NA	NA	NA
H6	30/M	Healthy Individual	NA	NA	NA
H7	22/M	Healthy Individual	NA	NA	NA
H8	24/F	Healthy Individual	NA	NA	NA
H9	27/M	Healthy Individual	NA	NA	NA
H10	30/F	Healthy Individual	NA	NA	NA
H11	30/M	Healthy Individual	NA	NA	NA
H12	42/F	Healthy Individual	NA	NA	NA

ASDs = Anti-seizure drugs, A = autopsy; CAR = Carbamazepine, CLN = clonazepam; CLO = clobazam, COD = cause of death; F=FCD patients, H=Healthy Individual; LEV = Leviteracetum, OXC = Oxcarbamazepine; PHE = Phenytoin, LTG = Lamotrigen; PBT = Phenobarbital, LCS = Lacosamide; TPR = Topiramate. T=tumor periphery; VAL = Valproate.

set was 10.65 ± 6.14 years. All patients with FCD were seizure-free post-resective surgery (Class I Engel outcome). Part of the resected samples from the FCD patients were stored in 4% paraformaldehyde for histopathological examination and the remaining part was immediately frozen and stored at -80°C until further use. All studies were conducted in accordance with the Declaration of Helsinki and were approved by the Institute Ethics Committee, AIIMS, New Delhi (File No. IEC-1011/03.10.2020). Informed and written consent was obtained from all patients, their parents, or legal representatives if patients were underage.

Resected cortical tissues obtained from the periphery of brain tumors during surgical resection, which was also a part of planned surgical resections, without any history of seizures were used as non-seizure controls (n=10) for experiments involving cellular electrophysiological studies. After resection, the brain tissues from FCD patients (F17 to F29) and non-seizure control tissue (from tumor periphery) were collected in well-carbogenated (95% O_2 + 5% CO_2) artificial cerebrospinal fluid (ACSF) and processed for electrophysiological experiments.

For quantification of microRNAs in blood, 5 ml blood samples were also collected from the patients undergoing surgery (n=16). For control, normal healthy individuals were enrolled (n=12).

Histopathology

Tissue was fixed in 4% paraformaldehyde and embedded in paraffin wax for preparing 5-µm thick tissue sections. Nissl staining with cresyl violet was used for this study. Briefly, sections were dehydrated and slides were consecutively rinsed in 95%, 80% and 70% ethanol solutions (v/v, in distilled water). Then slides were then rinsed in water and in cresyl violet solution (0.5%) for 30 min and then washed with distilled water. Subsequently, sections were dehydrated by consecutive washes of increased concentrations of ethanol solution and xylene. Slides were covered with glass coverslips and DPX (Sigma) as a mounting medium (Liberato et al., 2018). The stained sections were independently reviewed by two neuropathologists to confirm the pathology and to assess any damage in control tissue.

Blood processing and total RNA isolation

Up to 5 ml whole blood was collected in a serum separator tube from each participant and left in an upright position for 60 min at room temperature to allow clotting. After clotting, the blood tubes were centrifuged at 1500 × *g* for 15 min. The supernatants were transferred into microtubes and stored at -20°C and were

not thawed until use (Chan et al., 2023). The total RNA of serum was extracted and purified using miRNeasy Serum/Plasma kit (Qiagen) following the manufacturer's instructions. On-column DNase treatment was carried out to remove any DNA contamination. Qubit™ microRNA Assay Kit (Invitrogen) was used to measure the miRNA concentration by Qubit 3.0 fluorometer (Life Technologies).

$GABA_A$ receptor subunits and miRNA expression analysis by real-time PCR

Quantitative real-time PCR was performed to evaluate the mRNA levels of $GABA_A$ receptor α1 and α4 subunits and miRNAs (miR-155-5p, miR-186-5p and miR-24-3p) in FCD patients. Total RNA was extracted from surgically resected tissues using the miRNeasy kit (Qiagen) according to the manufacturer's protocol. qPCR was performed in 16 FCD (F1-F16) patients and 12 control samples (A1-A12). RNA integrity was determined by accessing the electropherograms and RNA quantity was determined using a Qubit 3.0 fluorometer (Life Technologies). The reverse transcription process of RNA was carried out using a high-capacity cDNA reverse transcription kit (Invitrogen). Real-time PCR amplifications was performed in CFX 96 Real Time Systems (Bio-Rad) with Bio-Rad CFX software manager with the following cycling parameters: an initial hot start of 95°C for 3 min followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. GAPDH was used as reference gene to normalize Ct expression values for each gene transcript. For miRNA quantification, cDNA synthesis was done using the miScript II RT Kit (Qiagen) in a total volume of 20 µl. miR16 was used as a reference gene for tissue-based studies, whereas U6SnRNA was used for serum-based studies. Reference genes for this study were selected based on previous studies, and their expression was also determined to ensure stable expression across the samples. Primer sequences for all the genes were obtained from previous studies (Srivastava et al., 2017; Wang et al., 2018; Liu et al., 2018; Sharma et al., 2021). The primers used in this study are listed in Table 2. cDNA was mixed with specific forward primer and miScript SYBR Green PCR Kit (Qiagen) plus the universal reverse primer. Real-time PCR amplifications was performed in CFX96 Real Time PCR detection System (Bio-Rad) with the following cycling parameters: initial hot start of 95°C for 15 min followed by 40 cycles of 95°C for 15 s and 55°C for 30 s followed by extension at 70°C for 30 s. The relative amount of $GABA_A$ receptor subunits and miRNA were assessed using the comparative Ct method normalized to the reference gene. At the end of PCR cycles, melting curve analyses were performed to validate the specific generation of the expected PCR products. Each sample was run in triplicates for analysis. The $2^{-\Delta\Delta Ct}$ method was used to measure the expression of a gene (Livak & Schmittgen, 2001).

In vitro electrophysiology

Tissue samples were obtained and submerged in ice-cold, carbogenated ACSF (artificial cerebrospinal fluid), which consisted of the following components: NaCl (125 mM), KCl (2.5 mM), $CaCl_2$ (2 mM), $NaHCO_3$ (25 mM), NaH_2PO_4 (1.25 mM), $MgCl_2$ (1 mM), and glucose (25 mM). Subsequently, 350-µm-thick slices were prepared using a vibratome (VT1000S, Leica). The slices were subsequently transferred to a recording chamber continuously perfused with ACSF (2 ml/min) at room temperature (20-22°C). The pyramidal neurons with thick soma and single tapering dendrite were identified visually using IR-DIC video microscopy. The patch pipettes, which had a resistance in the range of 3–5 MΩ, were loaded with an internal solution comprising the following components: HEPES (10 mM), MgCl2 (2 mM), Cs-methanesulfonate (130 mM), EGTA (10 mM), and CsCl (10 mM). Whole-cell patch clamp recordings were performed from those pyramidal neurons using an amplifier in voltage clamp mode (Axopatch 200B, Molecular Devices, CA, USA). Spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded at 0 mV (Banerjee et al., 2020; Sharma et al., 2021). The data were analysed in pCLAMP 10.0 software. Frequency, amplitude, and kinetic values (rise time, 10–90% and decay time con-

Table 2. Real-time PCR primers used in this study.

Gene	Sequence
GABRA1	CTGGACTCCGGACACATTTT
	TCTCACTGTCAGCCTCATGG
GABRA4	CCCAGTTTGGGTACTGTGCT
	CTCTAGTTTTGGCCCTGCTG
GAPDH	GAGTCAACGGATTTGGTCGT
	TTGATTTTGGAGGGATCTCG
mir-155	TTAATGCTAATCGTGATAGGGGTT
mir-186	CAAAGAATTCTCCTTTTGGGCT
mir-24	TGCCTACTGAGCTGATATCAGT
mir-16	TAGCAGCACGTAAATATTGGCG
U6SnRNA	CTC GCT TCG GCA GCA CA
	AAC GCT TCA CGA ATT TGC GT

stant, τ_d) of the synaptic events were measured. All recordings were visually inspected to find events, which show a steep rising and exponential decay phase for kinetic analysis of EPSCs. We have recorded sIPSCs from one neuron/slice from each patient. Consequently, the number of neurons represent the number of patients. Further to compare the kinetics of GABAergic events between FCD type I and type II we have correlated the findings from the current study with our previously reported *in vitro* electrophysiology data from FCD samples (F17 to F29) (Table 1; Banerjee et al., 2020).

Statistical analysis

Statistical analysis was performed using Sigma Plot software version 13.0 (Systat Software Inc.). All experiments were performed in triplicate and data are presented as the mean ± standard deviation. Data were analyzed using the student's t-test in independent experiments. Furthermore, the Pearson correlation coefficient was used to examine the relationship between gene expressions in samples and clinical features. The level of significance for statistical test was 0.05. Results were considered to be statistically significant with a significance level of $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****).

RESULTS

Histopathological examination of the resected brain specimens from FCD patients

Histopathological examinations were carried out on all the samples obtained for experiments (as mentioned in Table 1) to confirm the pathology (Fig. 1). Characteristic features of FCD patients were observed in all the patients. Tissue sections from FCD patients exhibited cortical malformations with severe cytoarchitectural abnormalities, including dysmorphic neurons with abnormal sizes and morphologies, as well as increased accumulation of neurofilament proteins, as depicted in Fig. 1B-C. Additionally, Fig. 1C revealed the presence of balloon cells with large, opalescent cytoplasm and poorly defined membranes. Cortical sections from autopsy showed normal cytoarchitecture (Fig. 1A).

Upregulation of $GABA_A$ receptor subunit expression in resected cortical specimens from FCD patients

To study the role of $GABA_A$ receptor α1 and α4 subunits in FCD, we analyzed the mRNA expression of α1 and α4 in the FCD brain specimens using RT-qPCR.

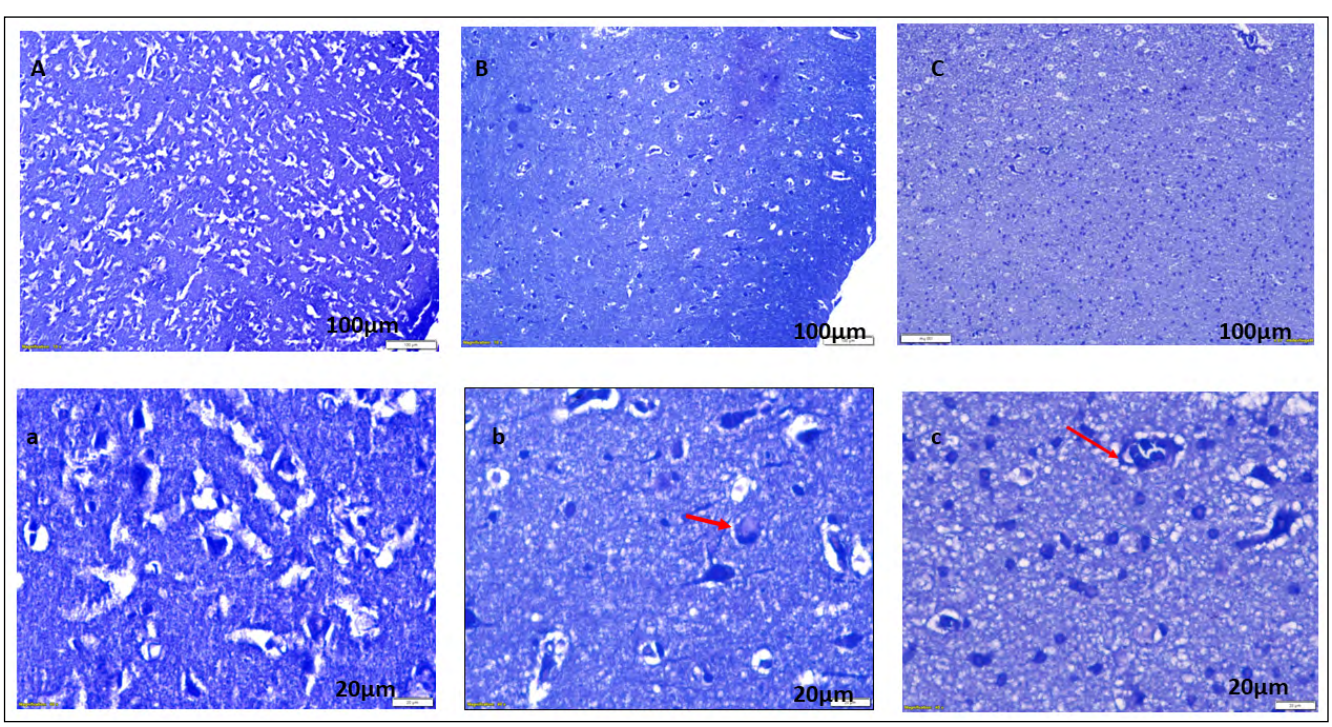

Fig. 1. Photomicrograph showing characteristic histopathological features of focal cortical dysplasia. Representative image of the cortical section from FCD patients showing dysmorphic neurons (B) CV staining, ×400; and dysmorphic neurons with balloon cells (C) CV staining, ×400. Cortical sections from autopsy showed normal cytoarchitechure. (A) CV staining, ×400.

The relative levels of the mRNA transcripts are shown in Fig. 2. Significant increase in α1 (4.06±1.46-fold, p<0.0001) and α4 (6.88±1.77-fold, p<0.01) expression were observed in FCD compared to autopsy control.

Altered expression pattern of miRNAs in resected cortical tissues and serum of FCD patients

The expression of miR-155-5p, miR-24-3p, and miR-186-5p were evaluated in both surgically resected specimens from FCD patients and serum of FCD patients. Increased expression of miR-155-5p (6.98±0.72 fold; $p < 0.0001$) while decreased expression of miR-24-3p (-2.20±0.63 fold; $p < 0.01$) and miR-186-5p (-2.74±0.69 fold; $p < 0.001$) was observed in FCD specimens compared with autopsy controls (Fig. 3).

The expression of miR-155-5p (2.51±0.64 fold; $p < 0.01$) was found to be increased in the serum of FCD patients compared to control serum (Fig. 4). No significant difference in the levels of miR-24-3p (0.73±0.48 fold; $p > 0.05$) and miR-186-5p (0.59±0.34 fold; $p > 0.05$) was observed in the serum of the two groups (Fig. 4).

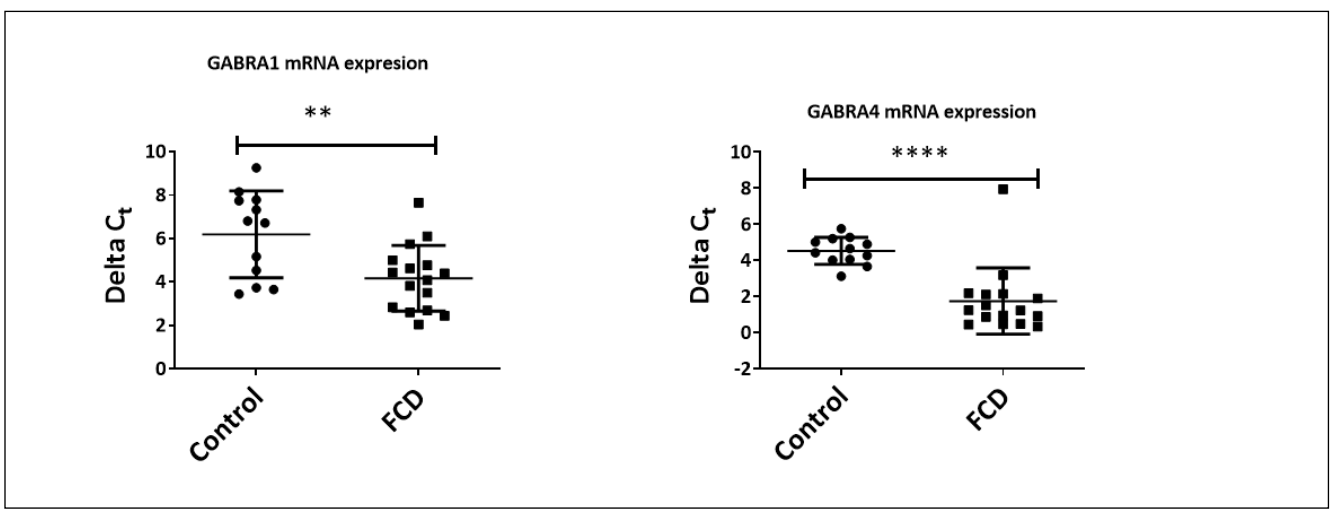

Fig. 2. The relative amount of mRNA transcripts in patients analyzed using quantitative real time PCR. mRNA levels of $GABA_A$ receptor α1 and α4 in FCD compared to control (t-test). Relative changes in gene expression were calculated using the ΔΔCT method with GAPDH as a reference gene. Error bar is ± SD based on 16 FCD patients and 12 control samples, and each sample was analyzed in triplicates. Mean increase in transcripts levels were statistically significant (**p<0.01; ****p<0.0001).

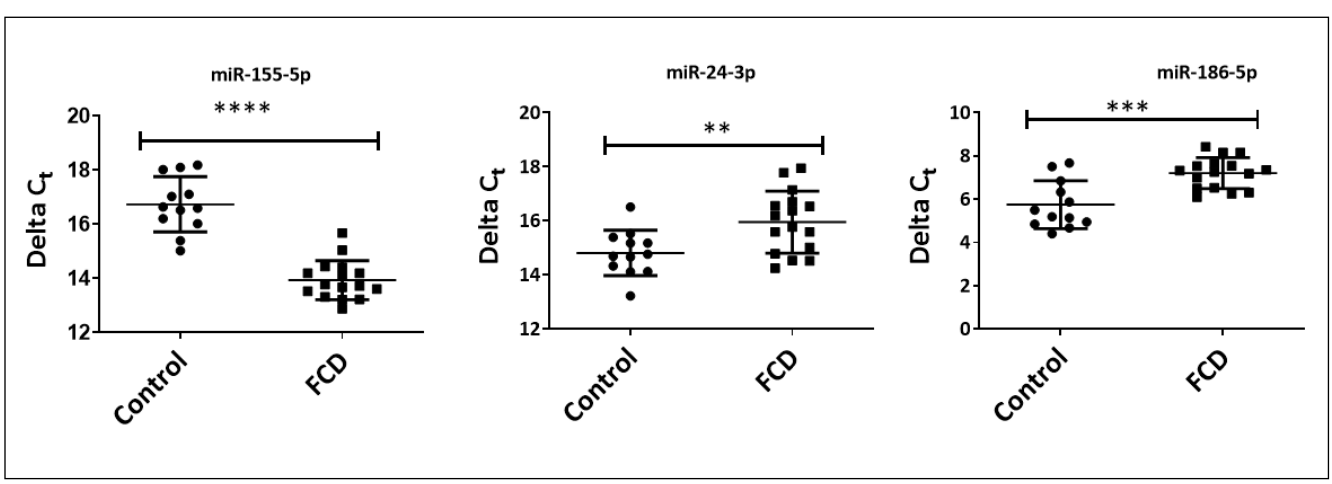

Fig. 3. Differential expression of miRNAs in surgically resected tissues of FCD patients. Error bar is mean ± SD based on sixteen patients from FCD and twelve control samples, and each sample is analyzed in triplicates. Relative changes in gene expression were calculated using the ΔΔCT method with miR16 as a reference gene. Mean increase in transcripts levels are statistically significant (t-test; **p<0.01; ***p<0.001; ****p<0.0001).

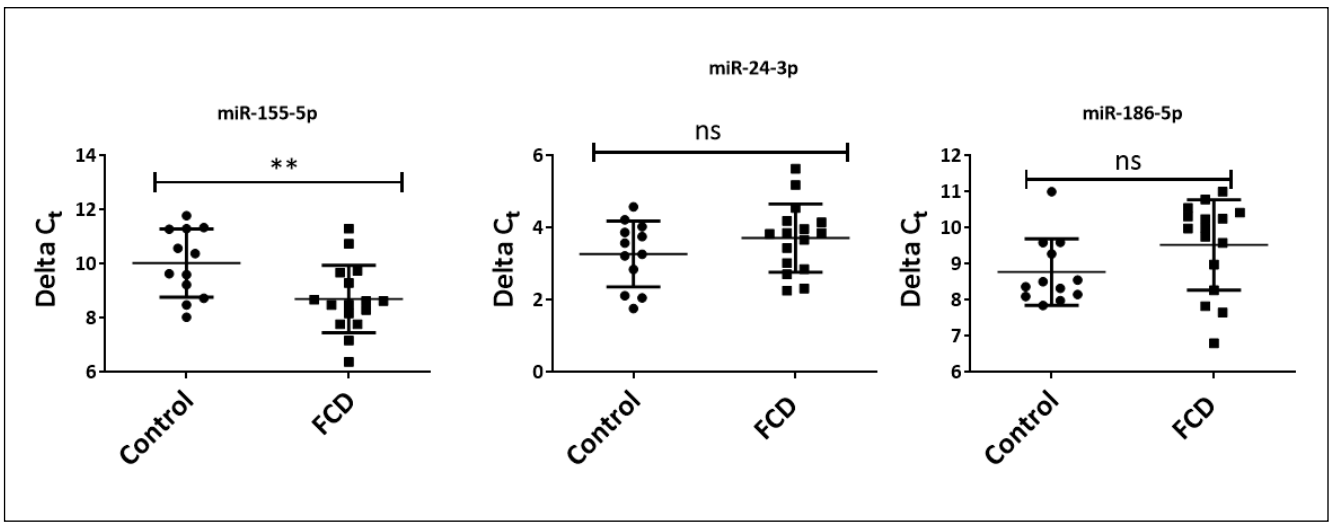

Fig. 4. Differential expression of miRNAs in serum of FCD patients. Error bar is mean ± SD based on sixteen patients from FCD and twelve control samples, and each sample is analyzed in triplicates. Relative changes in gene expression were calculated using the ΔΔCT method with U6SnRNA as a reference gene. Mean increase in transcripts levels are statistically significant (t-test; **p<0.01).

Potential regulatory role of miRNAs in $GABA_A$ receptor expression in FCD

Pearson's correlation analysis was performed to demonstrate the potential regulatory role of miRNAs in $GABA_A$ receptor subunit expression. The results demonstrated decreased expression of $GABA_A$ receptor α4 subunit as the expression level of miR-24-3p (r=-0.8835; p<0.0001; Fig. 5; Table 3) and miR-186-5p (r=-0.9206; p<0.0001; Fig. 5; Table 3) increased in resected brain specimens from FCD patients (Fig. 5; Table 3). No correlation was observed between miR-155-5p and $GABA_A$ receptor subunit α4 expression levels (r=-0.2577; p>0.05; Fig. 5; Table 3) in resected FCD specimens. The expression of miR-186-5p decreased with an increase in $GABA_A$ receptor subunit α1 mRNA expression (r=-0.6093; p<0.05; Fig. 5; Table 3), while no significant correlation was observed between miR-155-5p (r=0.02928; p>0.05; Fig. 5; Table 3); miR-24-3p (r=-0.4484; p>0.05; Fig. 5; Table 3) and $GABA_A$ receptor subunit α1 expression levels (r=0.028; P>0.05; Fig. 5; Table 3) in resected FCD specimens. In serum from FCD patients, the expression of miR-24-3p decreased as the expression of α4 expression increased (r=-0.5002; p<0.05; Fig. 5; Table 3).

Pearson correlation was used to correlate miRNAs (miR-155-5p, miR24-3p, and miR186-5p) and $GABA_A$ receptor subunit α1 and α4 mRNA expression with clinicopathological characteristics (age, gender, seizure frequency, duration of epilepsy and seizure onset). No significant correlation was observed except between $GABA_A$ receptor subunit α1 and age of FCD patients (r=-0.6373; p<0.01; Table 4).

$GABA_A$ receptor activity in resected cortical tissues of FCD patients

sIPSCs were recorded from pyramidal neurons of resected brain specimens obtained from patients with FCD and non-seizure controls. The sIPSCs were outward currents and blocked by bath perfusion with ACSF-containing 10 μM bicuculline for 15 min, implying that these events were mediated by $GABA_A$ receptors. These events were unaffected in the presence of 50 μM APV and 10 μM CNQX, implying that these events were not under regulation of NMDA and AMPA type glutamate receptors in these slice preparations. We have previously reported altered $GABA_A$ receptor activity in resected brain samples obtained from patients with FCD (Banerjee et al., 2020). Here we have pooled the *in vitro* electrophysiology data from the current study with that in our previous study and compared the kinetics of $GABA_A$ receptor activity in FCD type I and II with non-seizure controls. Our analysis did not reveal any significant differences in the GABAergic event kinetics between FCD type I and II. However, significant differences were observed in the kinetics of GABAergic event when comparing both FCD type I and II cases with non-seizure controls. The frequency, amplitude and decay time constant (τ_d) of the sIPSCs were significantly increased in the FCD samples (Fig. 6). Quantitative analysis showed that the normalized cumulative distribution of inter-event intervals shifted significantly to the left, whereas peak amplitudes shifted significantly to the right, in the brain specimens obtained from FCD patients (Fig. 6).

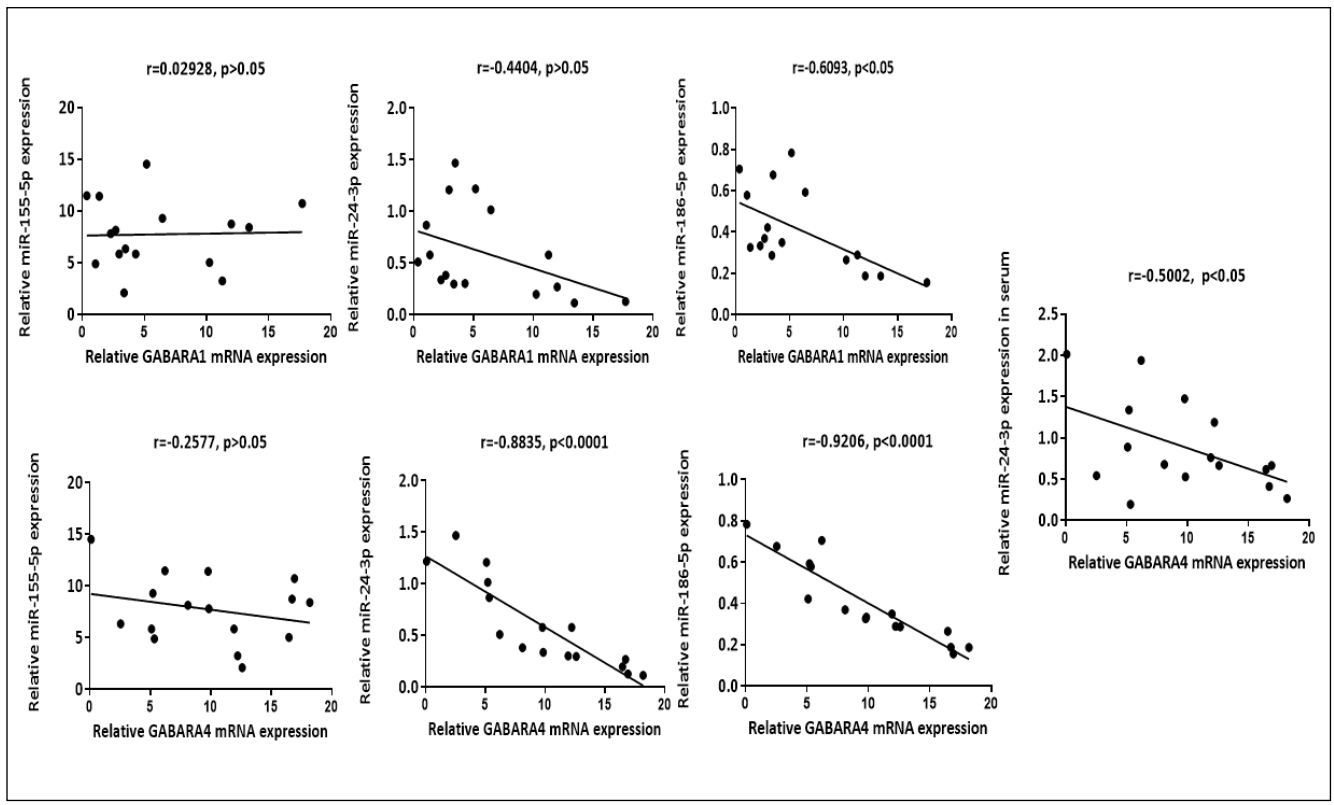

Fig. 5. Correlation between miRNAs and $GABA_A$ receptor subunit α1 & α4 mRNA expression in FCD patients. The relationships between miRNAs and its corresponding target prediction were assessed by Pearson's correlation coefficients. Negative correlation was observed between the expression level of miR-24-3p (r=-0.8835; p<0.0001) and miR-186-5p (r=-0.9206; p<0.0001) in resected tissues with the expression of $GABA_A$ receptor subunit α4 expression in FCD patients. No significant correlation was observed between miR-155-5p and $GABA_A$ receptor subunit α4 expression levels (r=-0.2577; p>0.05) in FCD resected tissues. An inverse correlation was observed between the expression of miR-186-5p and $GABA_A$ receptor subunit α1 mRNA expression (r=-0.6093; p<0.05), while no significant correlation was observed between miR-155-5p (r=0.02928; p>0.05) and miR-24-3p (r=-0.4484; p>0.05;) and $GABA_A$ receptor subunit α1 expression levels (r=0.028; p>0.05) in FCD resected tissues. In serum, only expression of miR-24-3p showed negative correlation with $GABA_A$ receptor subunit α4 expression (r=-0.5002; p<0.05).

Table 3. Correlation analysis of miR-155-5p, miR-24-3p, and miR-186-5p with $GABA_A$ receptor subunit expression in FCD patients.

Expression	$GABA_A$ receptor α1 expression	$GABA_A$ receptor α4 expression
miR-155-5p expression (tissues)	r=0.02928 p=0.9143	r=-0.2577 p=0.3353
miR-24-3p expression (tissues)	r=-0.4484 p=0.0815	r=-0.8835 p=<0.0001
miR-186 expression (tissues)	r=-0.6093 p=0.0122	r=-0.9206 p=<0.0001
miR-155-5p expression (serum)	r=-0.1504 p=0.5782	r=-0.2717 p=0.3087
miR-24-3p expression (serum)	r=-0.2764 p=0.3001	r=-0.5002 p=0.0485
miR-186 expression (serum)	r=-0.01731 p=0.9493	r=0.05481 p=0.8402

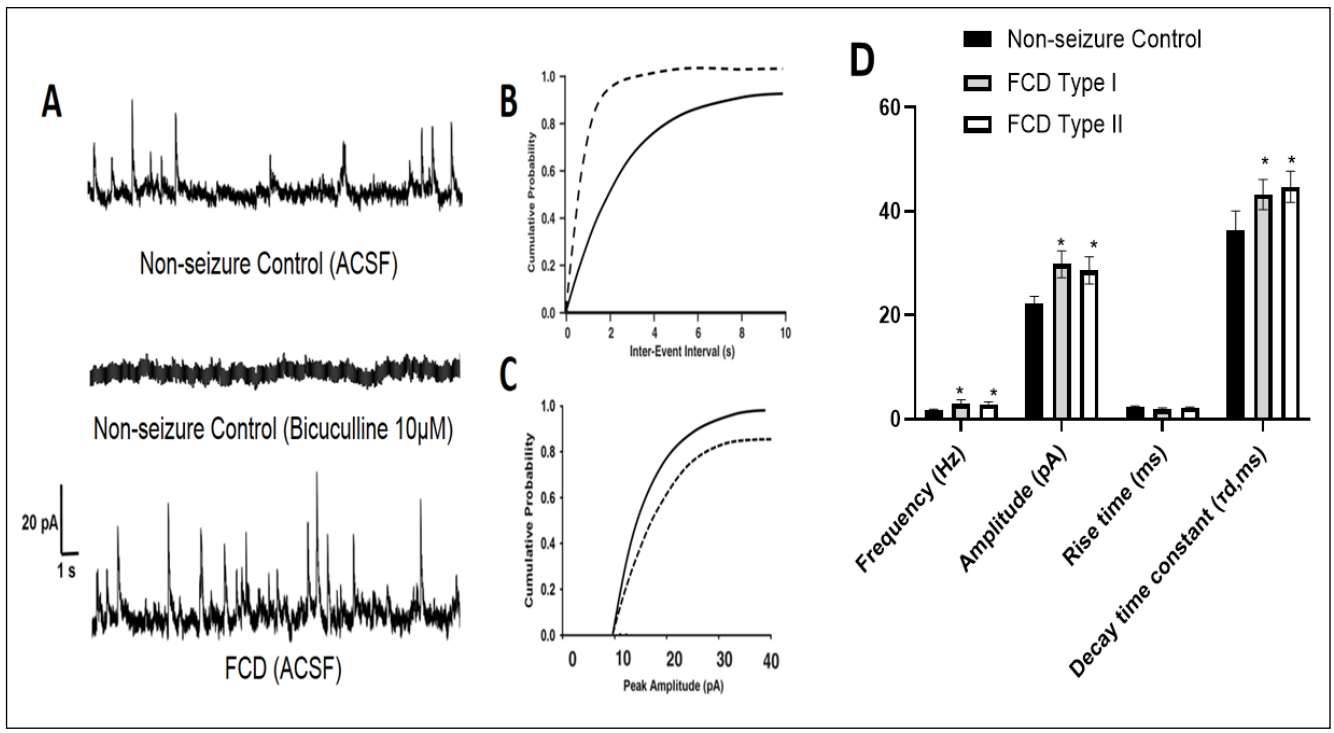

Fig. 6. GABAergic activity in non-seizure controls and FCD patients. The sample recordings of spontaneous GABAergic PSCs at 0 mV obtained from pyramidal neurons of non-seizure control samples (top trace). Second trace shows representative recordings at 0 mV, 15 min following superfusion of the slice with ACSF containing $GABA_A$ receptor antagonist bicuculline (10 μM). Third trace shows representative recordings of spontaneous IPSCs from pyramidal neuron at 0 mV from resected brain sample obtained from patient with FCD. In the FCD brain tissues, cumulative distribution of inter-event interval displaced towards lower intervals (K-S test, $p<0.001$) and (C) peak amplitude displaced towards longer amplitude (K-S test, $p<0.01$). (D) Characteristics of sIPSCs recorded from pyramidal neurons in resected brain samples obtained from patients with non-seizure control (n=10), FCD type I (n=6) and type II (n=7). The data are presented as the mean ± S.E.M. *$p<0.05$ compared to non-seizure control according to one-way ANOVA followed by Tukey *post hoc* test.

Table 4. Correlation analysis of $GABA_A$ receptor subunit expression and microRNA expression with clinicopathological characteristics of FCD patients.

Expression	Age	Gender	Seizure Frequency (per day)	Duration of epilepsy (in years)	Seizure onset
$GABA_A$ receptor α1 expression	r=-0.6373 p=0.0079	r=0.1746 p=0.5179	r=-0.2279 p=0.3960	r=-0.2041 p=0.4483	r=-0.4428 p=0.1021
$GABA_A$ receptor α4 expression	r=-0.3074 p=0.2468	r=0.03002 p=0.9121	r=-0.03805 p=0.8887	r=-0.2629 p=0.3252	r=-0.4236 p=0.66
miR-155-5p expression (tissues)	r=-0.07176 p=0.7917	r=0.3152 p=0.2344	r=0.2363 p=0.3783	r=0.2349 p=0.3811	r=0.07415 p=0.7849
miR-24-3p expression (tissues)	r=0.2148 p=0.4244	r=-0.04842 p=0.8587	r=0.07888 p=0.7715	r=0.04453 p=0.8699	r=0.1928 p=0.4743
miR-186-5p expression (tissues)	r=0.2401 p=0.3704	r=0.02428 p=0.9283	r=-0.1029 p=0.7045	r=0.3238 p=0.2212	r=0.4735 p=0.0640
miR-155-5p expression (serum)	r=0.0045 p=0.9866	r=0.4260 p=0.0999	r=0.05449 p=0.8411	r=-0.1517 p=0.5749	r=0.3035 p=0.2532
miR-24-3p expression (serum)	r=0.2910 p=0.2742	r=0.3484 p=0.2788	r=0.2710 p=0.3100	r=0.4327 p=0.1872	r=0.4747 p=0.0632
miR-186-5p expression (serum)	r=0.2806 p=0.2926	r=0.3832 p=0.1429	r=0.3037 p=0.2528	r=-0.1419 p=0.6000	r=0.3954 p=0.1295

DISCUSSION

Findings from the current study indicate that the dysregulation of miRNAs potentially involved in the alterations in $GABA_A$ receptor configuration resulting in aberrant $GABA_A$ receptor-mediated synaptic activity in FCD. The present study assessed miR-155-5p, mi-R24-3p, and miR-186-5p expression in patients with FCD with recorded pathological characteristics. We observed a significant increase in the expression of α1 and α4 $GABA_A$ receptor subunits. Upregulation of α4 subunit while decreased miR-24-3p, and miR-186-5p expression in FCD suggests a possible interplay between these miRNAs and $GABA_A$ receptor α4 subunit. Increased expression of α1 $GABA_A$ receptor subunit while decreased miR-186-5p expression suggests a possible deregulatory mechanism at the posttranscriptional level in the expression of α1 subunit in FCD. We also observed decrease in α1 $GABA_A$ receptor subunit expression with increase in the age of FCD patients. We have earlier reported the association between $GABA_A$ receptor function and age at epilepsy onset in patients with FCD (Banerjee et al., 2020). A decrease in the expression of α1 $GABA_A$ receptor with age in FCD patients may contribute to an altered ratio of α1/ α4, leading to changes in $GABA_A$ receptor composition that may underlie resistance to the benzodiazepine class of drugs in FCD (Sharma et al., 2021). Additionally, this receptor subtype desensitizes rapidly and may contribute to the process of epileptogenesis (Lagrange et al., 2007), resembling the immature neuronal cortex (Taylor et al., 1971; Palmini et al., 2004; Abdijadid et al., 2015). It has been reported that the replacement of α1β3γ2 with α4β3γ2 receptors at synaptic sites is likely to impair inhibitory transmission (Lorenz-Guertin & Jacob, 2018). Furthermore, receptors from the epileptic tissues, including the α4βγ2 receptors, have been shown to function relatively normally under baseline conditions but have no effect when enhanced inhibitory responses are required (Treiman, 2001). Pyramidal neurons that appear normal in epileptic lesions of FCD patients retain immature GABAergic inputs involved in the development of dysmature neuronal network (Cepeda et al., 2014). Here we have observed that the frequency, amplitude, and decay time constant (τ_d) of sIPSCs recorded from normal-looking pyramidal neurons of resected brain samples obtained from patients with FCD type I and II were significantly higher than that in case of non-seizure control samples obtained from patients with low-grade glioma without any history of seizures. This suggests a robust $GABA_A$ receptor mediated epileptiform synchronization in FCD samples. Our findings indicated enhanced spontaneous GABAergic input to the normal-looking pyramidal neurons in resected brain samples, possibly due to immaturity of neuronal circuits associated with FCD type I and II (Abdijadid et al., 2015; Banerjee et al., 2020). Increased amplitude of spontaneous IPSCs in samples obtained from patients with FCD indicates enhanced synaptic transmission through increased $GABA_A$ receptor density on the postsynaptic membrane of neurons. Prolonged decay time constants of spontaneous IPSCs indicated reinforced GABAergic activity under basal conditions in FCD type I and II which could be due to increased α4 subunit-containing $GABA_A$ receptors at the synaptic sites (Lagrange et al., 2007; Andre et al., 2010). It may be possible that GABAergic input to the normal looking pyramidal neurons in FCD type I and II are comparable due to similar subunit configuration of $GABA_A$ receptors in both pathologies. But the possibility of differences in the kinetics of GABAergic events on to abnormal pyramidal neurons and dysmorphic neurons in FCD type I and II cannot be ruled out.

We have demonstrated the upregulation of miR-155-5p in resected brain tissues and serum of FCD patients similar to previous studies (Lee et al., 2014; Srivastava et al., 2017). Unlike, previous studies we did not observe any significant correlation between the expression of miR-155-5p and the increased expression of α1 subunit of $GABA_A$ receptor (D'Urso et al., 2012). Upregulated miR-155-5p expression was found to be associated with increased expression of inflammatory cytokines and decreased expression of suppressor of cytokine signalling protein (SOCS1) (Srivastava et al., 2017). Zhang et al. (2019) also demonstrated the miR155 upregulation was accompanied with increased IL-1β, IL-6 and TNF-α, while, GAT-1, GAT-3 and GABA were reduced in rats following post-ischemic seizures. Earlier, we have also demonstrated the up regulation of IL-6, IL-1β, STAT-3 and miR-155-5p in FCD patients. An increase in miR-155-5p levels in FCD patients might influence the inflammation as evidenced by previous studies rather than regulating the $GABA_A$ receptor α1 expression. Further studies on a greater number of samples are needed to explore the role of miR-155-5p in the pathology of FCD.

Various studies have demonstrated the regulatory role of miR-186-5p and miR-24-3p on α4 subunit of $GABA_A$ receptors (Babenko et al., 2012; Bekdash & Harrison, 2015; Barker & Hines, 2020). Likewise, an increase in $GABA_A$ receptor α4 and a decrease in miR-24-3p and miR-186-5p have been demonstrated in surgically resected brain tissue samples of FCD patients (Bekdash & Harrison, 2015). In addition, a relatively lower level of miR-24-3p and increased expression of $GABA_A$ receptor α4 subunit has been demon-

strated in the serum of FCD patients. The binding site for miR-186-5p is predicted for α1 subunit of $GABA_A$ receptors (Zhao et al., 2012). The present study indicates that miR-186-5p might possibly regulate the expression of $GABA_A$ receptor subunit α1. In addition to their involvement in regulating $GABA_A$ receptor subunits, these microRNAs also play a crucial role in neuronal differentiation and the regulation of the GluA2 AMPA receptor subunit. They mediate synaptic scaling, which is initiated by a prolonged blockade of synaptic activity. Furthermore, alterations in the expression of microRNAs can impact these activities as well (Kang et al., 2019; Silva et al., 2019).

This preliminary study suggests a possible relation between dysregulation of microRNAs and expression of α1 and α4 subunit-containing $GABA_A$ receptors in FCD. However, this human study has few limitations. The main limitations of this study are the small sample size and the lack of age and gender-matched controls. Due to the small sample size used in the present study, the multivariate analysis could not be performed, thus the effects of potential confounding factors could not be assessed. Conceptually the potential control tissue could be a human brain sample resected from a similar age group with non-seizure pathologies such as tumour or autopsy controls. The tumor periphery tissue resected from patients with brain tumors might show abnormalities in levels of miRNAs in the tumorogenic tissue or peripheral blood. In addition, *post mortem* delays can affect the quality of miRNA and mRNA. Due to various limitations associated with autopsy and tumor periphery tissues, tumor periphery tissues were used for patch clamp-based studies and region-specific autopsy tissue was used as controls for molecular studies to overcome the disadvantages associated with either control. For blood-based studies, blood from healthy individuals was obtained. Prospective studies on larger sample sizes in order to provide sufficient evidence to determine the regulatory role of these miRNAs in the GABAergic activity of FCD patients is very much needed. Our study provides preliminary evidence, even though indirect, that miR-24-3p and miR-186-5p might play a regulatory role in the expression of $GABA_A$ receptor subunits in FCD patients. However, further functional studies, including *in vitro* transfections with miRNA mimics and inhibitors in acute slice preparations from freshly resected FCD samples and *in vivo* experiments using transgenic models, are essential to confirm these regulatory relationships and their impact on neuronal activity. These future studies will provide more comprehensive insights into the mechanisms by which these miRNAs contribute to the pathophysiology of FCD.

CONCLUSION

Our study demonstrates that miR-24-3p and miR-186-5p expressions are significantly downregulated while the expression of $GABA_A$ receptor α1 and α4 subunits is increased in resected brain samples from FCD patients. The observed altered expression of $GABA_A$ receptor subunits and the miRNAs provides preliminary evidence suggesting that these miRNAs might play a regulatory role in FCD. However, more extensive *in vivo* and *in vitro* experiments are required to establish this regulatory relationship.

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