NEUROPATHOLOGICAL ASSESSMENT OF BETA-AMYLOID AND TAU PATHOLOGY IN HUMAN FOCAL CORTICAL DYSPLASIA WITH DRUG-RESISTANT EPILEPSY

by

Alisha Aroor

A Thesis

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Master of Science

Department of Psychological Sciences

West Lafayette, Indiana

August 2021

THE PURDUE UNIVERSITY GRADUATE SCHOOL

STATEMENT OF COMMITTEE APPROVAL

Dr. Amy L. Brewster, Chair

Department of Psychological Sciences

Dr. Kimberly P. Kinzig

Department of Psychological Sciences

Dr. Edward A. Fox

Department of Psychological Sciences

Approved by:

Dr. Kimberly P. Kinzig

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Rationale: Focal cortical dysplasia (FCD) is a neurodevelopmental disorder that is associated with abnormal cortical development and is one of the most common drug-resistant epilepsies. The mechanistic target of rapamycin (mTOR) pathway is a highly complex pathway associated with cell proliferation, synaptic plasticity, neuroinflammation, and cortical development. Hyperactivation of this pathway has also been implicated in hyperexcitability, seizures, and accumulation of beta-amyloid (Aβ) plaques and neurofibrillary tangles (NFT) through hyperphosphorylation of tau. Interestingly, Aβ and hyperphosphorylated tau have been reported in both rodent models and human patients of temporal lobe epilepsy (TLE) and FCD however, the mechanisms through which this occurs are still yet to be defined. Therefore, to identify the possible link between Aβ and tau pathology in FCD, we determined the spatial distribution and protein levels of Aβ and phosphorylated tau (p-tau) along with mTOR signaling molecules. We hypothesized that there would be presence of Aβ and tau pathology as well as an increase in Aβ and p-tau protein levels that would be correlated with hyperactivation of the mTOR and GSK3 signaling pathways in tissue biopsies from human FCD patients compared to brain tissues from non-epileptic (NE) individuals.

Methods: Cortical brain samples surgically resected from patients with FCD were used and compared to NE samples surgically resected from glioblastoma patients with no history of seizures or epilepsy. Immunostaining was used to determine the distribution of phosphorylation of S6 (p-S6), a marker for mTOR activation, and NeuN, a marker for neurons, along with Aβ and p-tau. Additionally, western blotting (WB) was used to determine the levels of mTOR signaling through p-S6 and GSK3 (p-GSK) along with Aβ and p-tau.

Results: We found cortical dyslamination, mTOR activation, p-tau, and Aβ accumulation in cortices of patients with FCD with drug-resistant epilepsy. However, we did not find a significant difference in the protein levels of p-S6 (p = 0.422), p-GSK3 (p = 0.947), p-tau (p = 0.649), and Aβ (p = 0.852) in cortical tissue homogenates derived from FCD patients when compared to those of NE samples. Additionally, we did not find sex differences in the protein levels of p-S6 (p = 0.401), p-GSK3 (p = 0.331), p-tau (p = 0.935), and Aβ (p = 0.526). There was no significant correlation between age and p-S6 (p = 0.920), age and p-GSK3 (p = 0.089), age and p-tau (p = 0.956), and age and Aβ (p = 0.889). Moreover, there was no significant correlation

between mTOR activation (p-S6), Aβ (p = 0.586) and p-tau (p = 0.059) nor GSK3 activation (p-GSK3), Aβ (p = 0.326), and p-tau (p = 0.715). Lastly, there was no significant correlation within the mTOR and GSK3 pathway activation within the same patients (p = 0.602).

Conclusion: These data suggest that mTOR hyperactivation occurs alongside the presence of Aβ and tau pathology. However, several unknown factors such as medical and medication history may be altering the expression or suppression of these proteins. Additionally, there may be alternative pathways that crosstalk with mTOR signaling therefore influencing Aβ and tau pathology in FCD patients with drug-resistant epilepsy. Further investigation will need to be conducted to understand the detailed mechanisms through which Aβ and tau pathology occur in FCD.

INTRODUCTION

Epilepsy

Epilepsy is a neurological disorder characterized by the occurrence of two or more spontaneous seizures, with a seizure being an abnormal burst of electrical activity in the brain that can be accompanied by a behavioral output (e.g., convulsions). An initial insult to the brain can further trigger unprovoked seizures that can originate from cortical neurons and influence a series of cellular changes including cell loss, synaptic reorganization, and axonal sprouting in various brain regions. These events can eventually trigger an excitatory-inhibitory imbalance within neuronal networks that results in excessive neuronal firing and seizures (Stafstrom & Carmant, 2015). Epilepsy is a prevalent disorder affecting approximately 70 million people worldwide (Thijs, Surges, O'Brien, & Sander, 2019), suggesting the significance of investigating this disorder. Unfortunately, epilepsy has been stigmatized in many parts of the world leaving approximately 75% of people with epilepsy untreated further validating the need to understand this disorder and increase awareness.

Several risk factors that increase the development and progression of epilepsy include genetic influence, traumatic brain injuries, brain infections, stroke, excessive alcohol and drug use, or even through unknown causes (cryptogenic epilepsy) (Aroor & Brewster, 2021; Sirven, 2015), leaving the mechanisms through which some of them occur still a mystery. Different types of epilepsies vary based on their severity, location, frequency, cause, and consequently these can impact the course of treatment. The main characteristic is the occurrence of spontaneous seizures however, diagnosis can sometimes be difficult as behavioral seizures may be infrequent or absent. Over 50% of people with epilepsy experience comorbidities, which can exacerbate the disorder and impose further physical, emotional, and financial burden on the individual. Some of these comorbidities entail psychiatric disorders, cognitive disorders, and other neurological and neurodegenerative diseases. For instance, there is a 30-50% incidence of depression, approximately a 10-25% prevalence of anxiety, and a 5-40% occurrence of autism spectrum disorder in individuals with epilepsy (Paudel, Shaikh, Shah, Kumari, & Othman, 2018).

The primary treatment for epilepsy is through pharmacological therapy using anti-epileptic drugs (AEDs) that are specific to an individual’s epilepsy management plan. Treatment can vary

depending on age, sex, comorbidities, medical history, and seizure severity. However, not all AEDs typically target the comorbidities that occur with epilepsy. More importantly, approximately 30-40% of epilepsies that involve far more complex mechanisms have drug-resistant seizures (Xue-Ping, Hai-Jiao, Li-Na, Xu, & Ling, 2019). The International League Against Epilepsy states that drug-resistant epilepsies are refractory to pharmacological treatment and occur when there is a failure of seizure freedom after at least 2 AEDs specifically selected to treat a person's seizure type (Sirven, 2015). An alternative to this is through surgical resection of the seizure focus. However, surgical resection involves certain selection criteria and an extensive assessment to identify the epileptogenic zone, and the possible outcomes and risks. The two most common types of drug-resistant epilepsies that can benefit from surgical resection are temporal lobe epilepsy (TLE) and focal cortical dysplasia (FCD) with 80% and 33-75% seizure freedom post-surgery, respectively (Sheng, Liu, Qin, Li, & Zhang, 2018) (Aroor & Brewster, 2021; Lee & Kim, 2013). Interestingly, TLE and FCD have been shown to share a characteristic pathology that involves cell death, neuroinflammation, and synaptic instability, despite having different etiologies. As individuals may not attain 100% seizure freedom after surgery, further investigation on the mechanisms through which these epilepsies act is yet to be conducted.

Focal Cortical Dysplasia

Focal cortical dysplasia (FCD) is a neurodevelopmental disorder that is characterized by malformation of cortical development. This abnormal organization and cortical dyslamination is associated with neuronal loss and displacement, enlarged dysmorphic cells, and the occurrence of focal unprovoked seizures (Zimmer et al., 2020) (Siedlecka, Grajkowska, Galus, Dembowska-Baginska, & Jozwiak, 2016). These spontaneous recurrent seizures typically disrupt neuronal circuitries resulting in cell loss, synaptic reorganization, and neuronal hyperexcitability. FCD can be categorized into 3 main types depending on their histopathological profiles: Type I (a, b, c), Type II (a, b), and Type III (a, b, c, d) (Kim & Choi, 2019). Type Ia can be described as radial microcolumnar disorganization of neurons, Type Ib as abnormal tangential composition, and Type Ic as the combination of both Ia and Ib. Type IIa is characterized by the presence of dysmorphic neurons (DN) while Type IIb is described as having both DN and balloon cells. Type III, consisting of 4 subgroups, can be broadly defined as cortical dyslamination that is coupled with a primary lesion that is typically found adjacent to the respective cortical region, further affecting it. For

instance, Type IIIa occurs with hippocampal sclerosis, the pathological changes and neuronal death that occur within the hippocampus, Type IIIb which occurs adjacent to a glial or glioneuronal tumor, Type IIIc which is found adjacent to vascular malformation, and Type IIId which is adjacent to other lesions that were acquired early in life (Blumcke et al., 2011; Knerlich-Lukoschus, Connolly, Hendson, Steinbok, & Dunham, 2017). Genetic and acquired factors influence the onset of FCD and through extensive research, scientists identified the hyperactivation of the mechanistic target of rapamycin (mTOR) pathway to be a main molecular hallmark underlying FCD (Liu et al., 2014; Mirzaa et al., 2016).

The Mechanistic Target of Rapamycin Pathway

The mechanistic target of rapamycin (mTOR) pathway is a complex and essential signaling pathway that can be activated through various growth factors and proteins such as glycogen synthase kinase-3 beta (GSK3β) and protein kinase B (Akt) through the phosphatidylinositol 3-kinase (PI3k)/Akt and GSK3 signaling cascades (Switon, Kotulska, Janusz-Kaminska, Zmorzynska, & Jaworski, 2017). Regulatory molecules such as phosphatase and tensin homolog (Pten) and drugs such as Rapamycin that allow for the manipulation of this pathway have aided researchers in understanding the mechanisms through which mTOR operates (Brewster et al., 2013; Zimmer et al., 2020). Through these investigations, studies have found that mTOR is a regulator of a multitude of processes such as cell survival and proliferation, protein and lipid synthesis, dendritic stability, cortical development, neuroinflammation and microglia activation, and learning and memory (Hoeffer & Klann, 2010; Wyatt-Johnson & Brewster, 2020; Zimmer et al., 2020). However, extensive studies have also found that aberrant activation of the PI3k/Akt/mTOR pathway can negatively impact these physiological processes governing the instability of neuronal circuitries that contributes to cognitive decline, neuronal hyperexcitability, seizures, and malformation of the cortex (Nguyen, Mahadeo, & Bordey, 2019). Interestingly, it has also been found that abnormal activation of the mTOR pathway is implicated in the pathology of Alzheimer's Disease (AD) resulting in the accumulation of hyperphosphorylated tau tangles and beta-amyloid (Aβ) plaques (Oddo, 2012; Talboom, Velazquez, & Oddo, 2015). As there has been evidence that indicates a significant overlap between epilepsy and AD (Aroor & Brewster, 2020; Chin & Scharfman, 2013; Cortini, Cantoni, & Villa, 2018; Vossel, Tartaglia, Nygaard, Zeman, & Miller, 2017), the question arises if mTOR hyperactivation links the two diseases.

Alzheimer's Disease

AD is a common neurodegenerative disease that affects around 5.8 million people in the United States and around 50 million people worldwide ("2020 Alzheimer's disease facts and figures," 2020). An individual with AD experiences cognitive decline involving symptoms such as memory loss, confusion of time and place, speech disabilities, difficulty in problem solving and decision making, etc., as well as comorbidities with other neurodegenerative, psychiatric, and neurological disorders. AD can be classified into 2 main types: (1) Late-onset AD (LOAD) that occurs in individuals above the age of 65, and (2) Early-onset AD (EOAD) that affects individuals ages 65 and below. AD is an exceedingly widespread disease with EOAD affecting approximately 4% of people in the United States, which translates to around 200,000 people, suggesting how prevalent this disease is ("2020 Alzheimer's disease facts and figures," 2020). The neuropathology of EOAD is commonly associated with mutations in the amyloid precursor protein (App), and Presenilin 1 (Psen1) and Presenilin 2 (Psen2) genes. These mutations result in the formation of Aβ plaques that surround neurons and hinder the effective communication between neuronal networks. In addition to the plaques, hyperphosphorylation of the protein tau aggregates to form intracellular structures known as neurofibrillary tangles (NFT) (Chin & Scharfman, 2013; Vossel et al., 2017). These NFT form within the cell and inhibit cellular processes critical for nutrient and axonal transport, and cytoskeletal stability. Presence of this pathology initiates cell death, tissue damage, expansion of ventricles, and an overall reduction in brain volume, particularly in the hippocampus and cortex.

Overlap Between Epilepsy and Alzheimer's Disease

Apart from sharing comorbidities of cognitive decline and psychiatric disorders, epilepsy and AD have been shown to have a strong pathological correlation. Accumulation of Aβ plaques and hyperphosphorylation of tau have not only been reported in AD, but also in both experimental models of epilepsy and TLE patients (Alves, Kenny, de Leo, Beamer, & Engel, 2019; Aroor & Brewster, 2020; Gourmaud et al., 2020; Tai et al., 2016; Toral-Rios, Pichardo-Rojas, Alonso-Vanegas, & Campos-Pena, 2020). Interestingly, alterations in the App, Psen1, or Psen2 genes have been associated with neuronal network instability of the inhibitory-excitatory system. This can disrupt long-term synaptic depression and long-term potentiation resulting in abnormal neuronal

activity and seizures. Note that 60 mutations in Psen1 and 3 in Psen2 can contribute to neuronal hyperexcitability (Cortini et al., 2018). Furthermore, the neuropathology of AD has also been seen in parallel to neuronal hyperexcitability. For instance, a 10-22% incidence of seizures has been documented in patients with AD, and this has an 87-fold increase in EOAD (Aroor & Brewster, 2020; Reyes-Marin & Nunez, 2017). Studies using electroencephalographic (EEG) recordings have identified electrographic epileptiform activity originating from the hippocampus and cortex in patients with EOAD (Vossel et al., 2017) suggesting that epilepsy and AD may have common neuropathological features. Studies conducted using transgenic mouse models of AD with mutations in the App and Psen1/2 genes have Aβ plaque formation in addition to neuritic dystrophy, impairments in synaptic function, abnormal sprouting of axon terminals, and non-convulsive seizures evidenced by epileptiform activity obtained through EEG recordings (Palop et al., 2007; Reyes-Marin & Nunez, 2017). This provides further evidence in support of an overlap in the mechanisms underlying seizure activity and a common neuropathology in both AD and epilepsy.

The role of aberrant mTOR signaling in AD pathology is associated with the abnormal cleavage of App which aggregates to form Aβ fragments (Cai, Chen, He, Xiao, & Yan, 2015). Through this constant abnormal cleavage, the fragments accumulate and form plaques which trigger pathways such as the PI3k/Akt and GSK3 pathway, which in turn increases activity of the mTOR pathway. mTOR is responsible for the inhibition of autophagy, a process essential in eliminating the abnormally aggregated Aβ plaques. Therefore, hyperactivation of the mTOR pathway results in the dysfunction of autophagy further promoting the formation and build-up of the plaques (Cai et al., 2015). Similarly, phosphorylation of tau occurs through interactions within the PI3k/Akt/mTOR and GSK3 pathways and through multiple kinases, including GSK3β (Mueed et al., 2018; Toral-Rios et al., 2020). Therefore, through various growth factors, activity of proteins and kinases such as GSK3β within the PI3k/Akt/mTOR and GSK3 pathways is associated with the hyperphosphorylation of tau that leads to the development and accumulation of NFT.

Link Between Alzheimer's Disease Pathology and Epilepsy Through mTOR

Comprehensive studies support that inhibition of the mTOR pathway through the drug Rapamycin ameliorates cognitive deficits as well as reduces pathology and neurodegeneration in transgenic AD mouse models (Siman, Cocca, & Dong, 2015; Spilman et al., 2010). Additionally, inhibition of the mTOR pathway has also been shown to suppress seizure activity evidenced in

rodent models of both TLE and FCD (Huang et al., 2010; Nguyen et al., 2015; Sunnen et al., 2011; Zeng, Rensing, & Wong, 2009). Interestingly, a study conducted by Ngyuen et al. (2015) used the NS-Pten knockout (KO), a mouse model of FCD, and EEG recordings to elucidate the effect of mTOR inhibition with Rapamycin on spontaneous seizure activity (Nguyen et al., 2015). Results indicated that Rapamycin-mediated reductions in mTOR signaling suppressed epileptiform activity in the mouse model of FCD (Nguyen et al., 2015). Furthermore, a study conducted by Hodges et al. (2018) investigated the link between hyperactivation of the mTOR pathway and AD pathology in the same mouse model of FCD (NS-Pten KO) (Hodges et al., 2018). What was found were alterations in p-GSK3α as well as increased levels of Aβ, suggesting a correlation between mTOR activity and AD pathology in FCD. Taken together, the aforementioned findings suggest a strong association between mTOR activation and Aβ expression in epilepsy and AD. However, to our knowledge there have been no studies investigating the interaction between the extent of mTOR signaling and AD pathology in humans with FCD and drug-resistant seizures. This brings us back to the question, is hyperactive mTOR signaling associated with Aβ and tau pathology in human FCD with drug-resistant seizures?

HYPOTHESIS

In the current study, we investigated whether human brain specimens surgically resected from individuals with FCD with drug-resistant seizures have neuropathological features of AD, thereby suggesting an association between mTOR signaling, AD pathology, and FCD. We hypothesized that there would be presence of Aβ and tau pathology and an increase in Aβ and p-tau protein expression that is correlated with hyperactivation of the mTOR and GSK3 signaling pathways in tissue biopsies from human FCD patients compared to brain tissues from non-epileptic individuals.

<u>Aim 1:</u> To investigate the spatial distribution of beta-amyloid plaques, tau tangles, and mTOR signaling proteins in the cortex of FCD patients with drug-resistant epilepsy

a. To investigate the spatial distribution using antibodies against p-tau (AT8), Aβ, p-S6, and NeuN using immunohistochemistry (IHC). The color signal was visualized using a Leica DM500 microscope equipped with a high-resolution digital camera (Leica MC120 HD).

<u>Aim 2:</u> To determine protein levels of beta-amyloid, phosphorylated tau, and mTOR signaling associated with both neuronal hyperexcitability and AD pathology in human specimens derived from FCD patients with drug-resistant epilepsy.

a. To investigate the protein levels of beta-amyloid and p-tau in the cortex. Western blot was used with antibodies against Aβ, p-tau (AT8), and tau (TAU-5). Relative pixel intensity was measured using densitometry analysis of the immunoreactive bands. Proteins were normalized to Actin or GAPDH. Phosphorylated proteins were also normalized to the total protein of the same molecule in the same sample.

b. To investigate the protein levels of mTOR signaling in the cortex. Western blotting (WB) was used with antibodies against S6, p-S6, GSK3β, and p-GSK3β. Relative pixel intensity was measured using densitometry analysis of the immunoreactive bands. Proteins were normalized to Actin or GAPDH. Phosphorylated proteins were also normalized to the total protein of the same molecule in the same sample.

MATERIALS AND METHODS

Ethics Statement

All the tissue samples were collected with patients informed consent under the Institutional Review Board (IRB) protocol #1011004282 (Development of a Biorepository for Methodist Research Institute). All tissues were decoded from all identifiable information prior to processing for biochemical and histological analysis following guidelines from the Purdue University Human Research Protection IRB under the protocol #1507016240.

Human Brain Samples

The tissue samples (cortical tissues) were derived from patients with medically refractory epilepsy (FCD) who underwent resective surgery (n = 31). Non-epileptic (NE) specimens were from peritumoral non-pathological tissues derived from glioblastoma patients with no history of seizures or epilepsy (n = 6). Following resection, these brain tissues were placed into cryovials, submerged in liquid nitrogen, and stored at −80 °C until used for WB and IHC analyses. A total of 28 samples were available for WB and 12 were available for IHC. Table 1 shows the list of patients with the description of type of FCD, region of the brain surgically resected, age, and sex.

Table 1. Patient Information

Patient	Approach	FCD	Tissue Origin	Age	Sex
1	IHC	NA	Temporal Lobe	51	Male
2	WB/IHC	IIIa	Right Temporal Lobe	67	Male
3	IHC	NA	Left Temporal Lobe	59	Male
4	IHC	NA	Lateral Temporal Cortex	55	Male
5	IHC	NA	Lateral Temporal Lobe	36	Female
6	IHC	NA	Right Temporal Lobe	24	Male
7	IHC	NA	Right Temporal Lobe	44	Male
8	IHC	NA	Right Temporal Lobe	53	Female
9	IHC	Ic	Right Temporal Lobe	25	Female
10	IHC	NA	Right Anterior Temporal Lobe	44	Female
11	WB/IHC	NA	Left Lateral Temporal Lobe	25	Female
12	WB/IHC	IIIa	Left Lateral Temporal Lobe	28	Male
13	WB	IIIa	Lateral Right Temporal Lobe	33	Male
14	WB	IIIa	Lateral Temporal Lobe	24	Male
15	WB	NA	Left Lateral Temporal Cortex	33	Male
16	WB	Ia	Lateral Temporal Cortex	43	Male
17	WB	IIIa	Left Lateral Temporal Cortex	37	Male
18	WB	Ic	Right Lateral Temporal Lobe	43	Female
19	WB	Ic	Lateral Temporal Cortex	33	Male
20	WB	IIIc	Right Lateral Temporal Lobe	44	Female
21	WB	Ic	Lateral Temporal Lobe	28	Female
22	WB	NE	Left Temporal Lobe	65	Female
23	WB	IIIa	Lateral Temporal Lobe, Cortex	23	Female
24	WB	IIIa	Right Lateral Temporal Lobe	21	Female
25	WB	IIIa	Right Medial Temporal Cortex	33	Male
26	WB	IIIa	Temporal Lobe Cortex, Right Lateral	32	Female

Table 1 continued

Patient	Approach	FCD	Tissue Origin	Age	Sex
27	WB	IIIa	Left Temporal Lobe	20	Male
28	WB	IIIa	Left Lateral Temporal Lobe	47	Male
29	WB	Ic	Left temporal Lobe	28	Male
30	WB	NA	Lateral Temporal Lobe	45	Female
31	WB	NE	Right Occipital Lobe	53	Female
32	WB	NE	Right Temporal Lobe	69	Female
33	WB	NE	Right Temporal Lobe	51	Male
34	WB	NA	Right Temporal Lobectomy	63	Female
35	WB	NA	Temporal Lobectomy	68	Male
36	WB	NE	Left Temporal Lobe	28	Male
37	WB	NE	Left Temporal Lobe	65	Female

Note. WB, western blot; IHC, immunohistochemistry; NE, non-epileptic; NA, not available.

Immunohistochemistry

Small blocks of brain cortical tissues (0.5 cm) were placed in 4% paraformaldehyde (PFA) (24 hours) followed by 30% sucrose/1XPBS for cryoprotection at 4 °C (2-3 days). Some samples were placed in formalin for fixation after surgical dissection. All these samples, PFA and formalin fixed were then frozen in dry ice and stored in the −80 °C freezer until used for IHC. Samples were then sliced (40 µm) using a Leica CM1860 cryostat and stored in 1XPBS + 0.1% with sodium azide (0.01%) at 4 °C. Three to four sections per brain were used for histology. Sections were first washed in 1XPBS + 3% Triton (1XPBS-3%T) overnight at 4 °C, followed by 3% H2O2 (30 min), and 1XPBS + 3% Triton (1XPBS-3%T) (5 mins) at RT. Then, all sections were placed in an immuno buffer (5% goat serum, 0.3% BSA, 0.3% triton diluted in 1XPBS) overnight at 4 °C. Primary antibodies were added and incubated overnight at 4 °C: Aβ (rabbit, 1:100; ab2539, Abcam), p-tau (Ser202, Thr205) (AT8) (mouse, 1:100; MN1020, Invitrogen), p-S6 (Ser 240/244) (rabbit, 1:00; 5364, Cell signaling), and NeuN (rabbit, 1:100; Abcam). Tissues were then washed in 1XPBS + 0.1% Triton (1XPBS-T) (3x10 mins), followed by incubation in biotinylated secondary antibody (anti-rabbit or anti-mouse, 1:500; BA-1000 and BA-9200, respectively, Vector Laboratories, CA, USA) (1 hour), washed in 1XPBS + 0.1% Triton (1XPBS-T) (3x10 mins), and incubated with Avidin/Biotin Complex solution (30 mins). Next, sections were washed in 1XPBS + 0.1% Triton (1XPBS-T) (3x5 mins) and placed in DAB for detection. Lastly, brain sections were mounted on non-gelatin-coated glass slides. Once dry, sections were dehydrated in alcohol [50%, 70%, 95%, 100%], de-fatted in Xylene, and cover slipped using Permount mounting medium. The color signal was visualized using a Leica DM500 microscope equipped with a high-resolution digital camera (Leica MC120 HD). IHC was performed using previously described protocols (Wyatt, Witt, Barbaro, Cohen-Gadol, & Brewster, 2017).

Western Blot

A small block (0.5cm) of brain cortical tissue from each sample containing a mix of white and grey matter was homogenized and processed for immunoblotting. Protein concentrations were determined using the Bradford assay. Samples (15-20 µg/well) were loaded into 10% Tris-glycine gels (buffer, 30% acrylamide, 10% SDS, 10% ammonium persulfate, 10 µl TEMED) and run through sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to separate the

proteins within the samples according to their molecular weights. Gels were then transferred with mini trans-blot cells (Bio-Rad Laboratories) onto polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Chicago, IL). Membranes were blocked with 5% non-fat milk or 5% BSA diluted in 1XPBS+ 0.1%Tx at room temperature (RT) (1 hour) on a rotating platform. Membranes were incubated in primary antibodies overnight at 4 °C: S6 (rabbit, 1:1000; 2217, Cell signaling), p-S6 (Ser 240/244) (rabbit, 1:1000; 5364, Cell signaling), GSK3β (rabbit, 1:5000; 12456, Cell signaling), p-GSK3β (Ser9) (rabbit, 1:2000; 9323, Cell signaling), Aβ (rabbit, polyclonal, 1:10000; ab2539, Abcam), p-tau (Ser202, Thr205) (AT8) (mouse, monoclonal, 1:500; MN1020, Invitrogen), tau (TAU-5) (mouse, monoclonal, 1:500; AHB0042, Invitrogen), Actin (rabbit, 1:5000; A2066, Sigma-Aldrich), and GAPDH (rabbit, 1:50,000; G9545, Sigma-Aldrich). Membranes were washed in 1XTBS+0.1% Tween (3x5 min) and incubated (1 hour) with HRP-linked secondary antibodies (anti-rabbit or anti-mouse, 1:4000; Abcam). Immunoreactive bands were visualized using an enhanced chemiluminescence prime western blotting detection reagent (GE Healthcare) and captured on Double Emulsion Blue Autoradiography Film (MIDSCI, St. Louis, MO). The films were scanned and the relative pixel intensity of the immunoreactive bands were measured using Image J software V1.49. To obtain the relative pixel intensity, the background signal was measured and subtracted from the bands of proteins of interest, and normalized to loading controls such as Actin and GAPDH. Phospho-proteins were further normalized to the total protein for the same molecule in the same sample. WB was performed using previously described protocols (Wyatt et al., 2017).

Statistical Analyses

GraphPad Prism 9 software was used for WB statistical analyses. Unpaired t-tests were used to determine the difference in protein expression of our proteins of interest between FCD patients with drug-resistant epilepsy and the NE samples, and between females and males. Values are reported as means ± SEM. Additionally, linear regression and correlation analyses were performed to investigate the relationship between p-S6, Aβ and p-tau, and between p-GSK3, Aβ and p-tau within FCD samples and by age. Lastly, a linear regression analysis was conducted to determine the relationship between p-GSK3 and p-S6 activation.

RESULTS

Patient Information

Patient details are summarized in Table 1. The study consisted of examining 31 surgically resected FCD tissue, 3 of which were used for both WB and IHC, and 6 NE tissues surgically resected from glioblastoma patients with no history of seizures or epilepsy. Samples were derived from the right or left temporal lobes of 17 female and 20 male patients whose ages ranged from 20 to 69 years, with a mean age of 41.62 years. Of the 31 FCD samples, 1 was FCD type Ia, 5 were type Ic, 11 were type IIIa, 1 was type IIIc, and the FCD diagnosis for 13 samples was not available (NA). NE tissues were only available for WB. Note, as the information on whether the patients were asked their biological sex or gender at the time of surgery was unknown to us, we termed it as sex.

Cortical Dyslamination, mTOR Activation, Phosphorylated Tau, and Beta-Amyloid Accumulation was Found in Brain Biopsies of FCD Patients With Drug-Resistant Epilepsy

To investigate the spatial distribution of the proteins of interest within the cortex, we used IHC with antibodies against NeuN (neuronal marker), p-S6 (mTOR marker), p-tau, and Aβ in 12 different FCD brain samples. We were able to identify and confirm cortical dyslamination, neuronal loss, and abnormal cell size as well as areas of regular cortical organization within the same samples using NeuN in all the different tissue samples. Figure 1 shows representative images of normal appearing cortical regions that were found adjacent to areas of cortical dyslamination**.** Patients 1, 2, 3, and 9 revealed regular cortical layering (A, B, C, D) that can be seen in more detail (Ai, Bi, Ci, Di) along with normal looking cells of similar sizes (Aii, Bii, Cii, Dii). However, areas of cortical displacement were also found within these samples. Figure 2 shows representative images displaying characteristics of FCD from 3 different patients. Patient 1 exhibited displaced NeuN staining depicting neuronal displacement (Figure 2A) within the cortex that can be seen at higher magnification (Figure 2Ai), as well as presence of enlarged neurons (Figure 2Aii) (black arrowheads) compared to neurons at smaller sizes (white arrowheads). Additionally, Patient 2 displayed characteristics of neuronal loss (Figure 2B, 2Bi) that can be seen in greater detail at higher magnification (Figure 2Bii). Patient 3 showed radial microcolumnar disorganization

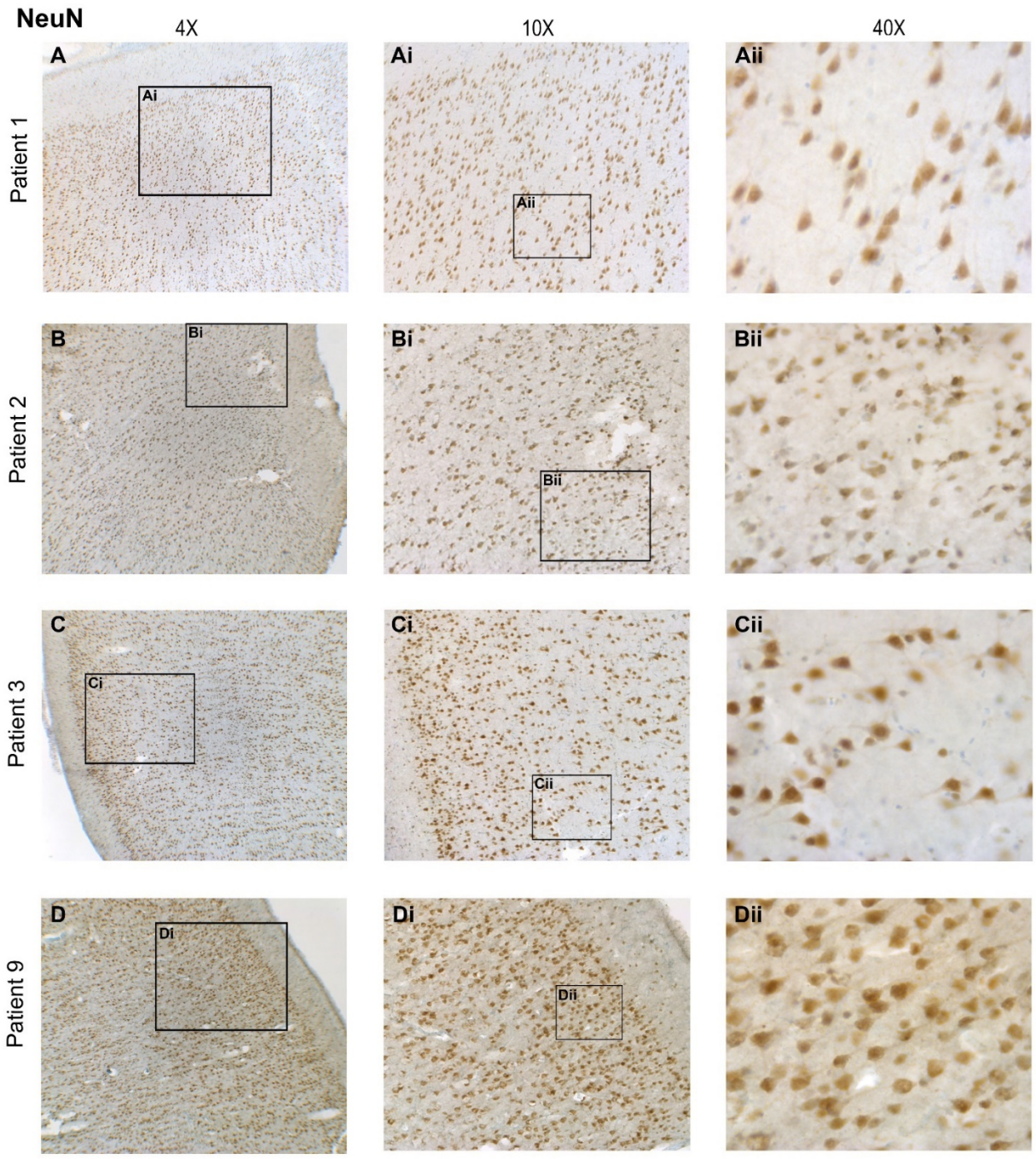

Figure 1. NeuN immunostaining shows regular cortical lamination in human focal cortical dysplasia **(FCD)**. Representative images of normal appearing cortical layering **(A-D)** adjacent to areas of dyslamination shown in Figure 2. NeuN staining is represented in brown while nuclear Nissl staining is shown in blue. Normal cortical lamination is seen at higher magnification **(Ai, Bi, Ci, Di)**. NeuN-positive neurons of similar sizes are found within areas of regular cortical organization **(Aii, Bii, Cii, Dii)**.

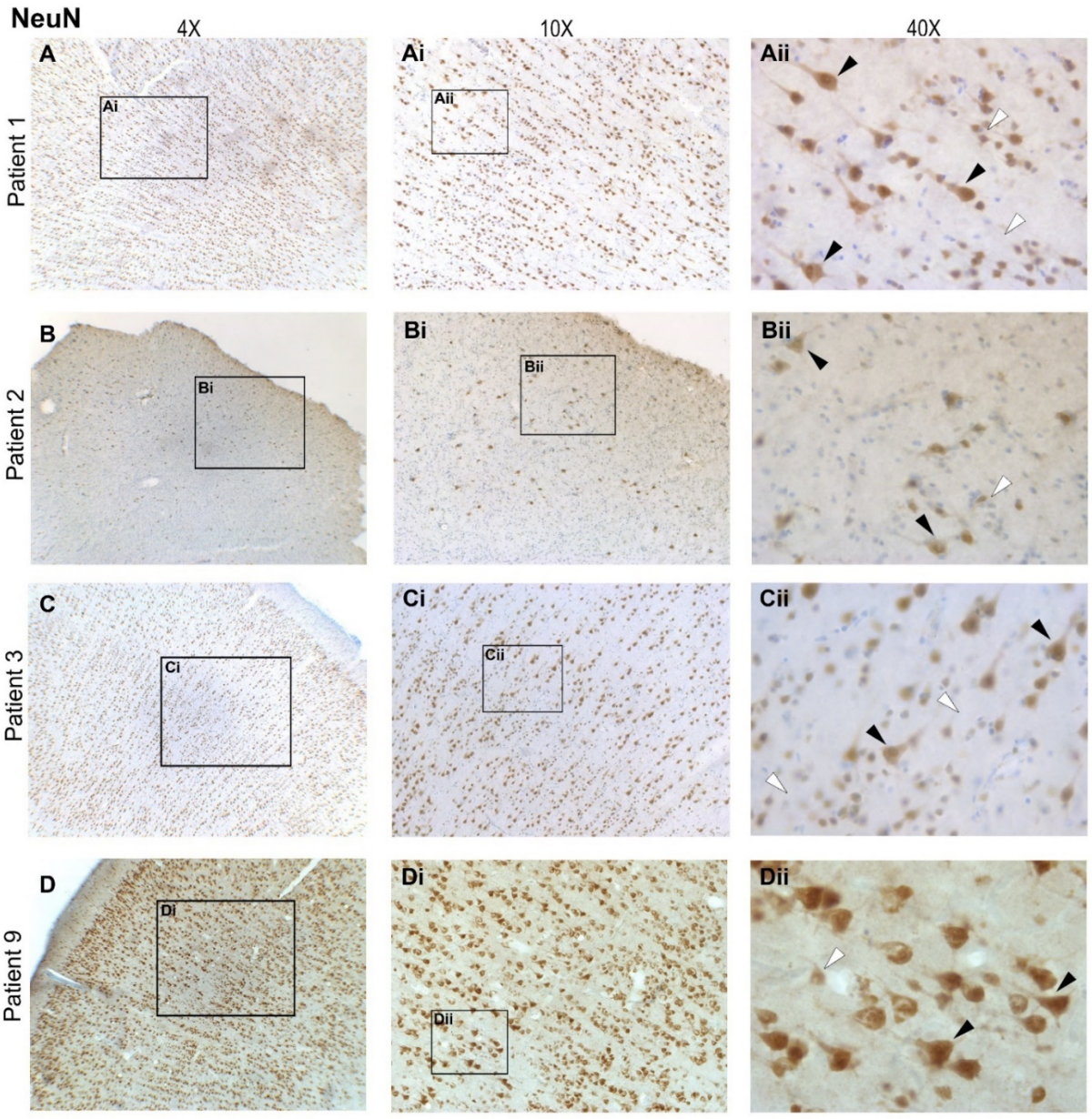

Figure 2. NeuN immunostaining shows cortical dyslamination in human focal cortical dysplasi**a (FCD).** Representative images of cortical dyslamination with neuronal displacement, neuronal loss, and enlarged cells are shown for different patients with FCD with drug-resistant seizures **(A-D)**. NeuN signal is shown in brown and nuclear Nissl staining is shown in blue. Characteristics of radial microcolumnar disorganization is shown in **(A, C, D)**, and neuronal loss in **(B).** NeuN-positive neurons are shown at higher magnification for all representative patients **(Ai, Bi, Ci, Di)**. Abnormal enlarged dysmorphic neurons (DN) (black arrowheads) are shown compared to smaller cells (white arrowheads) **(Aii, Bii, Cii, Dii)**.

(Figure 2C) which is also seen at higher magnification (Figure 2Ci). Also present, are dysmorphic neurons (black arrowheads) found scattered across the cortex in comparison to smaller neurons (white arrowheads) (Figure 2Cii). Lastly, Patient 9, aged 25, showed neuronal loss and displacement (Figure 2D, 2Dii) with dysmorphic neurons (black arrowheads) compared to smaller cells (white arrowheads) seen at higher magnification (Figure 2Dii). All patients displayed characteristics consistent with those of FCD.

Prominent immunostaining depicting p-S6-positive neurons was found within regions of dysplasia in the cortex in all the different patient samples. The presence of mTOR-activated neurons can be seen in all representative images (Figure 3). Moderate p-S6 signal can be seen in Patient 1 (Figure 3A, 3Ai) wherein some neurons displayed a stronger p-S6 staining (black arrowheads) signifying a higher mTOR activation compared to ones with a lower mTOR activation (white arrowheads) (Figure 3Aii). Furthermore, a robust p-S6 signal was noted in Patient 2 (Figure 3B, 3Bi) in a region that displayed neuronal loss (Figure 2B), supporting a higher mTOR activation in these neurons (black arrowheads) compared to a lower mTOR activation (white arrowheads) (Figure 3Bii). Patient 3 (Figure 3C/3Ci, 3D/Di) presented with p-S6 signal with some neurons (black arrowheads) depicting stronger mTOR activation while others showed a low activation (white arrowheads) (Figure 3Cii/, 3Dii). Note that in this same region (Figure 3C/Ci/Cii) we found extensive tau pathology (see Figure 4) while other regions did not display any beta-amyloid or tau pathology (3D/Di/Dii).

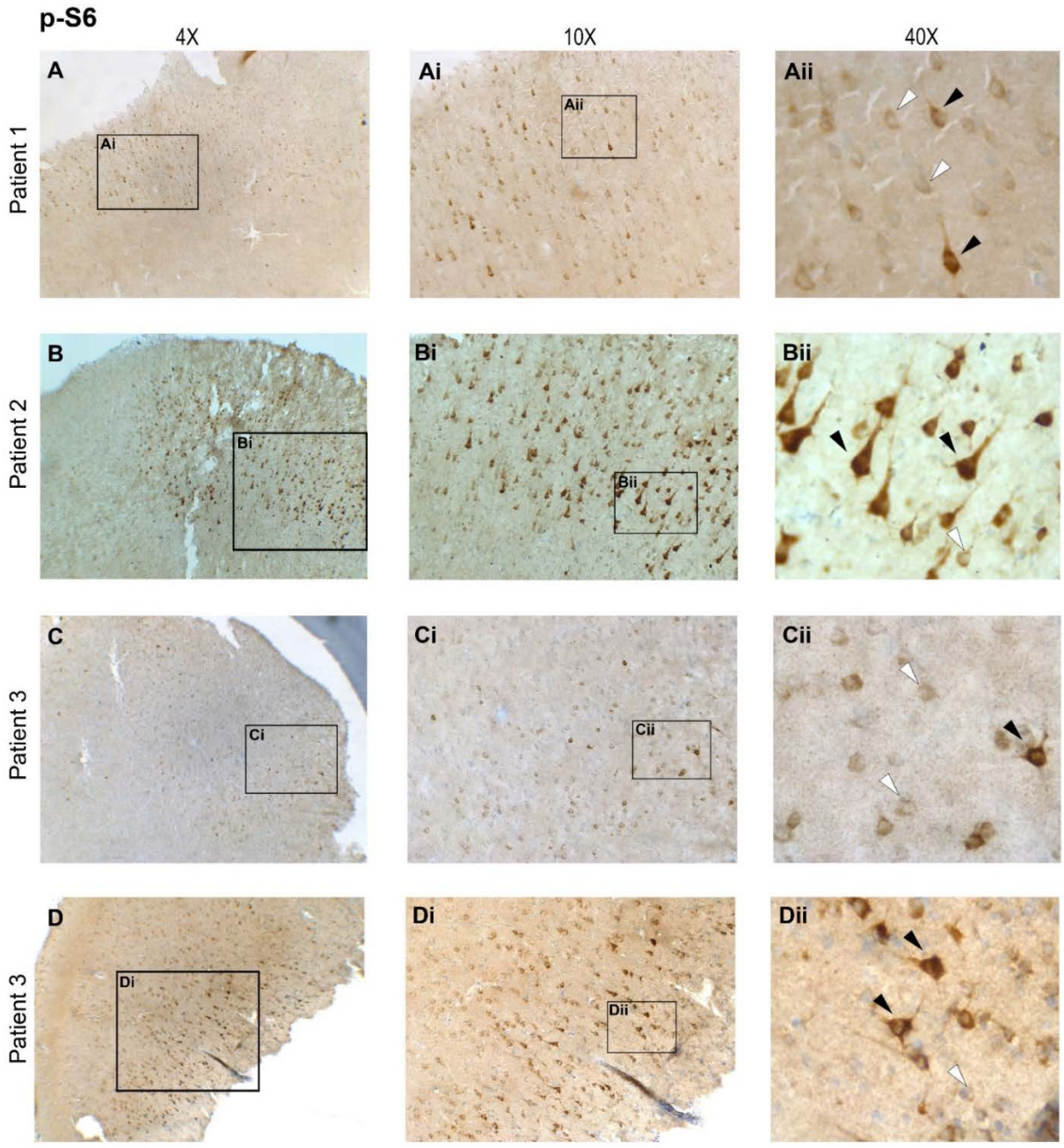

Figure 3. Phosphorylated ribosomal S6 (p-S6) protein is activated in human focal cortical dysplasia **(FCD).** Immunohistochemical staining of p-S6-positive neurons support activation of the mechanistic target of rapamycin (mTOR) pathway within the cortex of different FCD patients with drug-resistant seizures **(A-D)**. Brown staining represents p-S6 staining while blue signal represents nuclear Nissl staining. Areas with mTOR-activated (p-S6) neurons are localized within the cortex **(A, B, C, D)**. Higher magnification images of these areas are shown **(Ai, Bi, Ci, Di)**. Robust p-S6 signal is seen within some of the neurons **(Aii, Bii, Cii, Dii)**; Stronger p-S6 staining represents higher mTOR activation (black arrowheads) compared to cells with lighter p-S6 signal (white arrowheads).

Next, we were interested to investigate if there was tau pathology in the tissues from FCD patients with drug-resistant epilepsy (Figure 4). First, we examined the spatial distribution of p-tau molecules within the cortex. We used antibodies against p-tau (AT8) as hyperphosphorylated tau aggregates and forms NFT, therefore suggesting that more presence of p-tau would represent more accumulation of NFT within the cortex. Upon doing so, we found varying amounts of hyperphosphorylated tau signifying the presence of NFT in the temporal lobe biopsies. Figure 4 showed a representation of 4 different patients that displayed tau pathology. IHC of p-tau showed the presence of a low signal of tau pathology in Patients 1 and 2 (Figure 4A, 4B). Neuropil threads (white arrowheads) were found within these regions of cortical dysplasia, neuronal loss and high mTOR activation and can be seen at higher magnification (Figure 4Ai, 4Bi). Furthermore, some cases exhibited robust accumulation of pathological p-tau. Patient 3 (Figure 4C) showed many tangles (black arrowheads) with a dense meshwork of neuropil threads (white arrowheads) across the cortical laminae. Higher magnification revealed in greater detail the tau tangles (black arrowhead) and multiple neuropil threads (white arrowheads) (Figure 4Ci). Interestingly, Patient 9 also displayed a moderate accumulation of p-tau within the cortex (Figure 4D) with an isolated tau tangle (black arrowhead) and multiple neuropil threads (white arrowheads) (Figure 4Di). Based on the aforementioned results, this provides evidence of altering extents of tau pathology found in cortices of patients with FCD with drug-resistant epilepsy. Patients 5, 6, 11, and 12 did not show any p-tau staining.

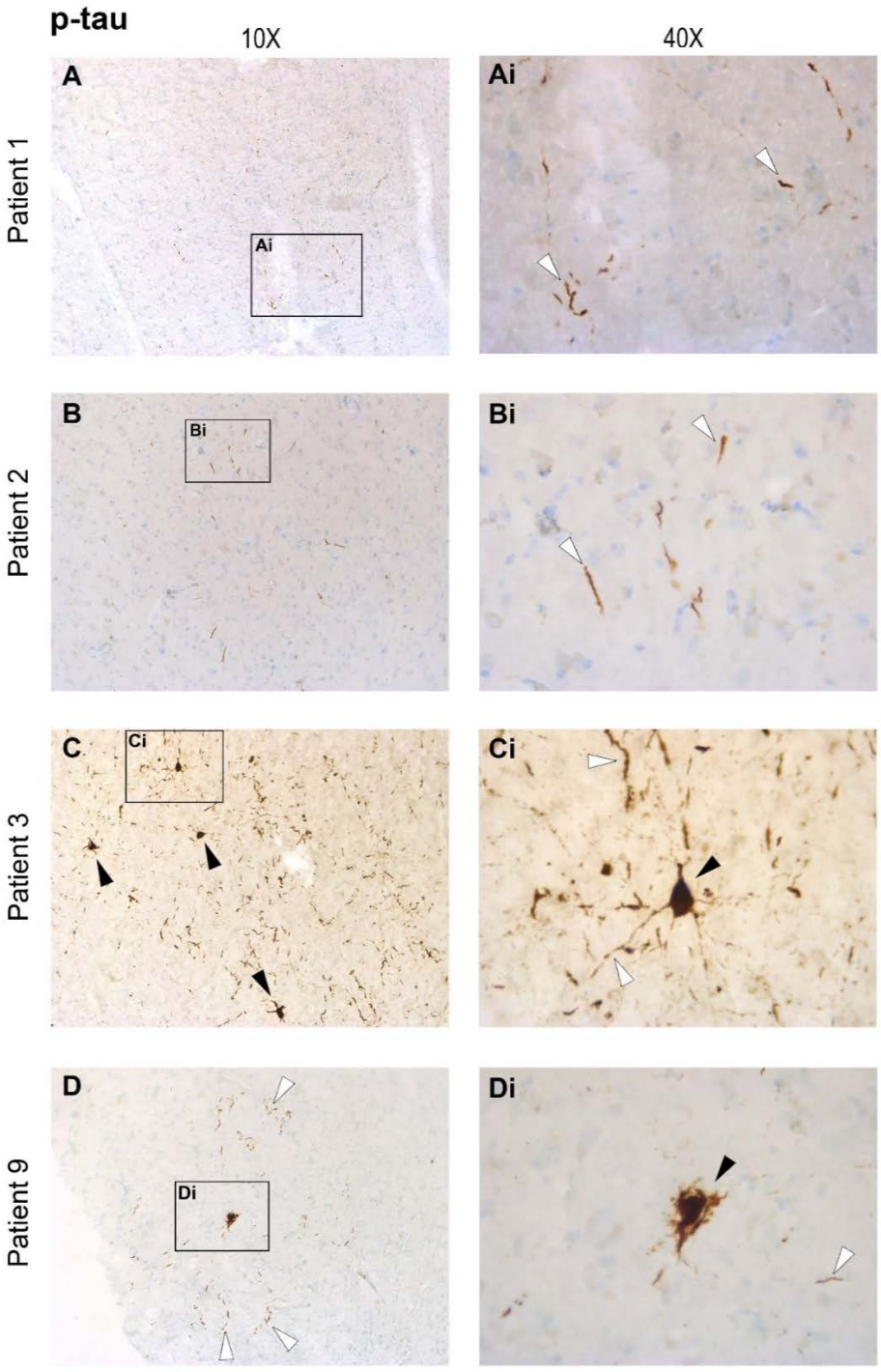

Figure 4. Phosphorylated tau (p-tau) is present in human focal cortical dysplasia **(FCD).** Immunohistochemical staining of p-tau detected with antibodies against AT8 show accumulation of neurofibrillary tangles (NFT) at different extents in the cortex of different FCD patients with drug-resistant seizures **(A, B, C, D)**. P-tau staining is shown in brown while nuclear Nissl staining is shown in blue. NFT immunostaining is evident at high **(C)**, moderate **(D)**, and low amounts **(A, B)** in different patients. Higher magnification images show p-tau signal within neuronal cell bodies with tangles (black arrowheads) and neuropil threads (white arrowheads) **(Ai, Bi, Ci, Di)**.

Lastly, we determined the spatial distribution of Aβ pathology within the cortex of FCD patients with drug-resistant epilepsy and found varying extents of Aβ accumulation. Figure 5 shows representative images of the different degrees of Aβ accumulation. Patient 1 (Figure 5A) showed a prominent accumulation of Aβ pathology found across the cortex that corresponded with regions of dysplasia, mTOR activation (Figure 3) and low tau pathology (Figure 4). Higher magnification confirmed that the plaques surrounded cells labeled in blue Nissl staining (Figure 5Ai). Patient 2 (Figure 5B) demonstrated weak Aβ signaling suggesting a low pathology (black arrowheads) that was found in a cortical region of neuronal loss (Figure 2), high mTOR activation (Figure 3), and low tau pathology (Figure 4). Finally, IHC staining in Patient 3 (Figure 5C) revealed a moderate Aβ pathology with Aβ (black arrowheads) localized between neurons (blue Nissl staining) at higher magnification (Figure 5Ci). Patients 4, 5, 6, and 7 did not show any Aβ staining. Taken together, this evidence suggests that both tau and Aβ pathology can be found in human brain cortical tissues derived from patients with FCD.

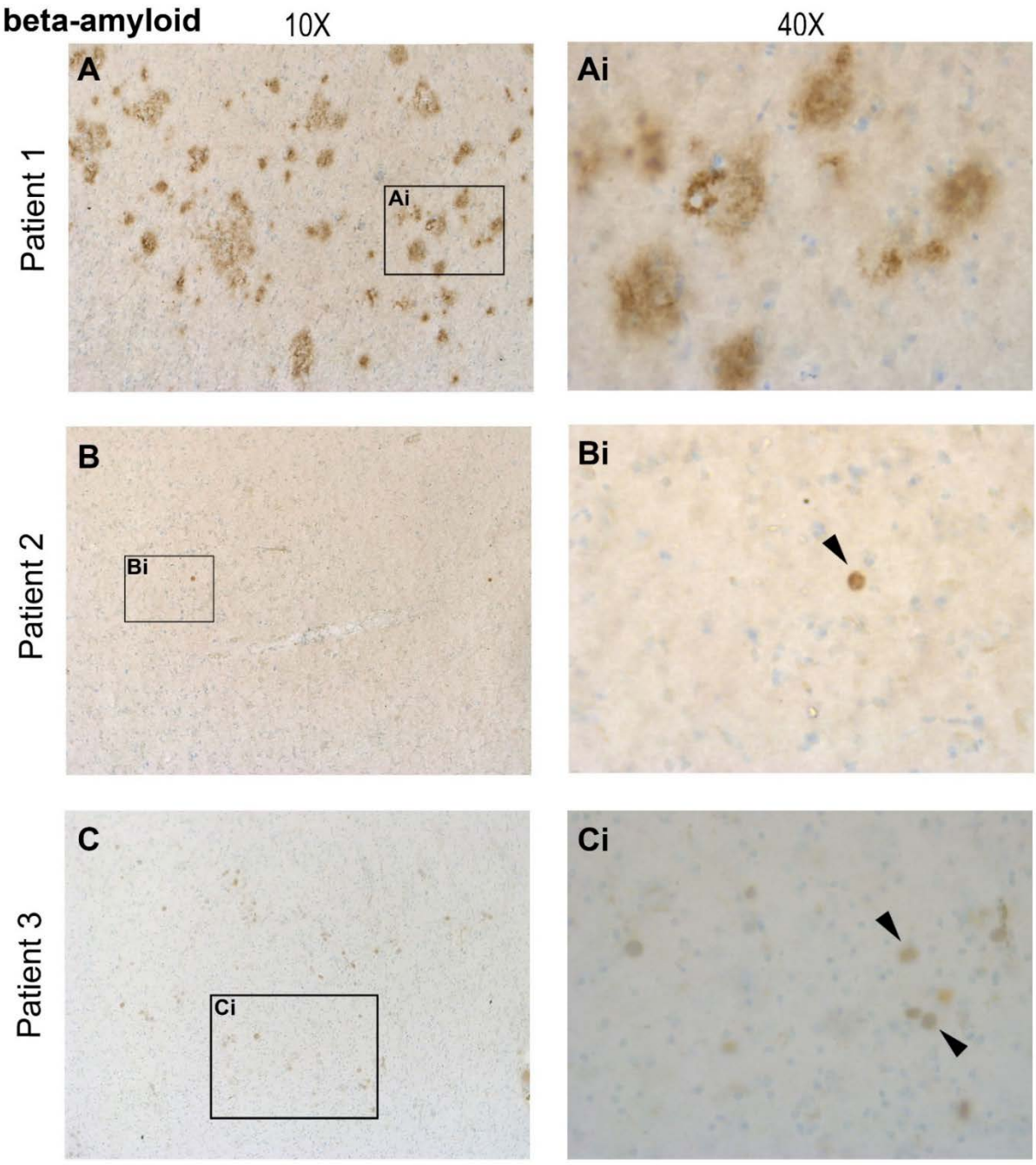

Figure 5. Beta-amyloid pathology is present in human focal cortical dysplasi**a (FCD).** Immunohistochemical staining of beta-amyloid is localized in the cortex of different FCD patients with drug-resistant seizures. Brown staining represents beta-amyloid plaques while the blue signal represents nuclear Nissl staining. Differing degrees of beta-amyloid plaque accumulation, high **(A)**, moderate **(C)**, and low **(B)** are seen in the cortex of different FCD patients. Beta-amyloid signal is localized surrounding and in between neurons. Higher magnification images reveal beta-amyloid staining (black arrowheads) at varying extents from high **(Ai)**, moderate **(Bi)**, and low **(Ci)** accumulation.

Detailed Analysis of FCD Patients With Drug-Resistant Epilepsy Using Immunoblots

To further understand the relationship between mTOR and GSK3 pathways activation, and AD pathology associated with Aβ and p-tau, WB was used to determine the protein levels of S6, p-S6, GSK3β, p-GSK3β, tau, p-tau, and Aβ in cortical tissue homogenates derived from FCD patients with drug-resistant epilepsy and NE patients. Figure 6 shows representative immunoblots for all these proteins of interests. Figures 7-11 show the densitometry/quantitative analysis and the associated statistical analyses for comparisons between FCD and NE groups (Figure 7), females and males (Figure 8), age-dependent analyses (Figure 9), correlations within samples between p-S6, Aβ, and p-tau (Figure 10A) as well as p-GSK3β, p-tau, and Aβ (Figure 10B), and correlation between the activation of p-S6 and p-GSK3 (Figure 11).

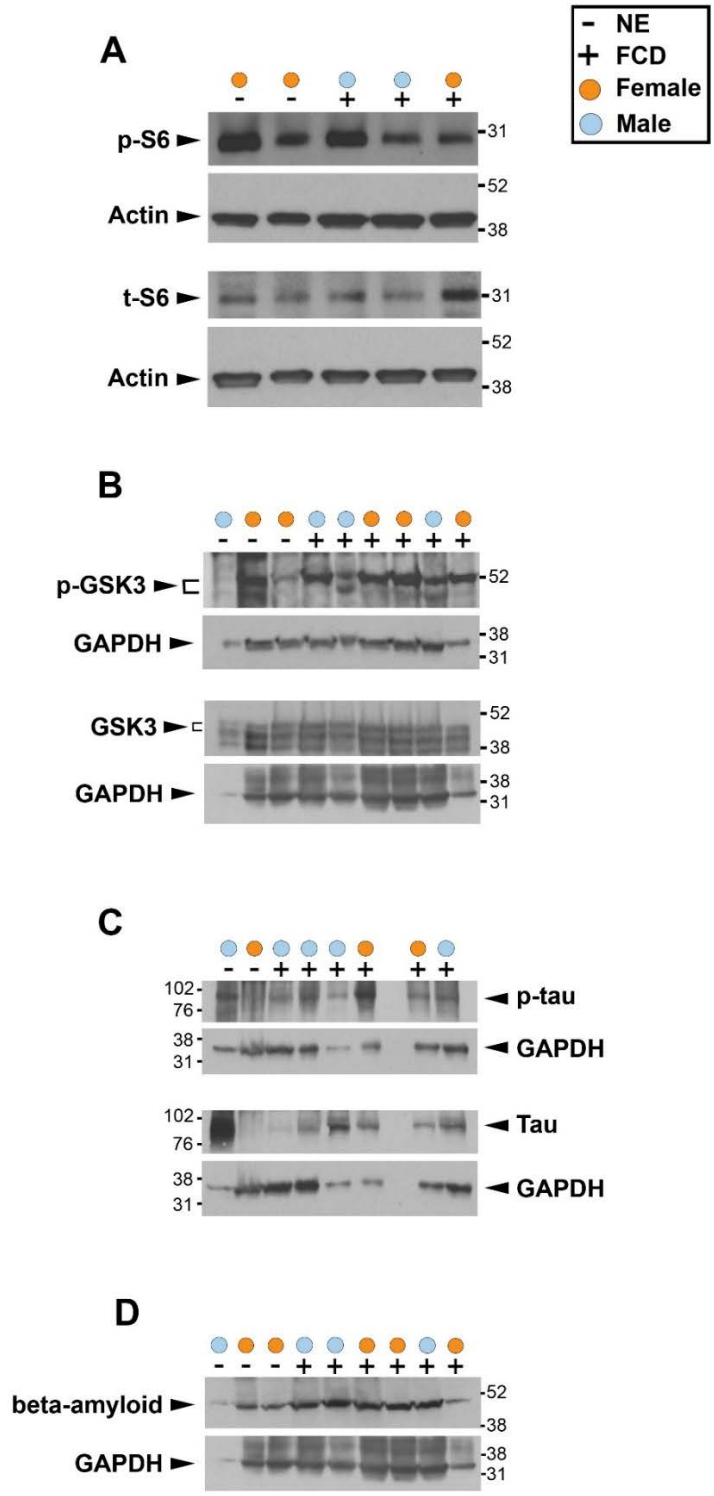

Figure 6. mTOR, beta-amyloid and tau pathology were examined using western blotting. **A-D)** Representative immunoblots for markers of mTOR activation, phospho-S6 (p-S6) **(A)** and phospho-GSK3 (p-GSK3) **(B)** are shown along with markers of Alzheimer's disease pathology, phospho-tau (p-tau) **(C)** and beta-amyloid (Aβ) **(D)**, and the corresponding gapdh/Actin (loading control).

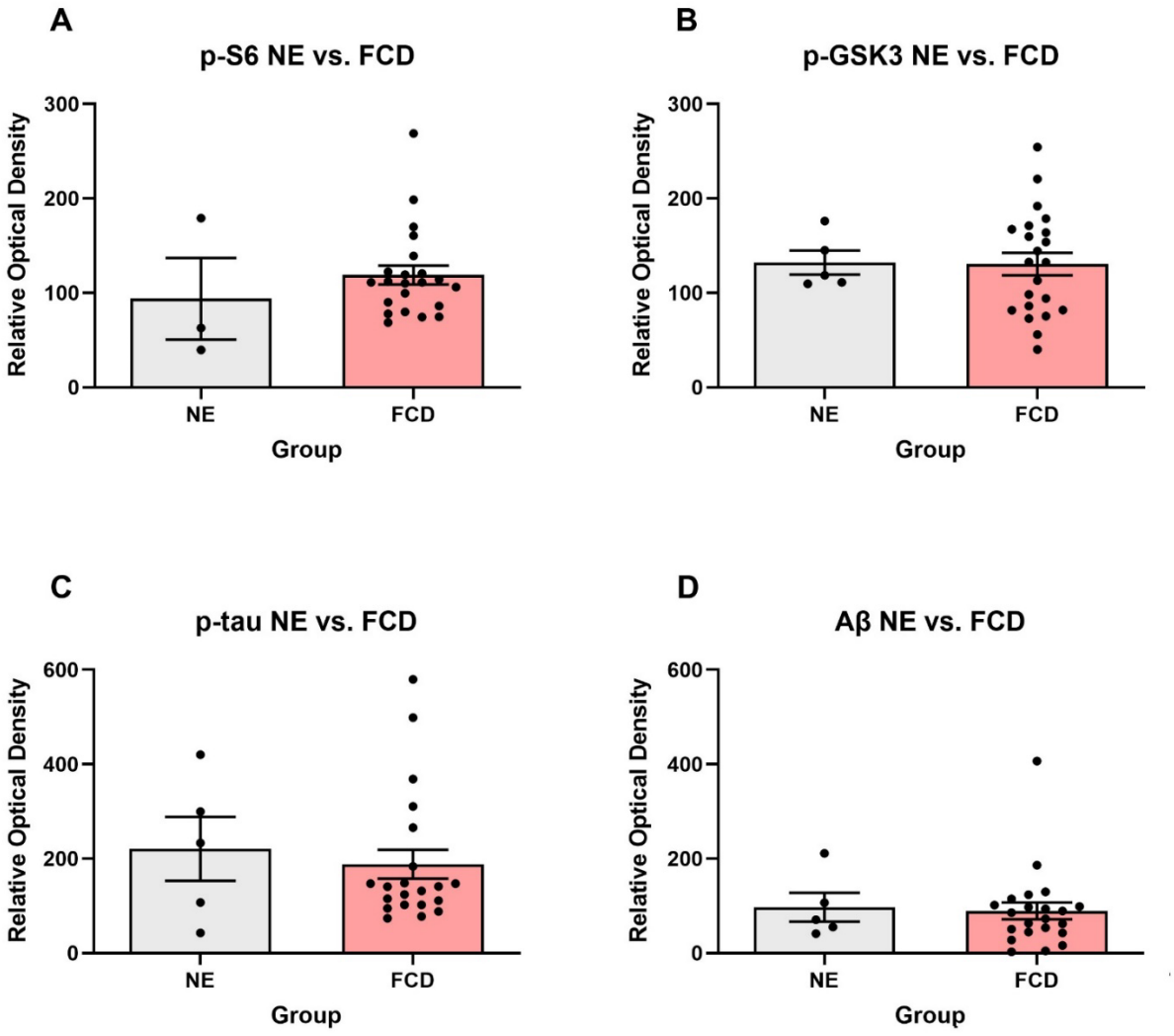

Figure 7. mTOR, beta-amyloid, and tau protein levels were not significantly different between focal cortical dysplasia (FCD) and non-epileptic (NE) tissue biopsies. **A-D)** Quantitative analysis of the mean pixel intensity shown as relative optical density units for p-S6/t-S6 **(A)**, p-GSK3/t-GSK3 **(B),** p-tau/tau **(C)**, and beta-amyloid/gapdh (Aβ) **(D)**. Data are shown as mean ± SEM. *n* = 22 for FCD and *n* = 5 for NE.

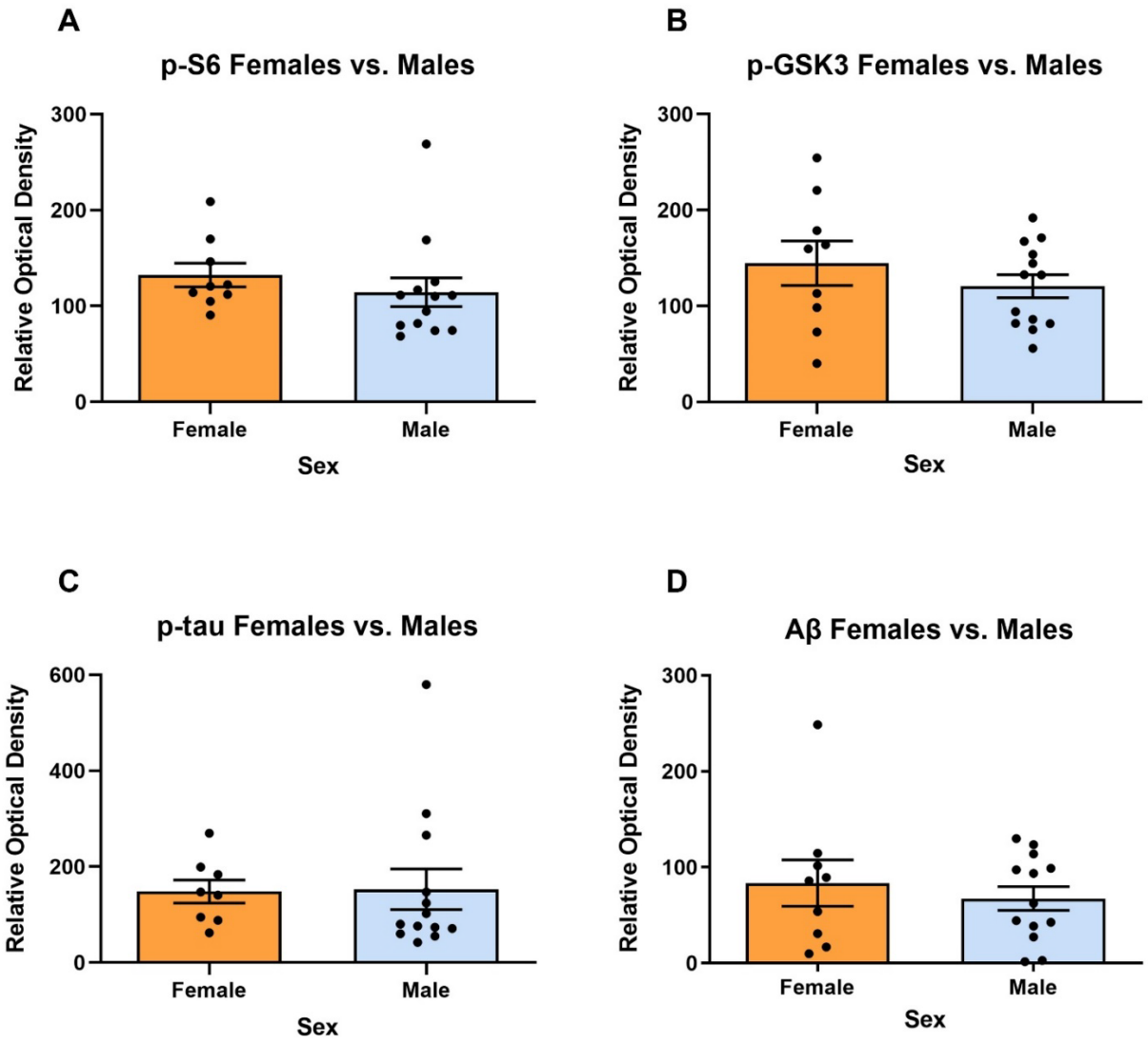

Figure 8. mTOR, beta-amyloid, and tau protein levels were not significantly different between female and male patients with FCD. **A-D)** Quantitative analysis of the mean pixel intensity shown as relative optical density units for p-S6/t-S6 **(A)**, p-GSK3/t-GSK3 **(B)**, p-tau/t-tau **(C)**, and beta-amyloid/gapdh (Aβ) **(D)**. Data are shown as mean ± SEM. *n* = 13 for FCD males and *n* = 9 for FCD females.

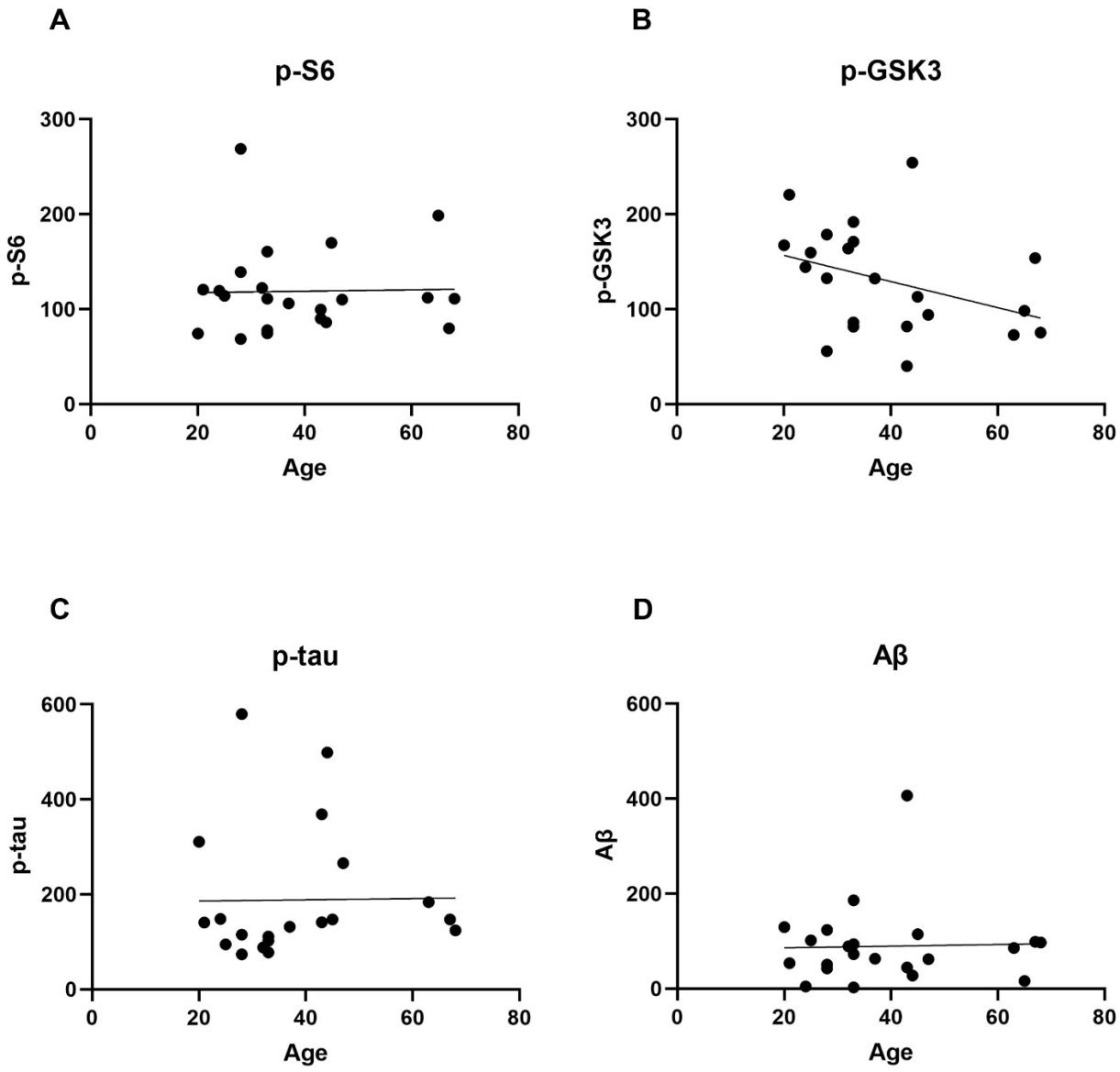

Figure 9. mTOR, beta-amyloid, and tau protein levels did not change with age in patients with FCD. Relative optical density data was plotted against age for linear regression analyses for p-S6/t-S6 **(A)**, p-GSK3/t-GSK3 **(B)**, p-tau/t-tau **(C)**, and beta-amyloid/gapdh (Aβ) **(D)**. Data are shown as mean ± SEM. *n* = 22 for FCD.

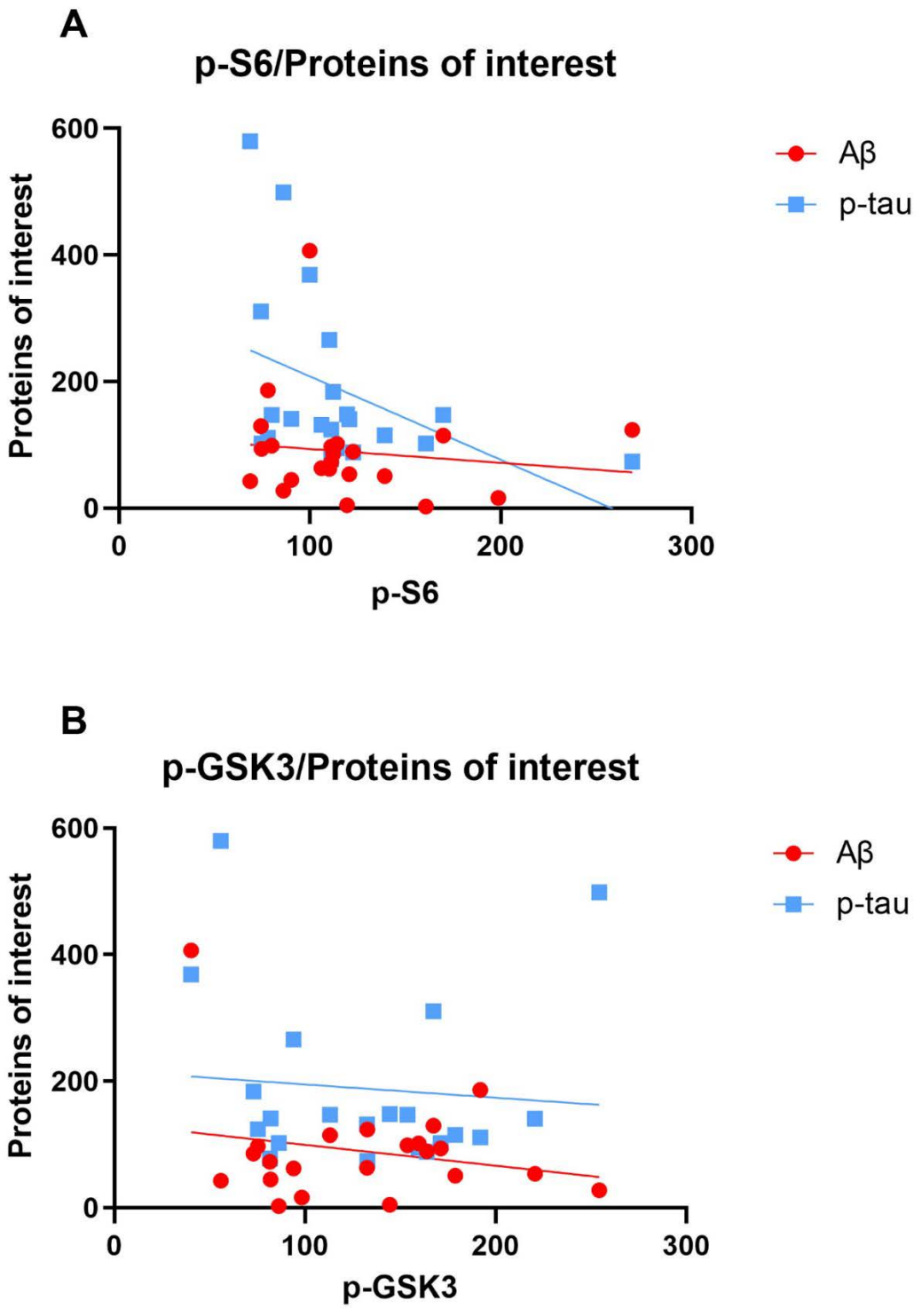

Figure 10. mTOR and GSK3 activation did not correlate with beta-amyloid (Aβ) and tau protein levels in patients with FCD. Relative optical density data of p-S6/t-S6 against beta-amyloid/gapdh (Aβ) and p-tau/tau **(A)** and p-GSK3/t-GSK3 against beta-amyloid/gapdh (Aβ) and p-tau/tau **(B)** were plotted. Data are shown as mean ± SEM. *n* = 22 for FCD.

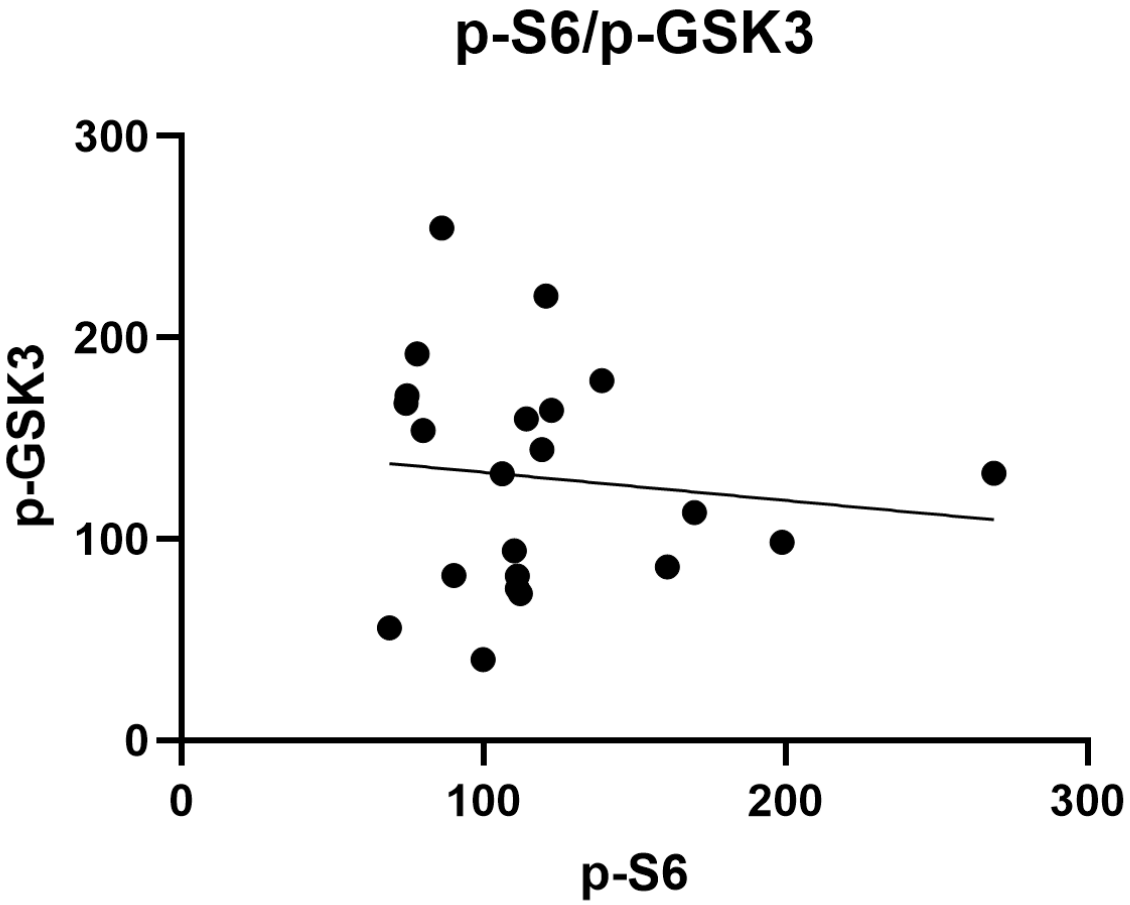

Figure 11. mTOR did not correlate with GSK3 activation in patients with FCD. Relative optical density of p-S6/t-S6 against p-GSK3/t-GSK3 was plotted. Data are shown as mean ± SEM. *n* = 22 for FCD

mTOR Activation, Beta-Amyloid Levels and Tau Levels Were not Different Between FCD and NE Patients

Densitometry analysis followed by t-test analyses revealed no significant differences between the FCD epilepsy and NE tissue samples for p-S6 (p = 0.422; Figure 7A), p-GSK3 (p = 0.947; Figure 7B), p-tau (p = 0.649; Figure 7C), and Aβ (p = 0.852; Figure 7D).

mTOR activation, Beta-Amyloid Levels and Tau Levels Were not Different Between FCD Females and Males

To determine potential sex differences, WB analyses were performed using antibodies against S6, p-S6, GSK3β, p-GSK3β, tau, p-tau, and Aβ in the group of FCD patients with drug-resistant epilepsy (Figure 8). *T*-tests comparing FCD females and males showed no significant differences for p-S6 (p = 0.401; Figure 8A), p-GSK3 (p = 0.331; Figure 8B), p-tau (p = 0.935; Figure 8C), and Aβ (p = 0.526; Figure 8D). However, it was noted that levels of p-S6 and p-GSK3 were trending to be higher in females compared to males.

No Age Correlation was Evident Between mTOR Activation, Beta-Amyloid, and P-Tau

As we obtained FCD samples from patients whose ages ranged from 20-68 years old, we were interested in investigating the age relationship between markers of mTOR activation (p-GSK3 and p-S6), and levels of Aβ and p-tau protein expression (Figure 9) because it is well established that the risk of beta-amyloid and tau pathology increases with age**.** We found that there was no significant correlation between age and p-S6 (r^2 = 0.0005, p = 0.920; Figure 9A), age and p-GSK3 (r^2 = 0.1373, p = 0.089; Figure 9B), age and p-tau (r^2 = 0.0001, p = 0.956; Figure 9C), and age and Aβ (r^2 = 0.0009, p = 0.889; Figure 9D).

mTOR Activation did not Correlate With the Levels of Beta-amyloid or P-Tau in the Cortex of FCD Patients

Furthermore, statistical analyses were performed to investigate the relationship between p-S6, Aβ and p-tau as well as the correlation between p-GSK3 Aβ, and p-tau protein levels within the same samples (Figure 10). We found that there was no significant correlation between p-S6 and Aβ (r^2 = 0.0150, p = 0.586), and p-S6 and p-tau (r^2 = 0.1745, p = 0.059) (Figure 10A) nor

between p-GSK3 and Aβ (r^2 = 0.0481, p = 0.326), and p-GSK3 and p-tau (r^2 = 0.0071, p = 0.715) (Figure 10B).

No Correlation Between mTOR and GSK3 Signaling

Lastly, GSK3 is an upstream regulator in the mTOR pathway. Therefore, we were interested in investigating whether activation of GSK3 and mTOR occurred similarly in the samples, which would suggest that these pathways depend on each other in the FCD tissues. We determined the relationship between p-S6 and p-GSK3 (Figure 11). Results revealed that there was no significant correlation between the two proteins (r^2 = 0.01383, p = 0.602) suggesting that other signaling cascades may crosstalk with mTOR and GSK3 in epilepsy, as has been shown for the mitogen activated protein kinase/extracellular-signal-regulated kinase (MAPK/ERK) pathway (Zimmer et al., 2020).

DISCUSSION

Previous studies reported the presence of Aβ and tau pathology in brain biopsies of both TLE and FCD patients (Gourmaud et al., 2020; Sen et al., 2007). For instance, the study conducted by Gourmaud et al. (2020) provided strong and extensive evidence of Aβ and tau alterations accompanied with cognitive impairments in biopsy tissue from patients with TLE, and compared these results to both NE tissue biopsies and post-mortem tissue from AD patients. Furthermore, the study conducted by Sen et al. (2007) claimed the presence of pathological tau tangles in older patients with FCD with drug-resistant epilepsy. Additionally, they found Aβ plaques in two of their patients aged 59 and 81. The aforementioned results therefore support that Aβ and tau pathology occurs not only in cases of human TLE, but also in human FCD. However, the mechanisms through which this pathology develops are still unknown. As mentioned previously, the mTOR pathway has been strongly associated with neurodevelopment, epilepsy and AD (Hoeffer & Klann, 2010; Nguyen et al., 2019; Oddo, 2012; Talboom et al., 2015; Wyatt-Johnson & Brewster, 2020; Zimmer et al., 2020) Therefore, in our study, we were interested in investigating the possible link between characteristic AD pathology in FCD through the mTOR pathway.

The aims of this study were to determine the spatial distribution of Aβ, p-tau, and mTOR activated neurons within the cortex. Additionally, we investigated the protein expression of Aβ and p-tau, and mTOR activation in cortical samples of FCD patients with drug-resistant epilepsy. The main findings were (1) Cortical dyslamination accompanied by mTOR activation in a subset of neurons localized to dysplastic regions was found along with Aβ and p-tau within cortices of FCD patients with drug-resistant epilepsy; (2) No significant difference in mTOR activation, and Aβ and p-tau expression between cortical samples from FCD patients with drug-resistant epilepsy and NE samples from glioblastoma patients with no history of epilepsy or seizures; (3) No significant difference in mTOR activation, and Aβ and p-tau expression between female and male FCD patients with drug-resistant epilepsy; (4) No significant correlation between mTOR activation, Aβ and p-tau expression, and age; (5) No significant correlation between mTOR activation, and Aβ and p-tau expression in FCD patients with drug-resistant epilepsy; (6) No significant correlation between the upstream regulator GSK3 and downstream ribosomal S6 protein in FCD patients with drug-resistant epilepsy.

Our results revealed cortical dyslamination through using NeuN to stain for neurons. In all 12 of our available tissue samples, we found pathological evidence characteristic of FCD, which is consistent with previous findings (Blumcke et al., 2011; Kim & Choi, 2019). Radial microcolumnar disorganization, neuronal loss and dysmorphic neurons were found within certain cortical regions (Figure 2) compared to other areas suggesting an insult to the region, most likely resulting from recurring seizures, therefore confirming characteristics of FCD in our samples. Staining with p-S6, an indicator of mTOR activation, further supported abnormal activity of p-S6-positive neurons within regions that also displayed cortical dyslamination (Figure 3). Interestingly, certain p-S6-positive neurons displayed a more robust signal implying higher mTOR activation compared to other p-S6 positive neurons that showed lower mTOR activity. This information paired with the results from our NeuN staining suggest mTOR hyperactivation played a role in abnormal cortical lamination as well as seizure activity. Furthermore, Aβ and p-tau staining revealed differing degrees of Aβ and tau pathology (Table 2). Within the regions of FCD samples that showed tau pathology, it was seen that there was moderate to low p-S6 signal depicting the level of mTOR activation, despite these same regions displaying cortical dyslamination (Figure 2, 3, 4). As several other growth factors and kinases such as Cyclin-dependent kinase 5 (Cdk5) play a role in the phosphorylation of tau (Sanchez, Garcia-Cabrero, Sanchez-Elexpuru, Burgos, & Serratosa, 2018), perhaps NFT accumulation could have been attributed to other signaling cascades that were not investigated in the current study. An interesting observation was that most tau pathology was seen in patients 44 years and older with the exception of Patient 9, aged 25 (Figure 4D/Di), which suggests the possibility of an age-dependent pathology. However, the extent of seizure frequency/duration was unknown/unavailable, and we can speculate that Patient 9 may have experienced a higher frequency of seizures in a shorter amount of time than the other samples around that age did. On the other hand, Aβ was seen in variable amounts in patients at different ages with minimal to no amount of accumulation in younger patients. Only the tissue biopsies of Patient 1, aged 51 (Figure 5A/Ai), and Patient 3, aged 59 (Figure 5B/Bi), showed high and moderate amounts of Aβ accumulation, respectively compared to other patients that showed low extents of Aβ accumulation. Additionally, the prominent Aβ accumulation was seen in cortices with moderate to low mTOR activation within the same dysplastic cortical region. However, other medical conditions, such as early-onset AD, that the patients may have been experiencing were unknown to us. Hence, we cannot say with certainty where the Aβ plaques derived from and

Table 2. Summary of IHC Staining of p-tau and Aβ

Patient	Age	Sex	FCD	p-tau	Aβ
1	51	Male	NA	Few neuropil threads and isolated tangles	High Aβ plaque accumulation
2	67	Male	IIIa	Few neuropil threads and isolated tangles	Few Aβ plaque accumulation
3	59	Male	NA	High amount of neuropil threads and tau tangles	Moderate Aβ plaque accumulation
4	55	Male	NA	Moderate amount of neuropil threads with isolated tangles	—
5	36	Female	NA	—	—
6	24	Male	NA	—	—
7	44	Male	NA	Few neuropil threads and isolated tangles	—
8	53	Female	NA	High amount of neuropil threads with few tau tangles	Few Aβ plaque accumulation
9	25	Female	Ic	Few neuropil threads	One Aβ plaque
10	44	Female	NA	Moderate amount of neuropil threads with isolated tangles	Few Aβ plaque accumulation
11	25	Female	NA	—	Very few Aβ plaque accumulation
12	28	Male	IIIa	—	Very few Aβ plaques accumulation

whether this was a result of mTOR hyperactivation, as medications the patients were taking could have altered mTOR expression by the time of surgery. Moreover, we did not have access to NE samples for immunostaining, which would have helped us further understand the extent of tau and beta-amyloid pathology in our FCD samples when compared to NE ones.

Activation of mTOR has been shown to differ between sexes (Baar, Carbajal, Ong, & Lamming, 2016). A study conducted by Baar et al. (2016), investigated sex and tissue-specific differences in mTOR signaling in C57BL/6J mice, and found increased mTOR signaling (p-S6) in the heart and liver of young female mice when compared to young male mice (Baar et al., 2016). This is consistent with our observation of FCD brain tissue from our female samples exhibiting slightly higher p-S6 and p-GSK3 levels (Figure 8A, 8B), and therefore activation, compared to males. Interestingly, it has also been found that estradiol is reliant on the phosphorylation of ERK or the mTOR pathway within the dorsal hippocampus in increasing CA1 dendritic spine density (Choleris, Galea, Sohrabji, & Frick, 2018). Another interesting point to note is that sex differences in severity of Aβ and tau pathology was seen not only in human female AD patients but also in mouse models expressing AD (Congdon, 2018). This could be due to the fact that autophagy, a process controlled by the mTOR pathway, has been reported to have lower levels of activity in females despite numerous autophagy-related genes being found on the X chromosome (Congdon, 2018). This evidence suggests that mTOR activation can be significantly altered based on the effects of hormonal proteins. Additionally, age can be implicated in the density of synaptodendritic structures, which is known to be regulated by mTOR activation (Brewster et al., 2013; Liu et al., 2014; Wong, 2013). Comprehensive studies involving both rodents and humans suggest that dendritic density decreases with age (Aroor & Brewster, 2021; Dickstein, Weaver, Luebke, & Hof, 2013), implicating that loss of dendritic spines could contribute to a higher seizure severity. While our western blot analyses do not provide support of the above-mentioned data, nor a link between AD pathology and FCD through the mTOR pathway, multiple possible explanations can be postulated as to why this may have occurred. For starters, working with human tissue has its limitations. As we were not able to obtain additional patient information such as duration of seizures, medication history, and other medical conditions, there could be other factors that influenced the levels of protein expression in FCD patients with drug-resistant epilepsy. For instance, some AEDs have been reported to produce adverse cognitive effects such as a decline on memory, attention, and motor function on patients that were prescribed certain medications (Eddy,

Rickards, & Cavanna, 2011). Moreover, AEDs can contribute to further modulation and suppression of mTOR signaling potentially disrupting other signaling pathways, therefore provoking additional side effects (Wong, 2013). Another limitation to the interpretation of our quantitative findings is related to the low number of NE samples available. A larger NE cohort would have been beneficial to better identity and quantify potential differences in protein expression between the FCD and NE groups. Furthermore, it has been reported that 20-50% of glioblastoma patients experience seizures (Prakash, Lukiw, Peruzzi, Reiss, & Musto, 2012). Although our NE group consisted of peritumoral tissue from glioblastoma patients that reported having no history of seizures and epilepsy, there could be a possibility that the patients experienced non-convulsive seizures which may have been unnoticed and therefore undiagnosed. Interestingly, mTOR has also been reported to play a role in tumor pathogenesis in glioblastoma patients (Duzgun, Eroglu, & Avci, 2016). As a result, some pharmacological therapies for glioblastoma target the inhibition of mTOR signaling further altering the expression of proteins within this signaling cascade. Therefore, future studies involving a different NE group or positive controls from AD patients could benefit in better defining the relationship between mTOR activation, AD pathology, and FCD in human epileptic tissue. It is important to note the complexity of the mTOR pathway and the other signaling pathways it interacts with (Switon et al., 2017), suggesting that alternative pathways may have influenced the alteration in protein expression. Therefore, it would be difficult to conclude the relationship between Aβ and tau pathology with mTOR activity in FCD solely based on our immunohistochemical findings. One of the limitations to this is that there were only 3 FCD samples used for IHC and WB that were from the same patients. Therefore, it would be beneficial to further investigate this relationship using samples from the same patients that are processed for both methods, immunoblotting and immunostaining to better define the relationship between mTOR, Aβ, and tau protein distribution and expression in FCD.

In sum, Aβ and tau pathology have been previously reported in both rodent models of FCD and FCD patients. The mTOR pathway has been shown to separately play a role in both AD pathology and abnormal cortical development in FCD. However, the question remains whether abnormal mTOR activity directly influences Aβ and tau pathology in FCD. Our present data provides varying extents of Aβ and tau pathology found within the cortex of FCD patients. This data correlated with different degrees of mTOR activation seen through IHC staining, suggesting potential support for the role of mTOR in Aβ and tau pathology in FCD. However, this evidence

is not enough to provide a definitive conclusion. Therefore, further research involving biochemical analysis of the same FCD tissue that underwent IHC staining would aid in better understanding the extent of mTOR activation, and Aβ and p-tau expression within the cortex. Additionally, future studies involving colocalization of mTOR activation with p-tau, and mTOR activation with Aβ in both mouse models of FCD and surgically resected human FCD tissue, along with extensive mapping of the proteins involved in the mTOR signaling cascade would be beneficial in understanding the mechanisms through which Aβ and tau pathology occur in FCD. It is important to note that treatment involving complete inhibition of the mTOR pathway could provoke adverse effects as mTOR is essential for several other processes. Therefore, detailed investigation of the association between mTOR signaling, and tau and Aβ pathology in FCD could provide more potential targets for pharmacological therapies.

REFERENCES

Alzheimer's Association Report (2020). 2020 Alzheimer's Disease Facts and Figures. *Alzheimers & Dementia, 16*(3), 391-460. doi:10.1002/alz.12068

Alves, M., Kenny, A., de Leo, G., Beamer, E. H., & Engel, T. (2019). Tau phosphorylation in a mouse model of temporal lobe epilepsy. *Frontiers in Aging Neuroscience, 11*, 308. doi:10.3389/fnagi.2019.00308

Aroor, A., & Brewster, A. L. (2020). Seizing the Alzheimer's brain: A role for sirtuin 3 in hyperexcitability. *Epilepsy Currents, 20*(4), 224-226. doi:10.1177/1535759720932157

Aroor, A., & Brewster, A. L. (2021). Dendritic and spine loss in epilepsy: What seizures got to do with It? *Epilepsy Currents, 21*(3), 186-188. doi:10.1177/15357597211003096

Baar, E. L., Carbajal, K. A., Ong, I. M., & Lamming, D. W. (2016). Sex- and tissue-specific changes in mTOR signaling with age in C57BL/6J mice. *Aging Cell, 15*(1), 155-166. doi:10.1111/acel.12425

Blumcke, I., Thom, M., Aronica, E., Armstrong, D. D., Vinters, H. V., Palmini, A., Jacques, T. S., Avanzini, G., Barkovich, A. J., Battaglia, G., Becker, A, Cepeda, C., Cendes, F., Colombo, N., Crino, P., Cross, J. H., Delalande, O.,Dubeau, F., Duncan, J., . . . Spreafico, R. (2011). The clinicopathologic spectrum of focal cortical dysplasias: a consensus classification proposed by an ad hoc Task Force of the ILAE Diagnostic Methods Commission. *Epilepsia, 52*(1), 158-174. doi:10.1111/j.1528-1167.2010.02777.x

Brewster, A. L., Lugo, J. N., Patil, V. V., Lee, W. L., Qian, Y., Vanegas, F., & Anderson, A. E. (2013). Rapamycin reverses status epilepticus-induced memory deficits and dendritic damage. *PLoS One, 8*(3), e57808. doi:10.1371/journal.pone.0057808

Cai, Z., Chen, G., He, W., Xiao, M., & Yan, L. J. (2015). Activation of mTOR: A culprit of Alzheimer's disease? *Neuropsychiatric Disease and Treatment, 11*, 1015-1030. doi:10.2147/NDT.S75717

Chin, J., & Scharfman, H. E. (2013). Shared cognitive and behavioral impairments in epilepsy and Alzheimer's disease and potential underlying mechanisms. *Epilepsy & Behavior, 26*(3), 343-351. doi:10.1016/j.yebeh.2012.11.040

Choleris, E., Galea, L. A. M., Sohrabji, F., & Frick, K. M. (2018). Sex differences in the brain: Implications for behavioral and biomedical research. *Neuroscience & Biobehavioral Reviews, 85*, 126-145. doi:10.1016/j.neubiorev.2017.07.005

Congdon, E. E. (2018). Sex differences in autophagy contribute to female vulnerability in Alzheimer's disease. *Frontiers in Neuroscience, 12*, 372. doi:10.3389/fnins.2018.00372

Cortini, F., Cantoni, C., & Villa, C. (2018). Epileptic seizures in autosomal dominant forms of Alzheimer's disease. *Seizure, 61*, 4-7. doi:10.1016/j.seizure.2018.07.015

Dickstein, D. L., Weaver, C. M., Luebke, J. I., & Hof, P. R. (2013). Dendritic spine changes associated with normal aging. *Neuroscience, 251*, 21-32. doi:10.1016/j.neuroscience.2012.09.077

Duzgun, Z., Eroglu, Z., & Avci, C. B. (2016). Role of mTOR in glioblastoma. *Gene, 575*(2), 187-190. doi:10.1016/j.gene.2015.08.060

Eddy, C. M., Rickards, H. E., & Cavanna, A. E. (2011). The cognitive impact of antiepileptic drugs. *Therapeutic Advances in Neurological Disorders, 4*(6), 385-407. doi:10.1177/1756285611417920

Gourmaud, S., Shou, H., Irwin, D. J., Sansalone, K., Jacobs, L. M., Lucas, T. H., Marsh, E. D., Davies, K. A., Jensen, F. E., & Talos, D. M. (2020). Alzheimer-like amyloid and tau alterations associated with cognitive deficit in temporal lobe epilepsy. *Brain, 143*(1), 191-209. doi:10.1093/brain/awz381

Hodges, S. L., Reynolds, C. D., Smith, G. D., Jefferson, T. S., Nolan, S. O., & Lugo, J. N. (2018). Molecular interplay between hyperactive mammalian target of rapamycin signaling and Alzheimer's disease neuropathology in the NS-Pten knockout mouse model. *Neuroreport, 29*(13), 1109-1113. doi:10.1097/WNR.0000000000001081

Hoeffer, C. A., & Klann, E. (2010). mTOR signaling: At the crossroads of plasticity, memory and disease. *Trends in Neuroscience, 33*(2), 67-75. doi:S0166-2236(09)00187-8 [pii] 10.1016/j.tins.2009.11.003

Huang, X., Zhang, H., Yang, J., Wu, J., McMahon, J., Lin, Y., Cao, Z., Gruenthal, M., & Huang, Y. (2010). Pharmacological inhibition of the mammalian target of rapamycin pathway suppresses acquired epilepsy. *Neurobiology of Disease, 40*(1), 193-199. doi:10.1016/j.nbd.2010.05.024

Kim, S. H., & Choi, J. (2019). Pathological classification of Focal Cortical Dysplasia (FCD): Personal comments for well understanding FCD classification. *Journal of Korean Neurosurgical Society, 62*(3), 288-295. doi:10.3340/jkns.2019.0025

Knerlich-Lukoschus, F., Connolly, M. B., Hendson, G., Steinbok, P., & Dunham, C. (2017). Clinical, imaging, and immunohistochemical characteristics of focal cortical dysplasia Type II extratemporal epilepsies in children: Analyses of an institutional case series. *Journal of Neurosurgery Pediatrics, 19*(2), 182-195. doi:10.3171/2016.8.PEDS1686

Lee, S. K., & Kim, D. W. (2013). Focal cortical dysplasia and epilepsy surgery. *Journal of Epilepsy Research, 3*(2), 43-47. doi:10.14581/jer.13009

Liu, J., Reeves, C., Michalak, Z., Coppola, A., Diehl, B., Sisodiya, S. M., & Thom, M. (2014). Evidence for mTOR pathway activation in a spectrum of epilepsy-associated pathologies. *Acta Neuropathologica Communications, 2*, 71. doi:10.1186/2051-5960-2-71

Mirzaa, G. M., Campbell, C. D., Solovieff, N., Goold, C., Jansen, L. A., Menon, S., Timms, A. E., Conti, V., Biag, J. D., Adams, C., Boyle, E. A., Collins, S., Ishak, G., Poliachik, S., Girisha, K. M., Yeung, K. S., Chung, B. H. Y., Rahikkala, E., Gunter, S. A., . . . Dobyns, W. B. (2016). Association of MTOR mutations with developmental brain disorders, including megalencephaly, focal cortical dysplasia, and pigmentary mosaicism. *JAMA Neurology, 73*(7), 836-845. doi:10.1001/jamaneurol.2016.0363

Mueed, Z., Tandon, P., Maurya, S. K., Deval, R., Kamal, M. A., & Poddar, N. K. (2018). Tau and mTOR: The hotspots for multifarious diseases in Alzheimer's development. *Frontiers in Neuroscience, 12*, 1017. doi:10.3389/fnins.2018.01017

Nguyen, L. H., Brewster, A. L., Clark, M. E., Regnier-Golanov, A., Sunnen, C. N., Patil, V. V., D'Arcangelo, G., & Anderson, A. E. (2015). mTOR inhibition suppresses established epilepsy in a mouse model of cortical dysplasia. *Epilepsia, 56*(4), 636-646. doi:10.1111/epi.12946

Nguyen, L. H., Mahadeo, T., & Bordey, A. (2019). mTOR hyperactivity levels influence the severity of epilepsy and associated neuropathology in an experimental model of tuberous sclerosis complex and focal cortical dysplasia. *Journal of Neuroscience, 39*(14), 2762-2773. doi:10.1523/JNEUROSCI.2260-18.2019

Oddo, S. (2012). The role of mTOR signaling in Alzheimer disease. *Frontiers in Bioscience (Schol Ed), 4*, 941-952. doi:10.2741/s310

Palop, J. J., Chin, J., Roberson, E. D., Wang, J., Thwin, M. T., Bien-Ly, N., Yoo, J., Ho, K. O., Yu, G.-Q., Kreitzer, A., Finkbeiner, S., Noebels, J. L., & Mucke, L. (2007). Aberrant excitatory neuronal activity and compensatory remodeling of inhibitory hippocampal circuits in mouse models of Alzheimer's disease. *Neuron, 55*(5), 697-711. doi:10.1016/j.neuron.2007.07.025

Paudel, Y. N., Shaikh, M. F., Shah, S., Kumari, Y., & Othman, I. (2018). Role of inflammation in epilepsy and neurobehavioral comorbidities: Implication for therapy. *European Journal of Pharmacology, 837,* 145-155. doi:10.1016/j.ejphar.2018.08.020

Prakash, O., Lukiw, W. J., Peruzzi, F., Reiss, K., & Musto, A. E. (2012). Gliomas and seizures. *Medical Hypotheses, 79*(5), 622-626. doi:10.1016/j.mehy.2012.07.037

Reyes-Marin, K. E., & Nunez, A. (2017). Seizure susceptibility in the APP/PS1 mouse model of Alzheimer's disease and relationship with amyloid beta plaques. *Brain Research, 1677,* 93-100. doi:10.1016/j.brainres.2017.09.026

Sanchez, M. P., Garcia-Cabrero, A. M., Sanchez-Elexpuru, G., Burgos, D. F., & Serratosa, J. M. (2018). Tau-induced pathology in epilepsy and dementia: Notions from patients and animal models. *International Journal of Molecular Science, 19*(4). doi:10.3390/ijms19041092

Sen, A., Thom, M., Martinian, L., Harding, B., Cross, J. H., Nikolic, M., & Sisodiya, S. M. (2007). Pathological tau tangles localize to focal cortical dysplasia in older patients. *Epilepsia, 48*(8), 1447-1454. doi:10.1111/j.1528-1167.2007.01107.x

Sheng, J., Liu, S., Qin, H., Li, B., & Zhang, X. (2018). Drug-resistant epilepsy and surgery. *Current Neuropharmacology, 16*(1), 17-28. doi:10.2174/1570159X15666170504123316

Siedlecka, M., Grajkowska, W., Galus, R., Dembowska-Baginska, B., & Jozwiak, J. (2016). Focal cortical dysplasia: Molecular disturbances and clinicopathological classification (Review). *International Journal of Molecular Medicine, 38*(5), 1327-1337. doi:10.3892/ijmm.2016.2760

Siman, R., Cocca, R., & Dong, Y. (2015). The mTOR inhibitor rapamycin mitigates perforant pathway neurodegeneration and synapse loss in a mouse model of early-stage Alzheimer-type tauopathy. *PLoS One, 10*(11), e0142340. doi:10.1371/journal.pone.0142340

Sirven, J. I. (2015). Epilepsy: A spectrum disorder. *Cold Spring Harbor Perspectives in Medicine, 5*(9), a022848. doi:10.1101/cshperspect.a022848

Spilman, P., Podlutskaya, N., Hart, M. J., Debnath, J., Gorostiza, O., Bredesen, D., Richardson, A., Strong, R., & Galvan, V. (2010). Inhibition of mTOR by rapamycin abolishes cognitive deficits and reduces amyloid-beta levels in a mouse model of Alzheimer's disease. *PLoS One, 5*(4), e9979. doi:10.1371/journal.pone.0009979

Stafstrom, C. E., & Carmant, L. (2015). Seizures and epilepsy: An overview for neuroscientists. *Cold Spring Harbor Perspectives in Medicine, 5*(6). doi:10.1101/cshperspect.a022426

Sunnen, C. N., Brewster, A. L., Lugo, J. N., Vanegas, F., Turcios, E., Mukhi, S., .Parghi, D., D'Arcangelo, G., & Anderson, A. E. (2011). Inhibition of the mammalian target of rapamycin blocks epilepsy progression in NS-Pten conditional knockout mice. *Epilepsia, 52*(11), 2065-2075. doi:10.1111/j.1528-1167.2011.03280.x

Switon, K., Kotulska, K., Janusz-Kaminska, A., Zmorzynska, J., & Jaworski, J. (2017). Molecular neurobiology of mTOR. *Neuroscience, 341*, 112-153. doi:10.1016/j.neuroscience.2016.11.017

Tai, X. Y., Koepp, M., Duncan, J. S., Fox, N., Thompson, P., Baxendale, S., Liu, J., Reeves, C., Michalak, Z., & Thom, M. (2016). Hyperphosphorylated tau in patients with refractory epilepsy correlates with cognitive decline: A study of temporal lobe resections. *Brain, 139*(Pt 9), 2441-2455. doi:10.1093/brain/aww187

Talboom, J. S., Velazquez, R., & Oddo, S. (2015). The mammalian target of rapamycin at the crossroad between cognitive aging and Alzheimer's disease. *NPJ Aging and Mechanisms of Disease, 1*, 15008. doi:10.1038/npjamd.2015.8

Thijs, R. D., Surges, R., O'Brien, T. J., & Sander, J. W. (2019). Epilepsy in adults. *Lancet, 393*(10172), 689-701. doi:10.1016/S0140-6736(18)32596-0

Toral-Rios, D., Pichardo-Rojas, P. S., Alonso-Vanegas, M., & Campos-Pena, V. (2020). GSK3beta and tau protein in Alzheimer's disease and epilepsy. *Frontiers in Cellular Neuroscience, 14*, 19. doi:10.3389/fncel.2020.00019

Vossel, K. A., Tartaglia, M. C., Nygaard, H. B., Zeman, A. Z., & Miller, B. L. (2017). Epileptic activity in Alzheimer's disease: Causes and clinical relevance. *Lancet Neurology, 16*(4), 311-322. doi:10.1016/S1474-4422(17)30044-3

Wong, M. (2013). A critical review of mTOR inhibitors and epilepsy: from basic science to clinical trials. *Expert Review of Neurotherapeutics, 13*(6), 657-669. doi:10.1586/ern.13.48

Wyatt-Johnson, S. K., & Brewster, A. L. (2020). Emerging roles for microglial phagocytic signaling in epilepsy. *Epilepsy Current, 20*(1), 33-38. doi:10.1177/1535759719890336

Wyatt, S. K., Witt, T., Barbaro, N. M., Cohen-Gadol, A. A., & Brewster, A. L. (2017). Enhanced classical complement pathway activation and altered phagocytosis signaling molecules in human epilepsy. *Experimental Neurology, 295*, 184-193. doi:10.1016/j.expneurol.2017.06.009

Xue-Ping, W., Hai-Jiao, W., Li-Na, Z., Xu, D., & Ling, L. (2019). Risk factors for drug-resistant epilepsy: A systematic review and meta-analysis. *Medicine (Baltimore), 98*(30), e16402. doi:10.1097/MD.0000000000016402

Zeng, L. H., Rensing, N. R., & Wong, M. (2009). The mammalian target of rapamycin signaling pathway mediates epileptogenesis in a model of temporal lobe epilepsy. *Journal of Neuroscience, 29*(21), 6964-6972. doi:29/21/6964 [pii]. 10.1523/JNEUROSCI.0066-09.2009

Zimmer, T. S., Broekaart, D. W. M., Gruber, V. E., van Vliet, E. A., Muhlebner, A., & Aronica, E. (2020). Tuberous sclerosis complex as disease model for investigating mTOR-related gliopathy during epileptogenesis. *Frontiers in Neurology, 11*, 1028. doi:10.3389/fneur.2020.01028