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ORIGINAL RESEARCH

Activation of the Reelin/GSK-3β/p-Tau Signaling Pathway in the Hippocampus of Patients with Temporal Lobe Epilepsy

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Purpose: In this study, we evaluated the presence of tau deposition and protein expression of Reelin/GSK-3β/p-Tau signaling pathway in the hippocampus of patients with temporal lobe epilepsy (TLE).

Methods: A total of 37 cases of TLE with and without hippocampal sclerosis (HS) were selected histopathologically for our study with 5 autopsy cases as the control group. Immunohistochemistry and the histelide assay, a novel technique quantifying antigens in paraffin section, were used to confirm the distribution of protein within Reelin/GSK-3β/p-Tau signaling pathway and validate the expression of GSK-3β and AT8 (hyperphosphorylated tau) in this study.

Results: Immunohistochemical staining for AT8 revealed punctate and filamentous positive expression in the CA1, CA2 and CA3 regions of the hippocampus with TLE under the ependyma, distributed in a band-like pattern. By contrast, the control group did not exhibit any immunopositivity. GSK-3β was strongly positive in the neuronal bodies, apical dendrites and axons in both groups of TLE, while all controls were negative. In addition, there was no significant difference in the immunohistochemical labelling of Reelin among all cases. The histelide assay indicated that the amounts of AT8 and GSK-3β were significantly increased in the two TLE groups ($P < 0.05$). Notably, there was a positive correlation between AT8 and GSK-3β in TLE without HS ($P < 0.05$).

Conclusion: The present data indicates that phosphorylated tau protein and GSK-3β are activated in the hippocampus of patients with TLE, and this is the first study to examine relevant proteins with the histelide assay in paraffin samples of human tissue. We consider that the regulatory network of tau protein between the two groups may be similar but not identical.

Significance: This study emphasized the Reelin/GSK-3β/p-Tau signaling pathway in TLE with a quantitative data of human tissues innovatively, revealing inspiration for mechanism exploration.

Keywords: epilepsy, temporal lobe epilepsy, hippocampal sclerosis, Reelin, GSK-3β, p-Tau

Introduction

Acquired epilepsy is one of the most common chronic neurological diseases, affecting approximately 50 million people worldwide.[1] Temporal Lobe Epilepsy (TLE), is the most common adult type of focal epilepsy, and about one-third of patients will develop into refractory epilepsy.

Hippocampal sclerosis (HS) is the most frequent histopathology in TLE patients. Usually, the formation of HS begins in childhood or adolescence, mostly due to febrile convulsion, trauma or infection.[2] ILAE classifies three types in anatomically well-preserved hippocampal specimens. HS type 1 refers always to severe neuronal cell loss and gliosis predominantly in CA1 and CA4 regions, compared to CA1 predominant neuronal cell loss and gliosis (HS type 2), or CA4

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predominant neuronal cell loss and gliosis (HS type 3).[3] HS may be the cause of epilepsy or the result of epilepsy. The relationship between the two is a focus of clinical discussion, but the specific mechanism of its occurrence remains unclear.

The MRI features of HS include reduced hippocampal volume, increased signal intensity on T2-weighted imaging, and disturbed internal architecture. Although the imaging changes are very similar to HS, approximately 20% of TLE cases do not show significant neuron loss with only reactive gliosis. As a consequence, this group was designated as "no hippocampal sclerosis with gliosis only (no-HS)"[3]. It was reported that patients with TLE no-HS tended to show worsen seizure outcome that those with HS type 1 and 2,[4] while despite many studies associating TLE with HS, there are few studies on no-HS. International consensus indicated that isolated gliosis potentially mediated through excitation and inflammation[3] and a recent study of a high-resolution single-cell transcriptomic atlas indicated a notable activation of inflammatory pathways in the TLE hippocampus.[5] Therefore, more researches are encouraged to focus on the mechanism of TLE no-HS.

Tau, a microtubule-associated protein, is a natively unfolded protein in human brains and has several roles including microtubule assembly and stabilization[6] and is a defined pathological feature of neurodegenerative diseases like Alzheimer's disease.[7] It was also reported that tau deposits would accelerate the cognitive decline of TLE,[7] and conditional depletion of tau in excitatory neurons or reduction of tau would reduce epilepsy.[8,9] The phosphorylation of tau is the prerequisite for its aggregation and toxicity, regulated by several certain protein kinases and phosphatases, among which glycogen synthase kinase 3beta(GSK-3β) is predominant kinase under physiological and pathological conditions.[10] It was suggested that hyperphosphorylated tau might play a potential mechanistic role in TLE.[11] A cohort study further proved that hyperphosphorylated tau was significantly higher in mesial TLE hippocampus than controls.[12] Therefore, there may be a potential correlation between phosphorylation of tau and epileptogenesis of TLE. In case of Reelin deficiency, the normal suppression of GSK-3β activity is deficient, so that GSK-3β is activated to increase tau phosphorylation.[13] It was reported that the Reelin/GSK-3β/p-tau signaling pathway is essential for the correct positioning of human hippocampal neurons, and therefore, may be involved in the pathological changes associated with epilepsy.

We wonder whether there is a connection between aggressive phosphorylation of tau and exists of HS in clinical practice for the pathology of TLE. To overcome the challenges of antigen quantification in formalin-fixed tissue, Nadia et al developed a new technique called histelide, based on immunohistochemistry (HIST-) and enzyme linked immunosorbent assay (-EL-) performed on a glass slide (-IDE).[14] Therefore, we can use histelide to validate protein expression and phosphorylation in sections of the hippocampus.

In this study, we aimed to assess the expression and significance of proteins in the Reelin/GSK-3β/p-Tau signaling pathway in the hippocampus of TLE patients using immunohistochemistry and the histelide assay with the hypothesis that the Reelin/GSK-3β/T-tau signaling pathway would mediate the pathogenesis of epilepsy in hippocampus of TLE patients.

Materials and Methods

Case Selection

A total of 37 cases of TLE was collected from the Department of Pathology, Xuanwu Hospital, Capital Medical University, from 2019 to 2020. All cases had a primary clinical diagnosis of TLE with history of chronic, drug-refractory disease. The 37 cases were divided into group A and group B according to the presence or absence of HS based on the histological findings.[3,15]

Clinical Data

A total of 22 cases of TLE patients with HS were classified into group A, including 9 males and 13 females, aged between 10 and 37 years old at the time of surgery, with disease duration of 3–30 years. A total of 15 cases of TLE patients without HS were classified into group B, including 9 males and 6 females, aged between 1 and 37 years old at the time of surgery, with disease duration of 1–28 years. We also selected 5 autopsy cases with nonepileptic disease as the control group. The age of the controls was 19–48 years old. Demographic data and the clinical history of each case were retrieved from clinical notes. More detailed information is summarized in Table 1.

Table 1 Clinical Presentation of TLE Patients With HS (Group A) and Without HS (Group B)

Group A								
Case	**Sex**	**Age**	**DE**	**History**	**CD**	**Side**	**FCD**	**HS Type**
1	F	27	23	Encephalitis at age 3	Memory, learning ability↓	L	–	1
2	F	29	8	–		L	–	2
3	F	25	13	Coma caused by brain injury at age 3	Memory↓	R	IIIa	1
4	F	26	20	–	–	R	IIIa	1
5	F	24	3	Grandfather had a history of epilepsy	–	L	–	3
6	M	37	30	Dystocia at birth	–	R	–	1
7	F	24	6	–	–	L	IIIa	1
8	M	21	18	Encephalitis at age 1	–	L	–	1
9	M	22	11	Dystocia at birth	–	R	–	1
10	M	31	8	Onset of Intracerebral hematoma after surgery	Memory↓	L	–	1
11	F	20	17	Febrile convulsions in childhood	–	R	IIIa	1
12	F	25	7	–	–	L	IIIa	1
13	M	28	17	Encephalitis at age 3	–	R	IIIa	1
14	F	15	10	–	–	L	IIIa	1
15	M	32	23	–	Memory, calculation↓	L	–	1
16	F	19	3	–	–	R	–	1
17	M	10	9	Cavernous hemangioma was removed at age 6	–	R	–	1
18	F	34	14	–	–	R	IIIa	2
19	M	31	18	–	–	R	IIIa	1
20	M	30	15	–	–	R	–	1
21	F	22	10	–	–	R	IIIa	1
22	F	17	4	–	–	R	–	1
Group B								
23	M	36	20	Encephalitis at age 5	–	R	Ia	–
24	F	1	1	–	Intelligence↓	R	Ia	–
25	M	34	7	–	–	R	–	–
26	F	24	18	Encephalitis at age 6	–	R	–	–
27	M	16	4	–	–	L	–	–
28	F	35	20	History of high fever at age 2	Memory, calculation↓	R	Ia	–
29	M	24	2	–	–	L	–	–
30	M	16	6	–	Memory↓	R	–	–
31	M	36	24	–	–	L	–	–
32	F	37	28	–	–	R	Ia	–

(*Continued*)

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Table 1 (Continued).

Case	Sex	Age	DE	History	CD	Side	FCD	HS Type
33	F	22	4	–	Bipolar disorder, memory↓	R	–	–
34	M	25	10	History of high fever at age 2	Memory, calculation↓	L	–	–
35	F	26	11	Encephalitis at age 10	–	L	Ib	–
36	M	28	10	–	–	L	Ib	–
37	M	17	9	–	–	R	–	–

Abbreviations: Age, Age at the time of surgery; DE, Duration of epilepsy; History, Clinical history; CD, Cognitive decline; FCD, Focal cortical dysplasia; F, Female; M, Male; L, Left; R, Right; –, No clinical history/diagnosis.

Immunohistochemistry

Paraffin-embedded sections were collected post-surgically, including hippocampus specimens from group A, group B and the control group. Following the routine immunohistochemical procedure, the following antibodies and dilution are used in the 4-μm tissue sections: Reelin (1:6000, Millipore, Clone:142), GSK-3β (1:400, OriGene, Clone:3D10) and AT8 (hyperphosphorylated tau, Ser202 and Thr205, 1:4000, Thermo, Clone: AT8). As a detection system, the EnVision FLEX+ kit (Dako) was utilized and diaminobenzidine as chromogen, executed either with an autostainer (Dako) or via cover plates (Thermo Fisher Scientific glass cover plates). The paraffin sections were counterstained with hematoxylin.

Histelide Method

Hippocampus sections of 10 cases from group A and group B, respectively, and 5 cases from group controls were used in the histelide assay.[14] 4-μm thick paraffin-embedded sections were deparaffinized and rehydrated according to the immunohistochemical methods, and then incubated with primary antibodies (AT-8 and GSK-3β diluted according to the instructions provided by the manufacturer for immunohistochemistry) for 8 h at room temperature, washed three times for 2 minutes each in blocking solution, and then incubated with goat anti-mouse IgG/Biotin (1:200, Zhongshan Golden Bridge Bio-technology, Beijing) for 2 h at room temperature. After washing three times for 2 minutes each in blocking solution, the slides were incubated with alkaline phosphatase for 2 h at room temperature, followed by washing three times of 2 minutes each in blocking solution. 200 μL of p-nitrophenyl phosphate (pNPP) was added on each section, and incubated for 30 minutes in darkness, after which 100 μL of reaction solution was transferred to a 96-well plate to record the absorbance at 405 nm (A405) by microplate reader. The average absorbance per unit area of tissue was calculated for quantitative analysis.

Statistical Analysis

The area of the paraffin tissue section of the histelide assay was measured using Image Pro Plus 6.0 software, and used to calculate the average absorbance per unit area. Statistical analysis was performed using SPSS 23.0, and Fisher's exact test was used for correlation analysis of counting data. Student's *t*-test was used to assess whether the difference between the mean values of two independent samples was significant. Pearson's test was used to analyze the correlation between two groups of data. For all statistical methods, differences with P-values of <0.05 were considered significant.

Results

Clinical Factors

Table 1 presented a list of clinical manifestation of enrolled patients, including presurgical data and pathological findings. Based on these details, patients were categorized based on with or without HS. The duration of epilepsy and ages indicated no clinical difference between group A and group B. According to ILAE classification system for HS, IHC indicated that in group A, 19 cases (86%) were HS type 1, 2 cases (9%) were HS type 2, and 1 case (5%) was HS type 3, meaning that most patients with TLE-HS tended to have severe neuron loss and obvious gliosis in CA1 and CA4 (Figure 1A and B), while there was no significant loss of hippocampal neurons in group B (Figure 1C and D). It was interesting to notice that all of the selected patients from group

A with dementia symptoms were histopathologically HS type 1. Notably, Neurofibrillary tangles (NFTs), which was made of hyperphosphorylated tau as one of the defining features of Alzheimer disease, were found in the hippocampal tissue of Case 12 from group A (Figure 1E–G). Therefore, a potential similarity between TLE and neurodegenerative diseases in morphology was suggested.

Pathology

Punctate and filamentous positive expression of AT8 was noted across the hippocampus with TLE under the ependyma (Figure 2A–C). We also found AT8 positivity in neurons (Figure 2D–G), mainly concentrated in the CA1 and CA3 regions, and negativity in controls. There were 12 cases (54.5%) in group A with AT8-positive neurons, compared to 2 cases (13.3%) in group B, while there was no clear positive staining in the dentate gyrus of any of the TLE cases (Supporting information Table 1 and Supporting information Table 2). Therefore, tau deposits may have a significant preference in TLE-HS.

GSK-3β was strongly expressed in neuronal bodies, apical dendrites and axons in both groups of epileptic specimens, similar to AT8. Different immunohistochemical findings in dendrites and axons between two groups resulted from the loss of neurons in group A (Figure 2H and I, Supporting information Table 3). However, the expression of GSK-3β was negative in the control specimens (Figure 2J). Compared to the positive expression in neurons from the three groups, there was a significant statistical difference (Supporting information Table 3, $P = 0.001 < 0.05$). IHC also presented that Reelin-

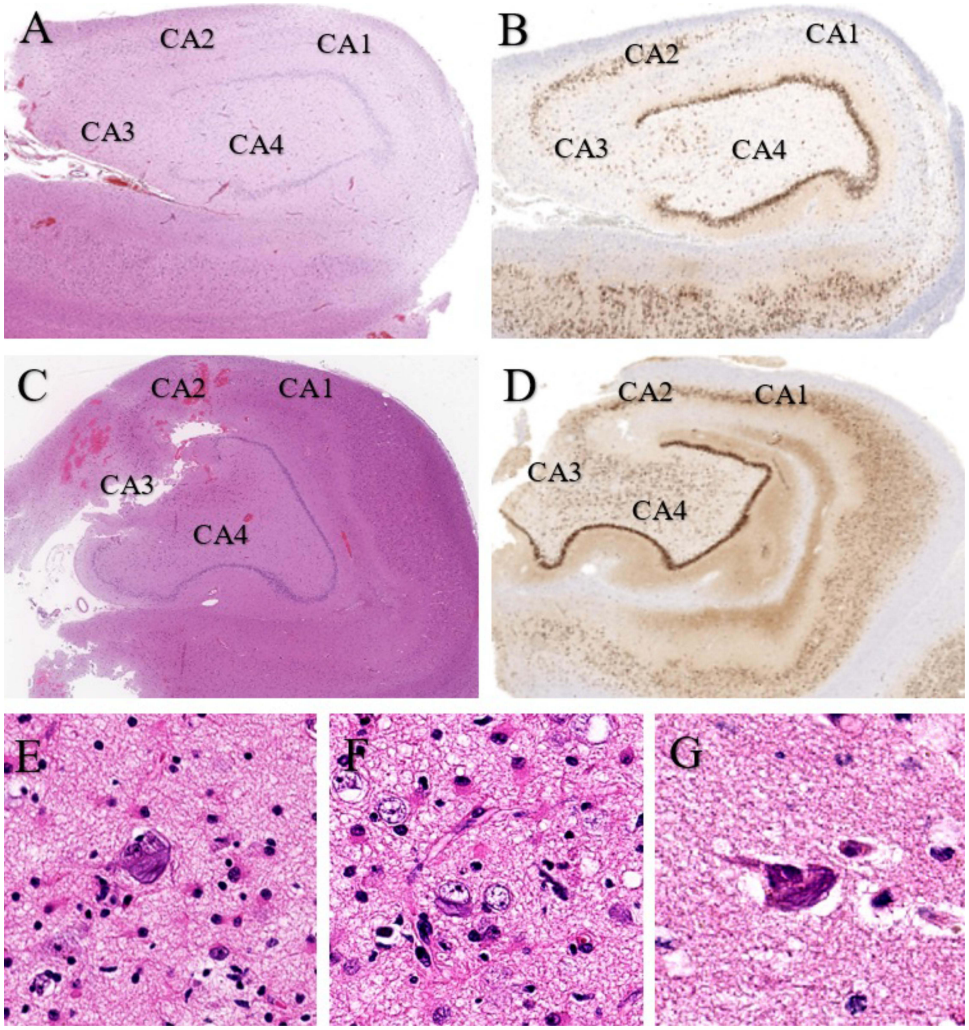

Figure 1 (**A**) HS type 1 involves neuronal loss in the CA1, CA3, and CA4 regions. **(B)** NeuN staining of figure A. (**C**) no neurons were lost in the hippocampus. **(D)** NeuN staining of figure C. (**E–G**) NFTs found in hippocampal tissue in Case 12 from group A.

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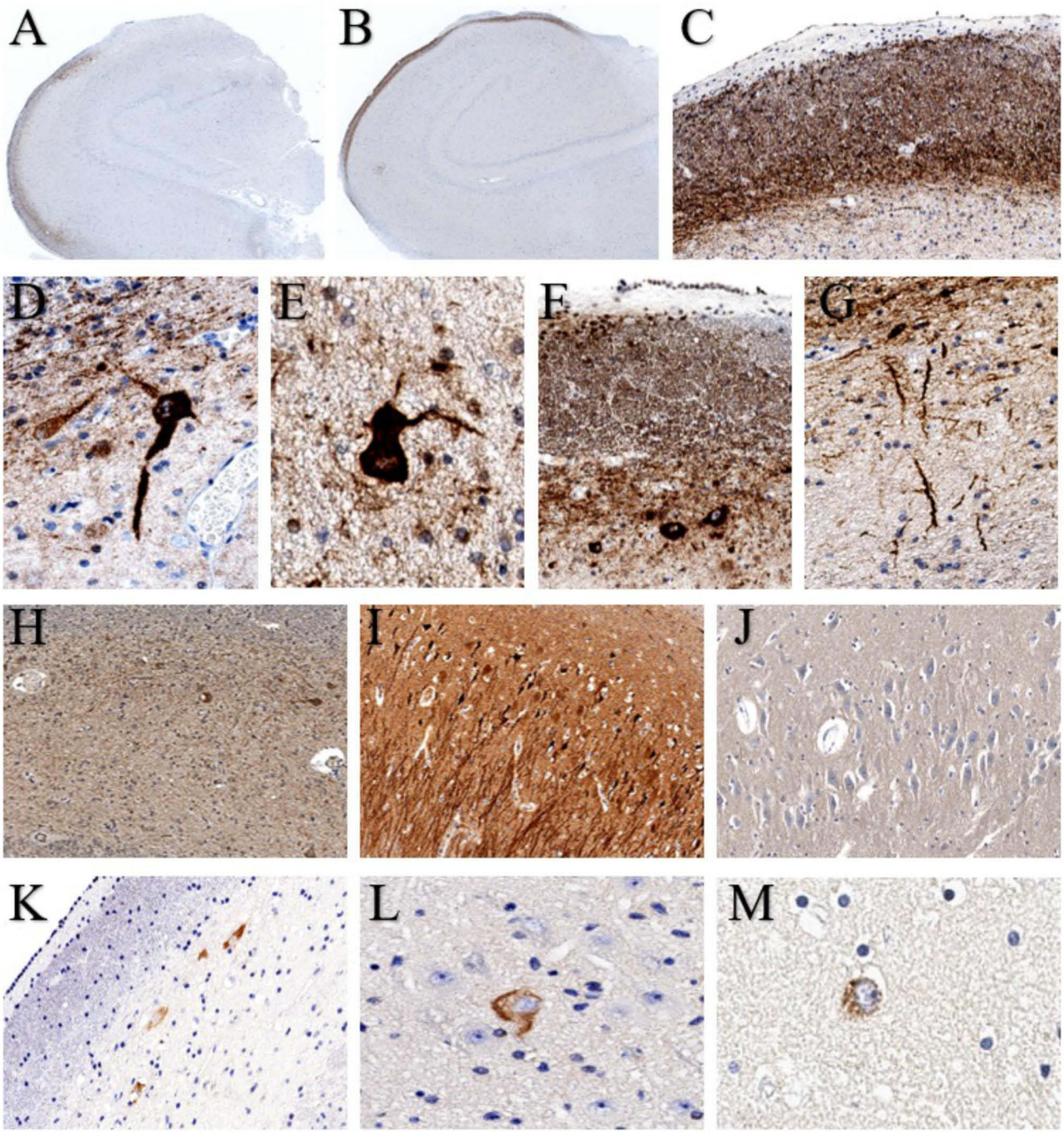

Figure 2 (**A** and **B**) The distribution of AT8 positivity exhibited a band-like pattern in groups A and B under the ependyma. (**C**) punctate and filamentous positive expression was noted in the CA1, CA2 and CA3 regions of the hippocampus of patients with TLE. (**D**–**G**) AT8 in neuronal cell bodies, threads and apical dendrites. (**H**) GSK-3β in CA1 of group A exhibited punctate or filamentous expression due to the loss of neurons. **(I)** GSK-3β in CA1 of group B exhibited complete and strong expression. (**J**) There was no GSK-3β expression in CA1 of the control group. (**K**) Reelin in CA1 of group A. (**L**) Reelin in CA4 of group B. **(M)** Reelin in the dentate gyrus of the control group.

positive neurons mainly distributed in the molecular layer of the dentate gyrus, CA1 and CA4 regions (Figure 2K–M). It was suggested that hyperphosphorylated tau and GSK-3β most deposits in hippocampal neurons in TLE.

Histelide Assay

For a quantitative analysis of protein expression on paraffin section, we introduce histelide assay and apply it within 25 cases for detection, including 10 TLE-HS cases, 10 TLE-NO-HS cases and 5 controls. Phosphorylated tau protein significantly increases in the hippocampus of the two TLE groups compared to the control group ($P < 0.05$; Figure 3A; Table 2 and Table 3).

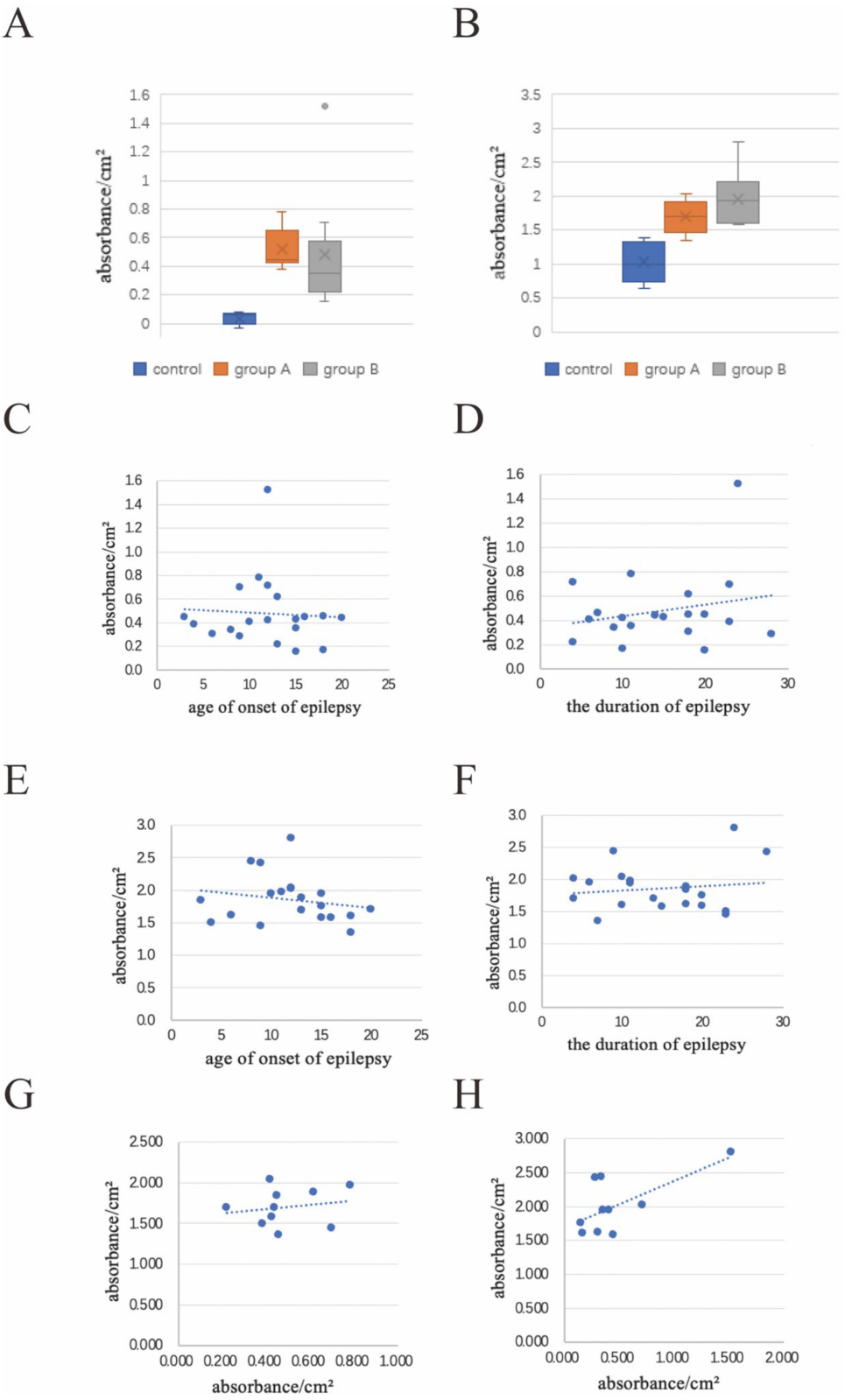

Figure 3 (**A**) Histelide assay of AT8. (**B**) Histelide assay of GSK-3β. Effects of (**C**) age of onset of epilepsy and (**D**) the duration of epilepsy on the value of AT8 in the epilepsy cohort. The correlation coefficient of (**E**) age of onset of epilepsy and (**F**) the duration of epilepsy with the value of GSK-3β. (**G** and **H**) There was no correlation between the values of AT8 and GSK-3β in group A, but there was a positive correlation between AT8 and GSK-3β in group B.

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Table 2 Histelide Assay for AT8 and GSK-3β

Grouping	AT8			GSK-3β		
	Group A	Group B	Control	Group A	Group B	Control
Case number	10	10	5	10	10	5
Mean value	0.488	0.469	0.039	1.699	2.012	1.029
Standard deviation	0.165	0.402	0.043	0.232	0.417	0.301
Standard error	0.052	0.127	0.019	0.073	0.132	0.135

Table 3 Histelide Assay for AT8 and GSK-3β (Absorbance/Cm²)

Grouping	Case	Sex	AT8			GSK-3β		
			Absorbance	cm²	Result	Absorbance	cm²	Result
Group A	1	F	0.421	0.450	0.383	1.134	0.558	1.495
	8	M	0.345	0.321	0.447	0.965	0.406	1.840
	9	M	0.394	0.295	0.783	0.883	0.352	1.972
	12	F	0.310	0.307	0.457	0.779	0.412	1.354
	15	M	0.437	0.350	0.696	1.044	0.527	1.444
	18	F	0.498	0.503	0.437	1.462	0.654	1.698
	19	M	0.354	0.303	0.615	0.987	0.408	1.882
	20	M	0.293	0.299	0.427	0.927	0.439	1.575
	21	F	0.338	0.348	0.418	1.081	0.420	2.037
	22	F	0.264	0.342	0.218	0.931	0.417	1.696
Group B	23	M	0.442	0.417	0.447	1.095	0.517	1.581
	26	F	0.424	0.494	0.305	1.353	0.628	1.617
	27	M	0.271	0.218	0.710	0.738	0.289	2.017
	28	F	0.308	0.437	0.152	0.941	0.411	1.753
	30	M	0.340	0.354	0.407	1.172	0.472	1.946
	31	M	0.544	0.262	1.523	1.092	0.327	2.802
	32	F	0.396	0.473	0.284	1.510	0.510	2.424
	35	F	0.299	0.330	0.353	1.099	0.443	1.944
	36	M	0.257	0.357	0.167	1.074	0.503	1.598
	37	M	0.286	0.320	0.341	1.364	0.458	2.441
Control	-	M	0.331	0.541	0.058	0.808	0.526	0.999
	-	F	0.306	0.525	0.030	0.919	0.665	0.845
	-	F	0.295	0.625	0.079	0.584	0.493	0.648
	-	M	0.254	0.415	0.059	0.864	0.479	1.267
	-	M	0.253	0.485	−0.031	1.015	0.528	1.385

However, we did not observe a significant difference between the two TLE groups ($P = 0.89 > 0.05$). In addition, there was no correlation between the age of onset, duration of epilepsy and value of AT8 in any of the groups ($P = 0.787 > 0.05$ and $P = 0.345 > 0.05$; Figure 3C and D).

Compared to the control group, GSK-3β was significantly increased in the two TLE groups ($P < 0.05$; Figure 3B; Table 2 and Table 3). Same as the results of AT8, there was no significant difference between the two TLE groups ($P = 0.053 > 0.05$), and there was no significant correlation between the age of onset, duration of epilepsy and histelide results of GSK-3β (Figure 3E and F). Notably, there was also no correlation between the values of AT8 and GSK-3β in group A ($P = 0.619 > 0.05$, Figure 3G), but there was a positive correlation between AT8 and GSK-3β in group B ($P = 0.038 < 0.05$, Figure 3H).

Discussion

Pathological phosphorylation of tau leads to its dissociation from microtubules and its aggregation into NFTs, the hallmark of several neurodegenerative cognitive disorders, including Alzheimer's disease.[16] Tau hyperphosphorylation has been previously reported to be increased in brain tissues of patients with drug-refractory TLE.[7,10] Mouse models of TLE confirmed that seizures affect tau hyperphosphorylation.[17] Epilepsy is not classically thought of as a neurodegenerative disease, but patients with refractory TLE have an increased risk of cognitive decline.[18] Among the cases we selected, 4 cases from group A (18.2%) and 5 cases from group B (33.3%) had impaired memory or computation abilities as the main manifestations of cognitive impairment. This was consistent with the idea that hyperphosphorylation of tau may be a potential mechanism contributing to increased states of hyperexcitability and cognitive decline. The hippocampus is a temporal brain structure belonging to the limbic lobe. It is fundamentally involved in memory processing, learning, and emotional processing. We found NFTs in the hippocampal tissue of a single case from group A. This is the most direct evidence of hyperphosphorylation of tau.

The hippocampus consists of two allocortical laminae, the dentate gyrus and the cornu ammonis (CA1, CA2, CA3, and CA4). The cornu ammonis consists of five layers, including the stratum alveolus, stratum oriens, stratum pyramidale, stratum radiatum, and stratum lacunosum-moleculare.[19] The stratum alveolus constitutes the deepest layer of the ventricular surface of the hippocampal cortex, and contains the dendrites of pyramidal neurons and a few scattered smaller neurons. Our immunohistochemical results revealed the presence of hyperphosphorylated tau (AT8) in neuronal cell bodies, neuropil threads and apical dendrites. All TLE cases exhibited a band-like pattern in the hippocampus under the ependyma (Supporting information Table 2). This suggests that phosphorylated tau protein accumulates in the hippocampus whether with or without sclerosis. The positive AT8 band-like pattern was mainly located in the stratum alveolus. Dendrites of pyramidal neurons seem to be the first to become affected by tau hyperphosphorylation, and special structures and components of the stratum alveolus may be responsible for this phenomenon.

The stratum pyramidale contains the principal excitatory neurons and a few interneurons. Excessive phosphorylation of tau protein in neurons may eventually lead to neuronal death.[20] Accordingly, the accumulation of hyperphosphorylated tau protein in neurons may be associated with HS. Both groups A and B had positive neurons in our study, but there was no obvious neuronal death in the hippocampus of group B. The relationship between the two is worth studying in the future. Patients with no-HS present clinically with epilepsy that lasts for many years, while no timeframe is available for no-HS and HS. In addition, different HS types have different pathological manifestations and surgical prognosis.[3,21] We therefore consider that the regulatory network of tau protein in the two groups may be similar but not identical.

Previous studies have demonstrated that GSK-3β affects axoplasmic transport and axonal growth through the phosphorylation of tau protein.[22] Huang et al found that the mRNA and protein expression of GSK-3β, as well as its activity, increased significantly with recurrent seizures in epileptic mice.[3] The histelide assay showed that the abundance of phosphorylated tau protein and GSK-3β was significantly increased in the two TLE groups. There was no correlation between the values of AT8 and GSK-3β in group A, but there was a positive correlation in group B. We therefore confirm and extend previous findings that tau pathology and GSK-3β are activated in patients with TLE, which may establish a key role in neuronal damage and development of epilepsy. However, without timeframes of the dynamic pathophysiological progression of surgical specimens, we cannot infer the disparity between the two groups. The underlying mechanism should be researched further.

Reelin is a key factor that regulates neuronal migration during cerebral development.[23] Its expression is crucial in the embryonic marginal zone, because mice with spontaneous RELN mutations develop an inverted cortex in which early neurons migrate to the superficial cortical plate instead of stopping at deeper layers.[24] Prolonged seizures decreased Reelin immunoreactivity in the adult rat dentate gyrus. Loss of Reelin expression in the epileptic hippocampus likely contributes to ectopic chain migration and aberrant integration of new dentate granule cells.[25] All cases in this study had Reelin positive neurons, without significant differences of expression among the three groups according to immunohistochemistry. It may be necessary to develop a more sensitive assay that can detect minor changes of Reelin expression.

Notably, we used the histelide method to measure phosphorylated tau and GSK-3β in the hippocampus for the first time. The experimental procedure is similar to immunohistochemical staining, which is practical and has broad application prospects. It was a novel method making it possible to obtain reliable data from formalin-fixed paraffin-embedded tissue,[14] what traditional methods fail to achieve. We also acknowledge that our study has limitations, as we have examined only hippocampal tissues resected during surgery. Quantitative analysis of Reelin was not performed. Our studies left an inspiration of TLE potential mechanism, while we did not relate significant changes of the Reelin/GSK-3β/p-Tau signaling pathway to TLE progression, given that molecular mechanism required further patient information. In summary, we show a pathological pattern of the Reelin/GSK-3β/p-Tau signaling pathway, which was activated in TLE. Further studies are needed to elucidate the exact mechanism underlying tau pathology, which may lead to new approaches for diagnosing and treating cognitive decline in temporal lobe epilepsy.

Conclusion

The present study indicates that phosphorylated tau protein and GSK-3β are activated in the hippocampus of patients with TLE both with and without sclerosis. Given that activation of GSK-3β might participate in epileptogensis[26] and the accumulation of hyperphosphorylated tau protein in neurons may be an important cause of HS and a promising trigger of epilepsy,[27] we consider that the regulatory network of tau protein between the two groups may be similar but not identical. The underlying mechanism should be researched further.

Ethics Declarations

All procedures performed in studies received approval by the Ethics Committee of Xuanwu Hospital, Capital Medical University (approval number [2021]068) in accordance with the Declaration of Helsinki. Informed consents have been waived by the Ethics Committee given that it was a retrospective study with no intervention of patients and the tissues used in the study were obtained from previous surgical sections in accordance to procedures.

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Disclosure

All authors report no conflicts of interest in this work.

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