Original Research

Hippocampal neuron loss and astrogliosis in medial temporal lobe epileptic patients with mental disorders

Jun Lu[1,*], Hongxing Huang[1], Qichang Zeng[1], Xinmei Zhang[2], Min Xu[3], Yi Cai[4], Qin Wang[1], Yahui Huang[1], Qiong Peng[5] and Lanqiuzi Deng[1]

[1] *Department of Neurosurgery, Hunan Provincial Brain Hospital, 410007, P. R. China*

[2] *Department of Neurology, Central South University, 410000, P. R. China*

[3] *Department of Pathology, Hunan Provincial Brain Hospital, 410007, P. R. China*

[4] *Department of Psychiatry, Hunan Provincial Brain Hospital, 410007, P. R. China*

[5] *Department of Neurology, Hunan Provincial Brain Hospital, 410007, P. R. China*

*Correspondence: lujundsa@163.com (Jun Lu)

Hippocampal neuron loss and reactive astrogliosis are pathological features of medial temporal lobe epilepsy. Here, the expression of hippocampal astrogliosis-associated genes are studied in subjects with medial temporal lobe epilepsy and mental disorders (such as depression, anxiety and psychiatric comorbidities). The relationship between functional changes in hippocampus astrocytes and concurrent mental disorders are discussed. Nissl staining identified medial temporal lobe epilepsy-induced neuronal loss in the CA1 region of hippocampus. Quantitative real-time polymerase chain reaction and immunofluorescence technology were used to detect hippocampus glial fibrillary acidic protein, metallothionein, and aquaporin-4. The hippocampus area of subjects with medial temporal lobe epilepsy (with or without mental disorders) were smaller than the control group. Hippocampal neuronal loss and astrogliosis were more obvious in groups of medial temporal lobe epileptic patients with mental disorders. Relative protein levels of glial fibrillary acidic protein, metallothionein-I/II, and aquaporin-4 were significantly higher in subjects with medial temporal lobe epilepsy than seen in controls. Medial temporal lobe epileptic patients with mental disorder or depression had elevated metallothionein-I/II protein level when compared to controls and medial temporal lobe epileptic patients without mental disorder. Protein levels of glial fibrillary acidic protein and aquaporin-4 in medial temporal lobe epileptic patients with mental disorders were significantly lower than that in medial temporal lobe epileptic patients with no mental disorder. It is concluded that functional changes in hippocampus astrocytes are associated with mental disorders in medial temporal lobe epileptic patients and the astrogliosis-related genes of glial fibrillary acidic protein, metallothionein-I/II and aquaporin-4, are involved in this process.

Keywords

1. Introduction

Epilepsy is a common neurological disorder that affects approximately 4%–8% of the population in developed countries (Bell and Sander, 2001; Jallon, 2002). It also affects five million people in China and has profound impacts on the daily life of patients (Zhou et al., 2007). About 70% of patients could benefit from antiepileptic drug (AED) therapy, while another third of patients have poor seizure control (Zhou et al., 2007). This treatment-resistant epilepsy is called temporal lobe epilepsy (TLE), where medial temporal lobe epilepsy (MTLE) is the most common type (Engel, 1996). MTLE is characterized by hippocampal sclerosis, neuronal loss, reorganization of synapses, reactive astrogliosis, and neuroinflammation (Falconer et al., 1964; Tellez-Zenteno et al., 2007). Gilal cell activation and proliferation is a pathological feature of MTLE hippocampal sclerosis. Astrocytes have been regarded as a key component of the tripartite synapse(Araque et al., 1999), which has a crucial role in the formation of synaptic networks, energy metabolism, and neurotransmitter homeostasis (Tian et al., 2005). An inflammatory response induces striking changes in cell mor-phology and function. Glial fibrillary acidic protein (GFAP) is a member of the intermediate filament structural protein family that is predominantly expressed in astrocytes. As a marker for astrogliosis, the level of GFAP is often measured and linked to activation of astrocytes and epilepsy pathogenesis (Hubbard et al., 2013). Metallothioneins (MTs) are a class of cystein-enriched pro-teins of low molecular weight, which can bind zinc and cadmium in cells (Kille et al., 1994). MTs are endogenous expressed in vari-ous tissues with four isoforms. Isoforms I, II and III are expressed in the central nervous system, of which MT-I/II are mainly ex-pressed in astrocytes (Wiese et al., 2006). Studies have shown that in pathological conditions, especially in patients with TLE, MT-I/II expression levels are increased (Peixoto-Santos et al., 2012), and

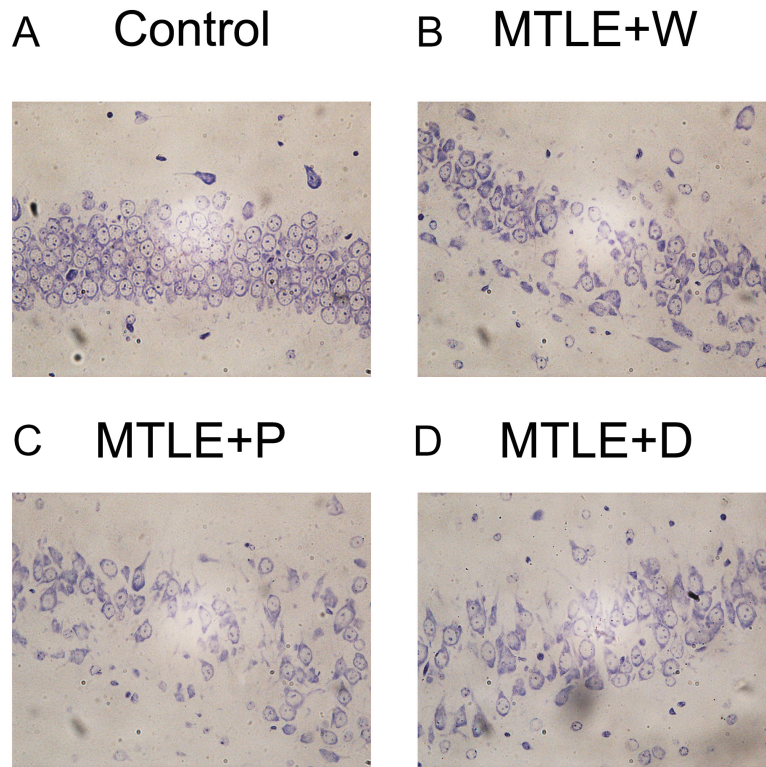*Journal of Integrative Neuroscience*

Figure 1. Hippocampus neuron loss in subjects with MTLE. Nissl staining identifies MTLE-induced neuronal loss in the CA1 region of hippocampus. (A) Control; (B) MTLE without mental disorder (MTLE + W); (C) MTLE with mental disorder (MTLE + P); (D) MTLE with depression (MTLE + D). Representative photomicrographs of Nissl staining (× 400) for each group.
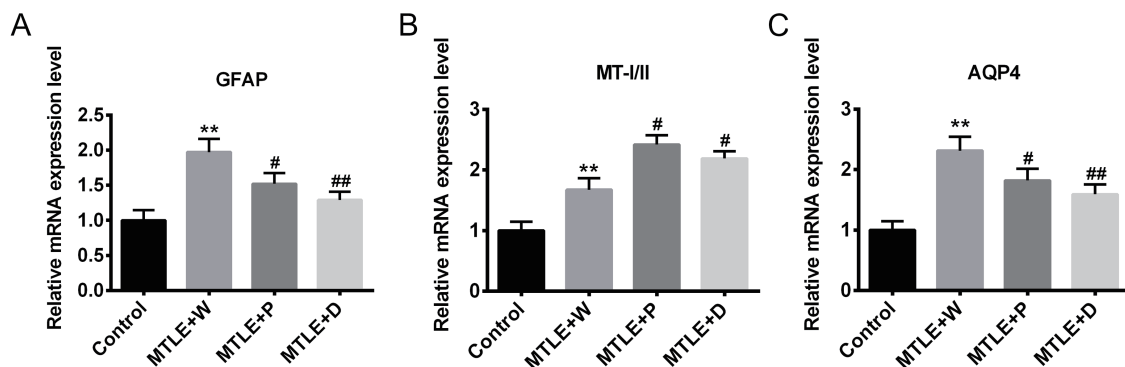

Figure 2. Relative hippocampal mRNA expression levels of GFAP, MT-I/II, and AQP4 elevated in patients with MTLE. Relative mRNA expression levels of (A) GFAP, (B) MT-I/II, and (C) AQP4, mean ± standard deviation (SD). $^*P < 0.05$, $^{**}P < 0.01$ compared with control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with MTLE + W. $F_{(3,8)} = 21.568$, $F_{(3,8)} = 51.175$, and $F_{(3,8)} = 24.893$, Fig. 2A-C, respectively.

elevated levels of MT-I/II are associated with reduced inflammatory response as well as neuronal death (Penkowa et al., 2005). Aquaporins (AQPs) are a series of small, intrinsic membrane proteins that function as water-selective channels in response to osmotic gradients and have an important role in water homeostasis (Binder et al., 2012). AQP4 is the predominant aquaporin found in the brain and is expressed in the cell membrane of astrocytes (Nagelhus et al., 2004). Previous studies have revealed that in mouse models of epilepsy AQP4 deficiency could cause increased

seizure duration (Binder and Steinhauser, 2006), indicating that it may participate in epileptogenesis (Binder et al., 2004). However, the alternative expression and regulation of AQP4 in epileptogenesis is not fully understood.

Epilepsy patients, including those with MTLE, experience many psychosocial problems, including depression, anxiety, and low self-esteem (Schiffer and Babigian, 1984; Tellez-Zenteno et al., 2007). Ertem et al. (2017) detected comorbid psychiatric disorders in 57% of patients with MTLE. Similarly, Nogueira et al.
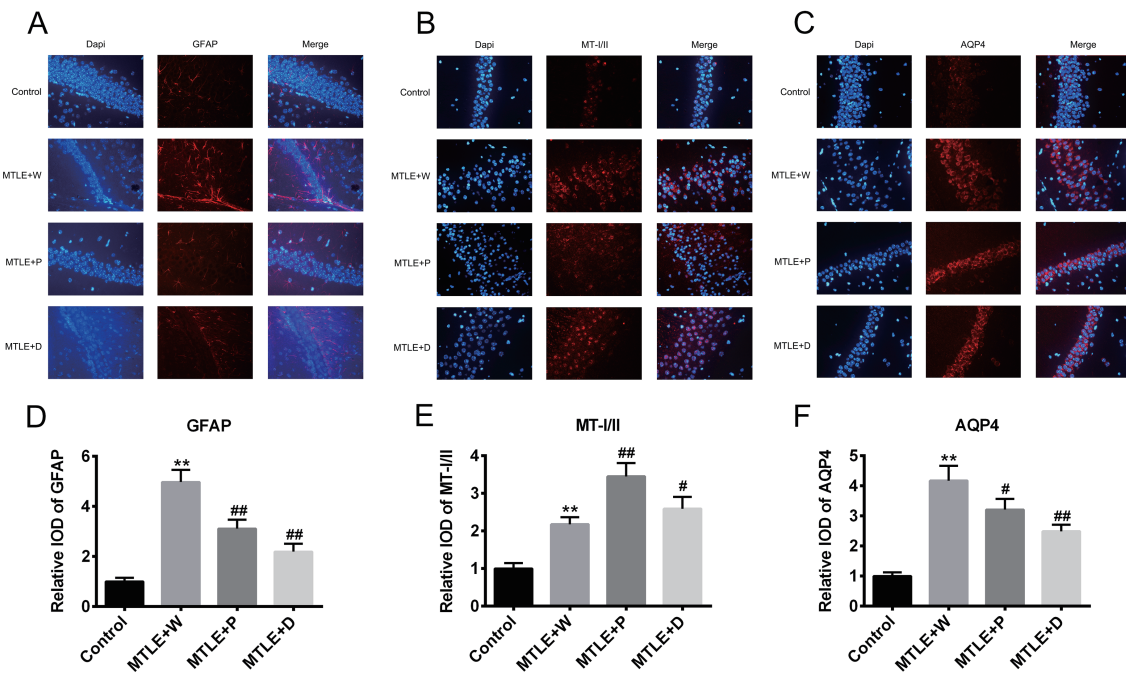
Figure 3. Elevated hippocampal GFAP, MT-I/II, and AQP4 level in subjects with MTLE. (A–C) Immunofluorescence of GFAP, MT-I/II, and AQP4 in hippocampal samples. (D–F) Integrated optic density (IOD) was analyzed for each section of A–C, mean $\pm$ standard deviation (SD). $^*P < 0.05$, $^{**}P < 0.01$ compared with control; $^\#P < 0.05$, $^{\#\#}P < 0.01$ compared with MTLE + W. $F_{(3,8)} = 69.032$, $F_{(3,8)} = 44.623$, and $F_{(3,8)} = 50.745$ for Fig. 3D-F, respectively.
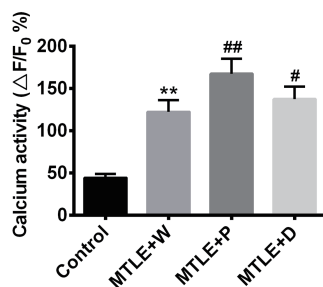

Figure 4. Elevated intracellular calcium signals of hippocampal astrocytes in MTLE subjects. Fluorescence calcium signals ($\triangle F/F_0$) from control, MTLE without mental disorder (MTLE + W), MTLE with mental disorder (MTLE + P), and MTLE with depression (MTLE + D) are shown. Data presented as the mean $\pm$ standard deviation (SD). $^*P < 0.05$, $^{**}P < 0.01$ compared with control; $^\#P < 0.05$, $^{\#\#}P < 0.01$ compared with MTLE + W $F_{(3,8)} = 44.108$.

(2017) found that 39.6% of MTLE patients met diagnostic criteria for mood disorders and 34.7% of MTLE patients met diagnostic criteria for anxiety disorders. Mental disorders can change with seizure recency and frequency (Thapar et al., 2009), indicating that psychopathological states in MTLE patients may relate to morphological alterations and functional changes in hippocampus. However, the relationship between epilepsy and mental disorders are complex and remain obscure.

Since GFAP, MT-I/II and AQP4 are suggested to be associated with astrogliosis, it was hypothesized that levels of these proteins may be altered in hippocampus of MTLE patients with mental disorders. Kandratavicius et al. (2015) has indicated the possible change of GFAP, MT-I/II and AQP4 in MTLE patients. In this study, GFAP, MT-I/II and AQP4 expression levels were examined by quantitative real-time polymerase chain reaction (RT-PCR) and immunofluorescence analysis in hippocampus of MTLE patients with or without mental disorders to further assess the potential relationship between hippocampal astrogliosis and disease pathogenesis. Whole-cell recording was also employed to measure intracellular calcium signals in hippocampal astrocytes.

2. Methods

2.1 Subjects and clinical data

Patients diagnosed with MTLE were recruited at our institution from January 2015 to June 2017. For each subject data was collected on epilepsy history, including antiepileptic treatment, dosage of drugs, age of disease onset, frequency of seizure, family history of epilepsy, history of brain injury (e.g. hyperpyretic convulsion, encephalitis, hypoxic ischemic encephalopathy, trauma), type of seizure and disease duration. Healthy controls are those who did not have a history of psychiatric disorders and five healthy controls were recruited in this study.

MTLE subjects met the following criteria: 1) more than four seizures per month, with poor seizure control after antiepileptic drug therapy; 2) no obvious cause of seizure and normal physical and neurological examinations; 3) according to MRI scanning no other pathological changes except hippocampal sclerosis; 4) epileptic seizure met the definitions proposed by the International League against Epilepsy (Salas-Puig, 2011); 5) patients un-

derwent several EEG recording and video-EEG assessments before surgery. Informed consent was given by subjects and their families before surgery. The study was approved by the Ethics Committee of Hunan Brain Hospital, Changsha, China. All subjects, including healthy controls and MTLE patients underwent a surgical procedure for hippocampal resection.

MTLE subjects were divided in three groups: 1) MTLE without mental disorders (MTLE + W, $N = 10$); 2) MTLE with mental disorders (MTLE + P, $N = 10$); 3) MTLE with depression (MTLE + D, $N = 10$). The clinical characteristics of all the participants are summarized in Table 1.

2.2 Immunofluorescence analysis

Hippocampal samples from MTLE patients and controls were repeatedly washed with ice-cold phosphate buffered saline (PBS) and fixed in 4% formaldehyde for over 24 hours. Samples were then cut into 50 μm sections. For immunofluorescence staining, sections were incubated with primary antibodies against GFAP 1 : 500 (Dako, Carpinteira), MT-I/II 1 : 500 (Dako, Carpinteira), and AQP4 1 : 1000 (Santa Cruz Biotechnology). Subsequently, sections were incubated with appropriate secondary antibodies conjugated to fluorophores Alexa Fluor 488 or Alexa Fluor 594 (Life Technologies).

Images of all hippocampal sections were taken under an Olympus microscope. The images were analyzed with image analysis software Image-pro plus 6.0. The overall integrated optic density (IOD) was obtained for each section for analysis.

2.3 Nissl staining

For Nissl staining, the sections were washed twice in PBS then stained in 0.1% cresyl violet staining solution dissolved in 0.01% glacial acetic acid at 37°C for 15 minutes. Following incubation, sections were washed in distilled water, then gradually dehydrated through graded ethanol (70%, 95%, and 100%). Sections were then fitted with cover slips and examined under the light microscope. Sections were imaged with an Olympus microscope and the number of stained cells counted. There were three to five slices per subject, and five different windows per image were chosen and analyed.

2.4 Quantitative real-time polymerase chain reaction (RT-PCR) analysis

Total RNA was harvested from hippocampal samples of MTLE subjects and controls using Trizol LS reagent (Invitrogen, USA). This RNA was then reverse-transcribed to cDNA by PrimeScriptRT reagent Kit with gDNA Eraser (Takara, Japan). mRNA levels for GFAP, MT-I/II, and AQP4 were measured by RT-PCR using SYBR Green PCR Kit (Takara, Japan). Expression levels of mRNAs were normalized to internal control GAPDH, and the $2^{-\triangle\triangle CT}$ method was used to calculate fold changes of mRNAs.

2.5 Statistical analysis

Data were presented as the mean $\pm$ standard deviation (SD). Statistical analysis was performed by SPSS software (version 17.0) and graphical presentations prepared using GraphPad Prism 5.0. Student's t-test and one-way analysis of variance were used to analyze paired and multiple groups, respectively. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Hippocampal neuronal loss in subjects with MTLE

Nissl staining was used to identify the extent of neuronal damage in the hippocampus CA1 region of controls and subjects with MTLE. In the control group, the Nissl-stained sections showed three to four layers of cells in area CA1. Cells were round or oval, had regularly shaped cell bodies, size, and clear cell boundaries (Fig. 1A). By contrast, neurons were severely degenerated, and morphological abnormalities were observed in the groups of MTLE without mental disorders (MTLE + W), MTLE with mental disorders (MTLE + P), and MTLE with depression (MTLE + D) (Fig. 1B-D). The number of surviving neurons was lower in the MTLE groups when compared to the control group.

3.2 Up-regulation of hippocampal GFAP, MT-I/II, and AQP4 level in subjects with MTLE

Relative expression levels of GFAP, MT-I/II, and AQP4 in hippocampus were analyzed from samples of MTLE subjects and controls using RT-PCR. Fig. 2 shows relative mRNA expression levels of GFAP, MT-I/II, and AQP4 were significantly upregulated in three groups of MTLE patients when compared with the control group. Notably, the MTLE + P and MTLE + D groups had lower GFAP and AQP4 level compared with the MTLE + W group ($^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, respectively, Fig. 2A, C), while the level of MT-I/II was much higher ($^{\#}P < 0.05$ compared with MTLE + W group, Fig. 2B).

Protein levels of these genes were then examined using immunofluorescence. The three groups of MTLE subjects displayed higher signals of GFAP, MT-I/II, and AQP4 in the CA1 region of hippocampus than the control group (Fig. 3). The MTLE + P and MTLE + D groups had lower signals for GFAP and AQP4 when compared with the MTLE + W group ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$, Fig. 3A, C, D, F). Immunofluorescence signals for the MT-I/II protein of MTLE + P and MTLE + D groups were higher than the control and MTLE + W groups ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$, Fig. 3B, E). The protein levels of these genes were consistent with the mRNA levels in hippocampal samples. These results indicated the abnormal expression of astrogliosis-related genes in patients with MTLE.

3.3 Elevated intracellular calcium signals of hippocampal astrocytes in MTLE subjects

Whole-cell recordings were obtained from the CA1 region of hippocampal brain slices of MTLE subjects and controls. The fluorescence calcium signals ($\triangle F/F_0$) from different groups are shown in Fig. 4. A significant increase in intracellular calcium signal was observed in groups of MTLE patients compared with the control group (Fig. 4). Meanwhile, the MTLE + P and MTLE + D groups displayed enhanced intracellular calcium when compared with the MTLE + W group ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$, Fig. 4), indicating that MTLE patients had abnormal functions in hippocampal astrocytes.

4. Discussion

Hippocampal sclerosis is a typical pathological symptom of temporal lobe epilepsy. Epilepsy patients with mental disorders often have disorders of consciousness, psychosis, and amnesia syndrome (Verrotti et al., 2014). Patients with epilepsy can also experience depression and attention deficits. Currently, it is generally believed that elevated levels of glutamate and its recep-

Table 1. Clinical characteristics of MTLE patients

Groups	MTLE + W N = 10	MTLE + P N = 10	MTLE + D N = 10
Gender			
Male	7 (70)	4 (40)	6 (60)
Female	3 (30)	6 (60)	4 (40)
Family history of epilepsy			
Yes	8 (80)	7 (70)	8 (80)
No	2 (20)	3 (30)	2 (20)
Age at epilepsy onset	45.7 ± 4.6	44.4 ± 7.2	46.4 ± 8.6
Disease duration (years)	15.6 ± 3.5	16.7 ± 1.2	18.5 ± 3.6
Monthly seizures (number)	4.1 ± 0.6	3.87 ± 0.02	4.02 ± 0.42

Data displayed as mean $\pm$ SD or mean number (N %).

tors in the brain are associated with neural impairment in patients with epilepsy (Devinsky et al., 2013; Binder and Carson, 2013). Astrocytes play supportive roles in the brain and the balance between glutamate and glutamine has an important impact on astrocytes. Abnormal astrocyte function may lead to alterations in both γ-aminobutyric acid uptake and K^+/Ca^{2+} signaling pathways, which in turn might contribute to increased neuronal excitability and bursts of inflammatory factors (Hubbard et al., 2013; Binder and Carson, 2013). Astrocytosis and neuroinflammation are thought to be associated with the onset of mental disorders. Damage to hippocampal neurons and astrocytes has been reported for epilepsy patients with mental disorders (Tian et al., 2005; Devinsky et al., 2013). This is consistent with the present work. In this study, CA1 cell loss and reactive astrocytosis was found in MTLE patients with mental disorders, indicating that psychopathological states in epilepsy patients may be related to morphological and functional changes in hippocampus, and temporal lobe epilepsy and mental disorders may have common underlying pathophysiological mechanisms.

GFAP is a intermediate filament structural protein which is uniquely expressed in astrocytes. When neural damage occurs, there is a burst of astrocyte proliferation and GFAP protein expression. In this study, the relative hippocampal GFAP level in MTLE patients is significantly increased when compared with controls ($P < 0.05$). This suggests a reactive hyperplasia of hippocampal astrocytes in MTLE patients. In the early stage of epilepsy, astrocytes proliferate in large numbers and contribute to brain repair. However, sustained astrogliosis can cause scar formations, which in turn attenuates synapse regeneration (Lavialle et al., 2011). In this study, MTLE patients with mental disorders expressed less GFAP than MTLE patients without mental disorders, at both the mRNA and protein level ($P < 0.05$). This may be attributed to attenuated astrogliosis during gradually developing mental disorders.

MT-I/II plays an important role in the protection and repair of oxidative damage in the central nervous system (Penkowa et al., 2005). Increased MT-I/II levels in MTLE patients may be associated with an imbalanced Zn^{2+} homeostasis and astrogliosis in hippocampus. There is evidences that brain MT-I/II levels could be regulated by catecholamines, glucocorticoids, inflammatory factors, and heavy metal ions such as Cu^{2+} and Zn^{2+} (Ugajin et

al., 2015; Jakovac et al., 2013). Results of this study showed that MTLE patients with mental disorders had a higher MT-I/II expression level than found in controls and MTLE patients without mental disorders ($P < 0.05$). Morphologically, the shrinkage of hippocampal neurons was observed in MTLE patients with mental disorders, suggesting that neuronal loss could lead to the compensatory expression of MT-I/II genes.

AQP4 plays a crucial role in the regulation of water and potassium homeostasis in the brain microenvironment, the formation of glial scars, as well as regulating nerve excitability (Binder et al., 2004). Increasing evidence has suggested dysregulation of AQP4 in the pathogenesis of epilepsy (Tian et al., 2005; Binder and Steinhauser, 2006). Previous studies have demonstrated that AQP4 knockout mice have significantly prolonged seizure duration (Lee et al., 2012; Haj-Yasein et al., 2015) and more severe symptoms of depression (Kong et al., 2014). In the current study, it was discovered that the hippocampal expression level of AQP4 in MTLE subjects was significantly higher than that of normal tissue ($P < 0.05$), along with hippocampal neuronal loss and a turbulant calcium signal in astrocytes. Thus, AQP4 may take part in the occurrence and development of epilepsy and mental disorders by regulating astrocyte function.

Kandratavicius et al. (2015) has indicated an increased GFAP immunoreactive area of MTLE + W and MTLE + P compared to MTLE + D patients, which were similar to those of observations reported here and further confirmed by quantitative RT-PCR. According to Kandratavicius et al. (2015), MTLE + W cases showed increased MT-I/II in the hippocampus granule cell layer and CA1 when compared to MTLE + P and in the parasubiculum, when compared to MTLE + D and MTLE + P. Quantitative RT-PCR and immunofluorescence results showed elevated MT-I/II for the MTLE + P and MTLE + D groups when compared to control and the MTLE + W group. The differences may be due to different sample regions. Kandratavicius et al. divided hippocampal samples into granule cell layer, CA1, and CA2 regions, while whole hippocampal samples were used in this study. Along with molecular experiments, functional studies were also applied in the current research. Taken together, results confirm abnormal changes of GFAP, MT-I/II, and AQP4 levels, and abnormal functions of hippocampal astrocytes in MTLE patients.

5. Conclusion

GFAP, MT-I/II, and AQP4 are of great significance in the development of mental disorders associated with MTLE. Changes of these protein levels may reflect abnormal astrocyte function and psychopathological states in MTLE patients. It also suggests that these three proteins are significant in the development of mental disorders in MTLE patients and may provide new therapeutic targets for MTLE treatment.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JL and YH designed the study. HH and QP collected the data, QZ analyzed the data, XZ, MX and YC analyzed the results and drafted the paper, QW prepared and finally approved the paper.

Ethics approval and consent to participate

Animal handling protocols were approved by the Ethics Committee of the Hunan Brain Hospital (Z2016003).

Conflict of interest

The authors declare that they have no competing interests.

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