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Research report

Investigation of synapses in the cortical white matter in human temporal lobe epilepsy

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ABSTRACT

Temporal lobe epilepsy (TLE) is one of the most common focal pharmacotherapy-resistant epilepsy in adults. Previous studies have shown significantly higher numbers of neurons in the neocortical white matter in TLE patients than in controls.

The aim of this work was to investigate whether white matter neurons are part of the neuronal circuitry. Therefore, we studied the distribution and density of synapses in surgically resected neocortical tissue of pharmacotherapy-resistant TLE patients. Neocortical white matter of temporal lobe from non-epileptic patients were used as controls. Synapses and neurons were visualized with immunohistochemistry using antibodies against synaptophysin and NeuN, respectively. The presence of synaptophysin in presynaptic terminals was verified by electron microscopy. Quantification of immunostaining was performed and the data of the patients' cognitive tests as well as clinical records were compared to the density of neurons and synapses.

Synaptophysin density in the white matter of TLE patients was significantly higher than in controls. In TLE, a significant correlation was found between synaptophysin immunodensity and density of white matter neurons. Neuronal as well as synaptophysin density significantly correlated with scores of verbal memory of TLE patients. Neurosurgical outcome of TLE patients did not significantly correlate with histological data, although, higher neuronal and synaptophysin densities were observed in patients with favorable post-surgical outcome.

Our results suggest that white matter neurons in TLE patients receive substantial synaptic input and indicate that white matter neurons may be integrated in epileptic neuronal networks responsible for the development or maintenance of seizures.

Abbreviations: AED, antiepileptic drug; AVLT, Rey auditory verbal learning test; BW, black and white; d, day; DAB, 3,3'-diaminobenzidine; F, female; FS, febrile seizure; GM, gray matter; HS, hippocampal sclerosis; IPI, initial precipitating injury; L, left; M, male; m, month; MRI, magnetic resonance imaging; MCD, malformation of cortical development; NeuN, neuronal nuclear antigen; PB, phosphate buffer; PFA, paraformaldehyde; R, right; ROCF, Rey-Osterrieth Complex Figure; SD, standard deviation; SYN, synaptophysin; TEM, transmission electron microscopy; TLE, temporal lobe epilepsy; TRIS, Tris-buffer; UPMS, University of Pécs Medical School; Y, year; w, week; WM, white matter.

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1. Introduction

Previous studies have shown the presence of neurons in the neocortical white matter (WM) of healthy adults (Meyer et al., 1992; Suárez-Solá et al., 2009). The majority of these neurons are remnants of the subplate, a transient layer during fetal cortical development (Chun and Shatz, 1989; Kostovic and Rakic, 1990; Mrzljak et al., 1988; Suárez-Solá et al., 2009). A portion of subplate neurons undergoes programmed cell death in the perinatal period, while many of them survive and are distributed in the subcortical WM as interstitial neurons (Chun and Shatz, 1989; Judas et al., 2010; Kostovic and Rakic, 1990; Suárez-Solá et al., 2009). Although, the role of WM neurons in adults is not clear, a potential role of them was proposed in schizophrenia or in Alzheimer disease (Akbarian et al., 1993; Eastwood and Harrison, 2005; Kirkpatrick et al., 1999, 2003; Kowall and Beal, 1988; Tao et al., 1999; Van de Nes et al., 2002).

In temporal lobe epilepsy (TLE), which is one of the most common form of focal epilepsies, a higher number of WM neurons have been found in the neurosurgically resected neo- and archicortex (Emery et al., 1997; Hardiman et al., 1988; Kasper et al., 1999; Liu et al., 2014; Richter et al., 2016; Thom et al., 2001). Etiology of TLE can be attributed to various pathological conditions including malformation of cortical development (MCD) and hippocampal sclerosis (HS). Heterotopic neurons are present in the WM of the temporal lobes of patients with MCD as well as with classical type of HS characterized by severe neuronal loss in Ammon's horn and in the hilus of the dentate gyrus (Liu et al., 2014; Richter et al., 2016; Thom et al., 2001). Characterization of WM neurons using specific markers for excitatory and inhibitory neurons has shown that these neurons comprise both excitatory and inhibitory cells in controls and TLE patients (Judas et al., 2010; Richter et al., 2016). The functional role of WM neurons in epilepsy has already been indicated by the association of the presence of WM neurons with favorable post-surgical outcome in TLE found by Hardiman et al. (1988) and by Thom et al. (2001). According to other studies, increased density of WM neurons was associated with worse outcome of patients following surgery and despite the removal of the cortical area containing large number of WM neurons, TLE patients did not become seizure free (Emery et al., 1997; Kasper et al., 1999). Therefore, it has been suggested that the presence of WM neurons is an epiphenomenon rather than the cause of seizure activity (Emery et al., 1997; Kasper et al., 1999).

Thus, the question about the role of WM neurons in the pathophysiology of epileptic seizures is still open. We hypothesize that due to their excess numbers, WM neurons form abnormal neuronal circuitry, which may have pathological significance and may play a role in the development and/or maintenance of epilepsy. Therefore, our aim was to investigate the possible synaptic connections of WM neurons, which would indicate that WM neurons are functionally active and may be integral part of epileptic neuronal networks. Therefore we have studied the distribution of neurons and synapses in the neocortical WM in surgically resected tissue samples of patients with pharmacotherapy-resistant TLE.

Visualization of WM neurons was performed by the detection of a neuronal nuclear antigen (NeuN) suitable for the study of the whole neuronal population present in the WM (Gusel'nikova et al., 2015; Mullen et al., 1992; Richter et al., 2016; Sarnat et al., 1998; Wolf et al., 1996). Synapses have been studied with an antibody against synaptophysin (SYN), a 38 kDa transmembrane synaptic vesicle protein that binds to another essential fusion molecule synaptobrevin II (Becher et al., 1999; Edelmann et al., 1995; Südhof, 1987). SYN is present in the presynaptic terminals of both excitatory and inhibitory neurons in the central nervous system and because of its general occurrence, SYN immunostaining can be used for quantification of synapses (Alder et al., 1992; Bragina et al., 2007; Calhoun et al., 1996; Gaardsvoll et al., 1988; Gronborg et al., 2010; Micheva et al., 2010; Rehm et al., 1986; Thomas et al., 1988; Wiedenmann and Franke, 1985). In addition to the distribution of WM neurons and synapses, we studied the correlation between the density of WM neurons and synapses that would indicate functional significance of WM neurons in the temporal neocortex of TLE patients. We further analyzed the possible correlation between the density of neurons as well as synapses and the clinical data of patients, including the age of onset, the duration of the epilepsy as well as the post-surgical outcome. Since WM abnormalities have been associated with cognitive performance (Alexander et al., 2014; Reyes et al., 2019; Riley et al., 2010; Rodríguez-Cruces et al., 2018), histological data were correlated with the scores of verbal and visual cognitive tests of TLE patients.

2. Results

In harmony with earlier studies and as demonstrated in Fig. 1, we detected larger number of neurons in the neocortical WM of TLE patients than in controls, although, the possible synaptic contacts and the functional impact of WM neurons remained to be elucidated (Emery et al., 1997; Richter et al, 2016; Thom et al., 2001). To identify and quantify synapses in the neocortical WM, we used immunohistochemistry to detect SYN, a vesicular protein present in presynaptic terminals (Alder et al., 1992; Wiedenmann and Franke, 1985).

2.1. Synaptophysin immunoreactivity in the white matter

Synapses were visualized with immunohistochemistry based on the SYN protein content of the presynaptic axon terminals in TLE patients and in control subjects who had no epilepsy. We used two different control groups. SYN immunohistochemistry was performed on sections of the middle temporal gyrus from surgically resected tissue blocks of patients who have been operated with intracranial tumor. The tissue samples examined in this study were completely tumor-free. In addition to biopsy, autopsy control samples were used as well. In both samples, SYN-immunoreactive profiles could be seen as small dots in the sections. As it was expected, dense SYN immunoreactivity was observed in the GM and the border between GM and WM was clearly outlined. In low numbers, SYN-immunoreactive puncta could be also found in the WM in the control group (Fig. 2A).

In TLE patients' samples, SYN immunoreactivity was also remarkably stronger in GM than in WM, and in a few HS and in MCD cases, the border between WM and GM appeared to be blurred (Fig. 2B). The most striking observation in TLE samples was the higher density of SYN-immunoreactive puncta in the neocortical WM (Fig. 2B). In the deep WM, distribution of SYN-immunoreactive profiles was variable (Fig. 2C, D). In many cases, SYN-immunoreactive dots were evenly distributed in the WM, while in other patients patchy appearance of SYN immunoreactivity could be observed. Occasionally, SYN-immunoreactive puncta were organized in parallel indicating the presence of synaptic terminals along dendrites.

In order to verify that SYN-immunoreactive puncta observed with light microscope signify synapses, we examined SYN-immunoreactive profiles with TEM. SYN-immunoreactivity was located in presynaptic axon terminals (Fig. 2E-F).

2.2. Quantification of synaptophysin immunoreactivity in the white matter

Quantification of SYN immunoreactivity was performed in the deep WM, approximately 500 μm below the border of WM and GM in both autopsy and biopsy controls, as well as in TLE samples. Although the average of optical density of SYN-immunoreactive puncta in biopsy preparations was slightly higher (6.73 ± 2.92) than in autopsy samples (5.09 ± 3.54), the difference between the two patient groups was not significant (Fig. 3A). Therefore, we pooled the control samples of both groups, and averaged the SYN-immunoreactive optical density values (5.91 ± 3.04) of autopsy and biopsy controls (Fig. 3B).

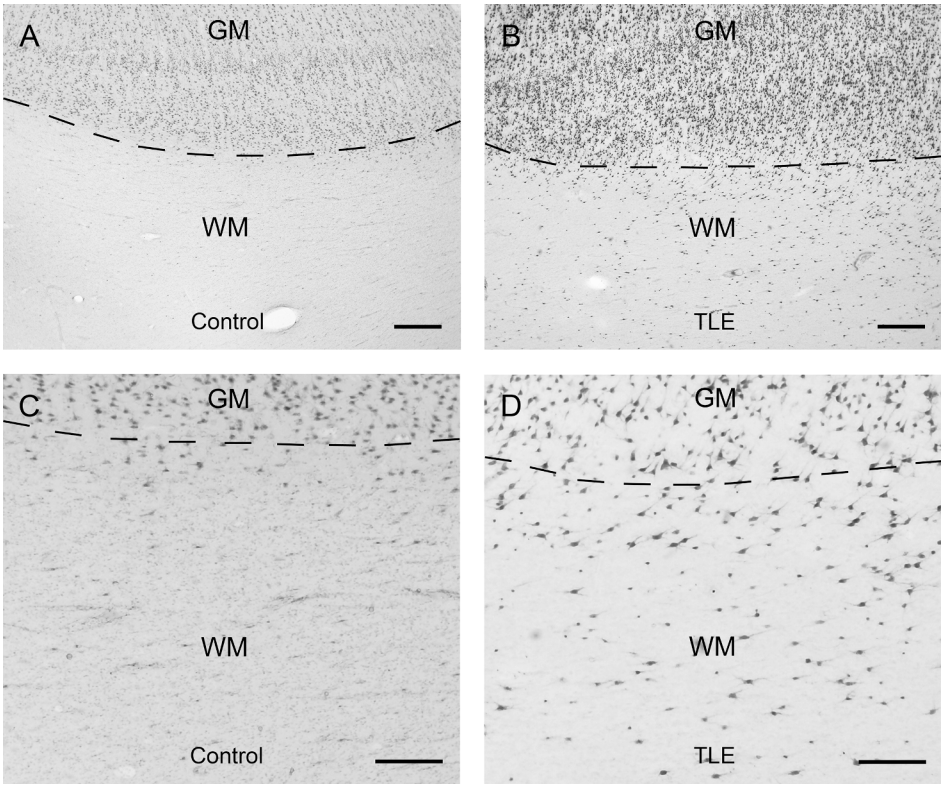

Fig. 1. NeuN immunoreactivity in the temporal neocortex NeuN immunoreactivity in a control sample (A and C) and in a patient with TLE (B and D). Dashed lines show the transition between gray matter (GM) and white matter (WM). Abbreviations: GM, gray matter; WM, white matter. Scale bar = 500 μm in A, B and 200 μm in C, F.

Quantification revealed that optical density of SYN-immunoreactive dots in the neocortical WM showed individual differences among TLE patients. The average density of SYN immunostaining in the neocortical WM of 14 TLE patients was 11.04 ± 3.13, which was significantly ($p = 0.007$) higher than that in control samples (Fig. 3B).

In TLE patients with HS, average of SYN optical density was 10.25 ± 2.43. The difference between SYN immunodensity in WM of the HS patients and that of controls were statistically significant ($p = 0.011$, Fig. 3C). In MCD, average of optical density of SYN-immunoreactive profiles was 13.04 ± 5.13, and the difference between SYN-immunoreactivity of controls and patients with MCD-related epilepsy was not statistically significant (Fig. 3D).

2.3. Correlation between optical density of synaptophysin immunoreactivity and density of neurons in the neocortical white matter

In this part of the study, we correlated the density of WM neurons with the optical density of SYN-immunoreactivity. In case of 13 TLE samples, we had data on both optical density of SYN immunostaining and density of NeuN-immunoreactive WM neurons. Spearman's analysis has revealed significant ($p = 0.001$) positive correlation between SYN optical density and density of neurons in the WM (Fig. 4A). In addition, significant ($p = 0.005$) correlation was observed in the WM of HS patients between SYN-optical density and density of neurons (Fig. 4B). Due to the low number of patients in the MCD group, association between SYN optical density and density of neurons in the WM was not statistically significant. However, larger SYN optical density was detected in those samples that contained larger number of neurons per unit area.

2.4. Correlation between histological findings and the clinical and cognitive data of TLE patients

We correlated SYN immunoreactivity in the neocortical WM with the following clinical parameters: age and gender of patients, age at onset of epilepsy and duration of the disease. Correlation analysis revealed no significant association between the above-mentioned parameters and the SYN immunoreactivity. We have to note, however, that age of autopsy controls were significantly higher than that of TLE patients, although, ages of individuals in the TLE group varied and significant deviation could be found in both directions from the average age of the patients. In contrast, no significant difference was observed in neuronal and SYN densities in the samples of those TLE patients who were younger than the average age of the whole TLE group when compared to those who were older.

In addition, association of SYN immunopositivity in the WM with the frequency of the seizures in the year before operation was examined, but no significant association could be found. No correlation was found between SYN immunoreactivity and the incidence of childhood febrile convulsions.

Regarding the postoperative outcome of patients, the Engel classification was used (Durnford et al., 2011; Engel et al., 1993). Twelve of our TLE patients became seizure-free and belonged to Engel class 1. One patient belonged to class 2, which indicate a slightly worse postsurgical outcome than for Engel class 1 patients. No meaningful improvement occurred in one patient who belonged to class 4. Engel class 1 can be further divided into subclasses. Patients with the most favorable outcome, who were completely seizure free without antiepileptic drugs (AED) belonged to class 1A. Those patients who had non-disabling simple partial seizures only since epilepsy surgery belonged to Engel class 1B, while patients with some disabling seizures after surgery, but who were free of disabling seizures for at least 2 years were registered in class 1C. Patients who had generalized convulsions with antiepileptic AED withdrawal only belonged to Engel class 1D. While analyzing the association between postsurgical outcome and histological findings, we separately examined those patients who became completely seizure free without AEDs (class 1A) and other patients who belonged to Engel class

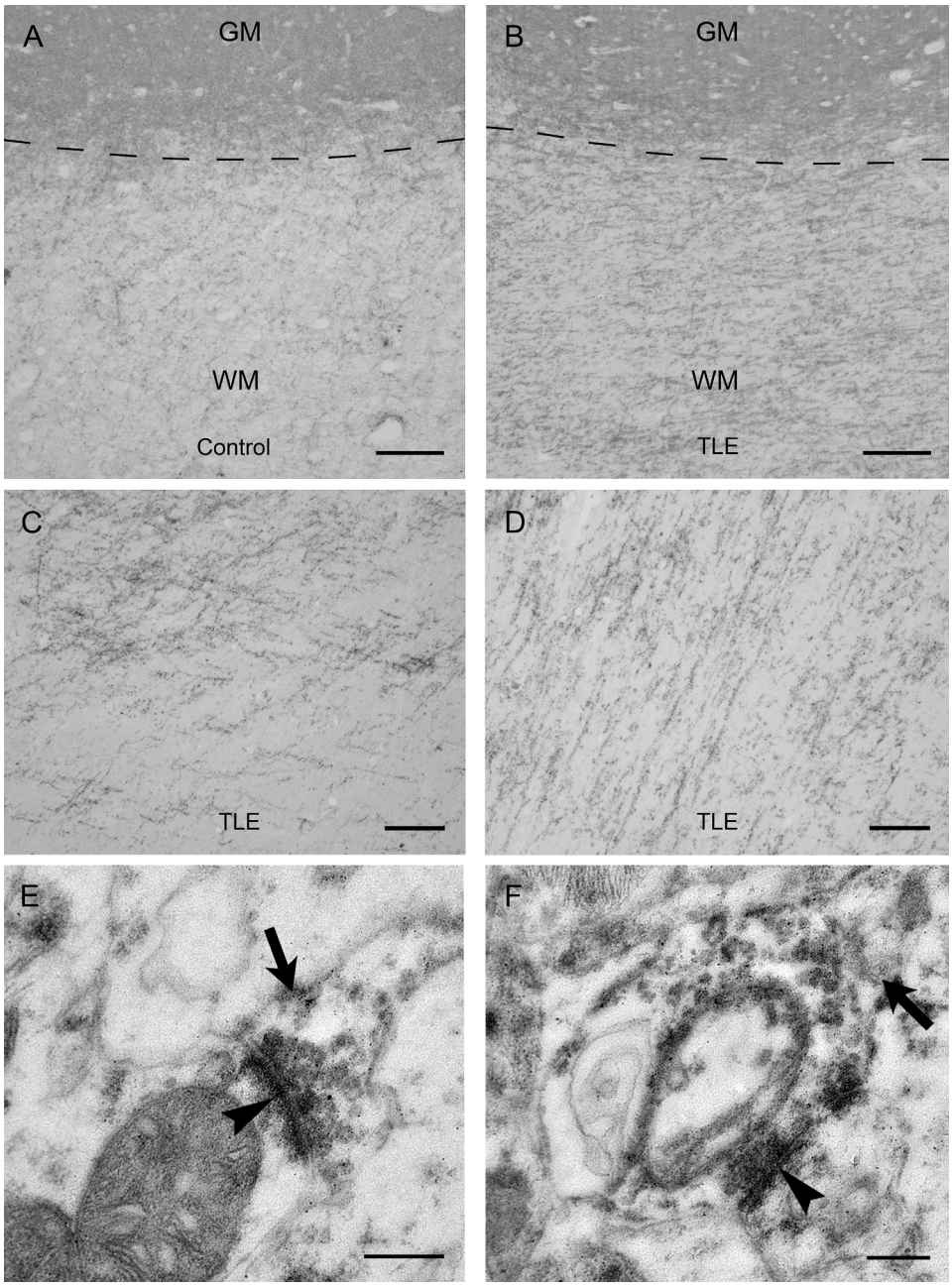

Fig. 2. Synaptophysin immunoreactivity in the white matter Synaptophysin (SYN) immunoreactivity in the gray matter (GM) and white matter (WM) of a control (A) and a patient with epilepsy (B). C. Dense SYN immunoreactivity in the WM of a TLE patient. D. Parallel-organized SYN-immunopositive profiles indicate presynaptic axon terminals terminating on dendrites or axon-initial segments of WM neurons in a TLE patient. E: Electron micrograph of an axo-dendritic synapse formed by a SYN-immunoreactive presynaptic terminal (arrow) in a TLE patient. F: Electron micrograph of a SYN-immunoreactive presynaptic terminal (arrow) forming an asymmetric synapse with a dendritic spine. Arrowheads in E and F point to post-synaptic density. Scale bars = 200 μm in A, B, 100 μm in C, D and 200 nm in E and F.

1B-D. We assigned numerical values to these two categories as follows: to 1A 0.5, to Engel class 1 other than 1A (1B-D) 1 were allocated. Regarding other Engel classes, subclasses were not considered and the numerical value assigned was identical with the number of the class. Our analyses revealed that post-surgical outcome significantly ($p = 0.03$) correlated with the optical density of SYN-immunoreactivity (Fig. 5A).

In the subgroups of TLE, all patients with HS became seizure free and belonged to Engel class 1. Analyzing association of SYN-immunoreactivity with the postsurgical outcome of HS patients, no significant association was found (not shown). Similarly, no significant association was observed between SYN-immunodensity and postsurgical outcome in the MCD group, however, trend showed that the higher the SYN immunodensity, the more favorable the postsurgical outcome was (Fig. 5B).

The density of NeuN-immunoreactive cells in the WM and the post-surgical outcome of TLE patients did not show significant association, although an interesting trend was observed indicating that the larger the density of neurons the better was the postsurgical outcome (not shown).

Linear regression analysis between the histological findings and the cognitive performance of patients indicated that neuronal and synaptic densities in the WM were associated with verbal memory (Fig. 6). The optical density of SYN immunoreactivity was significantly correlated with the interference in AVLT ($F(1,11) = 7.57$, $p < 0.05$) (Fig. 6A). Similarly, higher neuronal density was associated with lower scores of interference in AVLT, although the association was not statistically significant ($F(1,11) = 2.86$, $p = 0.11$, not shown). Regarding short-term verbal memory, a significant correlation was observed between scores of digit span forward test and SYN immunodensity ($F(1,11) = 4,81$, $p = 0.05$), as well as density of neurons ($F(1,11) = 11.4$, $p < 0.01$) (Fig. 6B and C). The scores of visual attention and memory tests including copy of ROCF did not show significant correlation with SYN-immunodensity and with the density of NeuN-immunoreactive cells, although a tendency ($p = 0.08$) could be observed when forward version of the Corsi Block-Tapping task scores and neuronal density were correlated (not

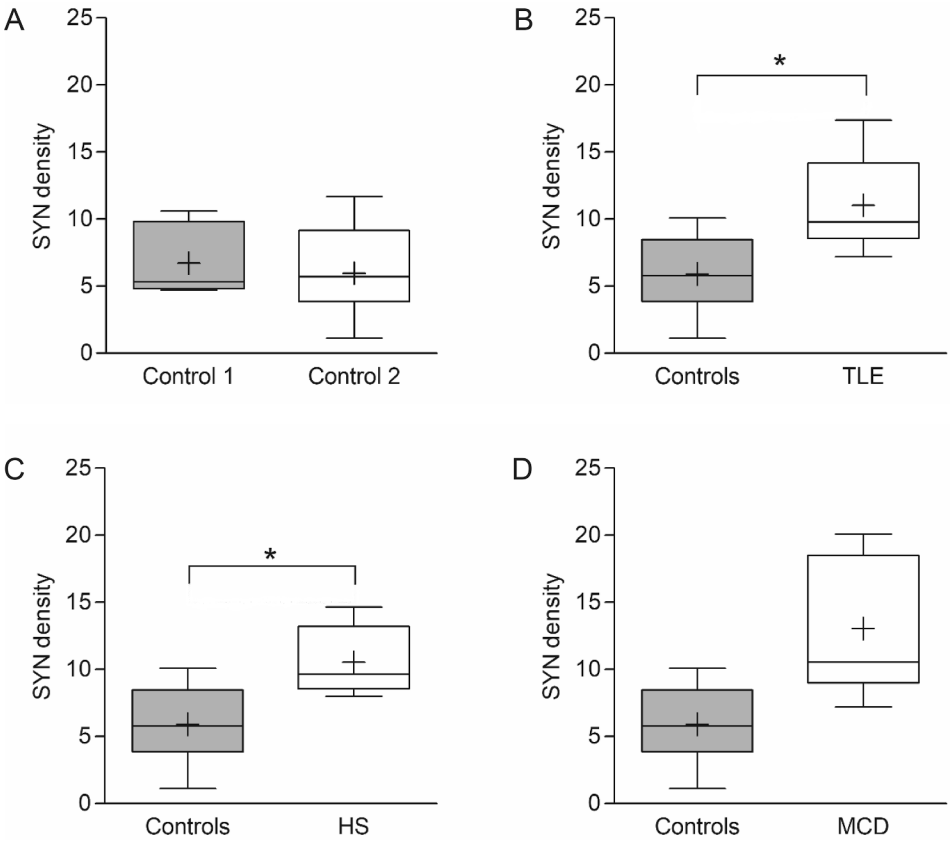

Fig. 3. Graphs showing optical density of synaptophysin immunoreactivity in controls and in TLE patients A. Mean of optical density of synaptophysin (SYN) immunoreactivity in the two control groups. No significant difference can be seen in SYN immunoreactivity in samples taken by biopsy (gray box) and after autopsy (white box). B. Mean optical density of SYN immunoreactivity measured in TLE patients' samples (white box) and in controls (gray box). C. Mean optical density of SYN immunoreactivity measured in HS (white box) and in controls (gray box). D: Mean optical density of SYN immunoreactivity measured in the group of MCD-induced TLE patients (white box) and in controls (gray box). Asterisks indicate significant differences. Whiskers show the 5th and 95th percentiles.

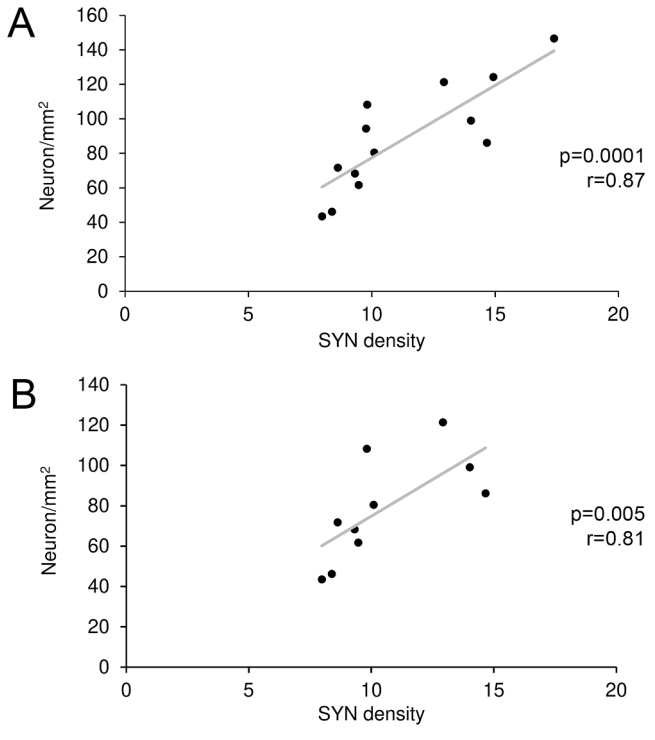

Fig. 4. Diagrams reveal the association between optical density of synaptophysin immunoreactivity and density of WM neurons in TLE patients' groups. A and B: Significant positive correlation was observed between the density of WM neurons and the optical density of synaptophysin (SYN) immunopositivity in all TLE patients (A) and in TLE patients with HS (B).

shown).

In the group of TLE patients with HS, no significant linear regression could be seen between optical density of SYN immunoreactivity and scores of verbal memory, and between density of NeuN-immunopositive cells and interference of AVLT (Fig. 7A, B, D). Analyzing the association between WM neuronal density and verbal memory performance of HS patients, a significant ($F(1,8) = 5{,}573$, $p = 0.046$) linear regression was found between the density of NeuN-immunopositive cells and the scores of digit span forward test (Fig. 7C). Although, the low number of samples of TLE patients with MCD did not allow statistical analysis, a trend was detected when the optical density of SYN-immunoreactivity and the scores of interference in AVLT and digit span forward tests were correlated.

3. Discussion

In the present study, we have shown that a significantly higher density of synapses could be found in the neocortical WM of TLE patients than in controls. In addition, we have observed a significant correlation between the density of synapses and the density of neurons in the WM of epilepsy patients. Density of synapses in the WM significantly correlated with the postsurgical outcome of TLE patients. In addition, the number of neurons and the density of synapses significantly associated with the verbal memory performance of patients. In groups of TLE patients with HS and with MCD, synapse density was separately examined. According to our findings, the density of synapses was significantly higher in the WM of TLE patients with HS than in controls. In addition, a significant correlation could be found between the density of synapses and the density of neurons in the neocortical WM of those TLE patients who had HS.

Neuronal numbers in the neocortical and archicortical WM have been reported to be increased in epileptic patients compared to non-epileptic controls (Emery et al., 1993; Richter et al., 2016; Thom

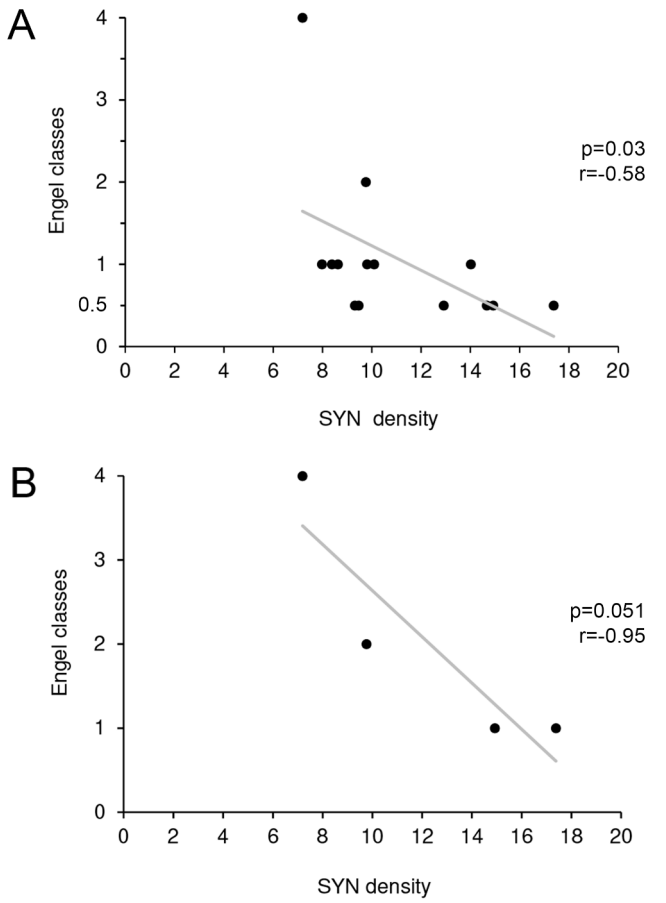

Fig. 5. Relationship between optical density of synaptophysin immunoreactivity in the WM and postoperative outcome of TLE patients. A: Chart demonstrates significant correlation between optical density of synaptophysin (SYN) immunoreactivity in the WM and TLE patients' postoperative outcome according to the Engel classification. Engel class 1 was further divided into subclasses 1A and 1B-D. For the analysis of the association between postsurgical outcome and histological findings, we assigned numerical values to these subclasses as follows: 1A – 0.5, 1B-D –1. These values are present also on the Y axis of the chart in A. B. Relationship between optical density of SYN immunoreactivity and patients' postoperative outcome according to the Engel classification in MCD-induced TLE.

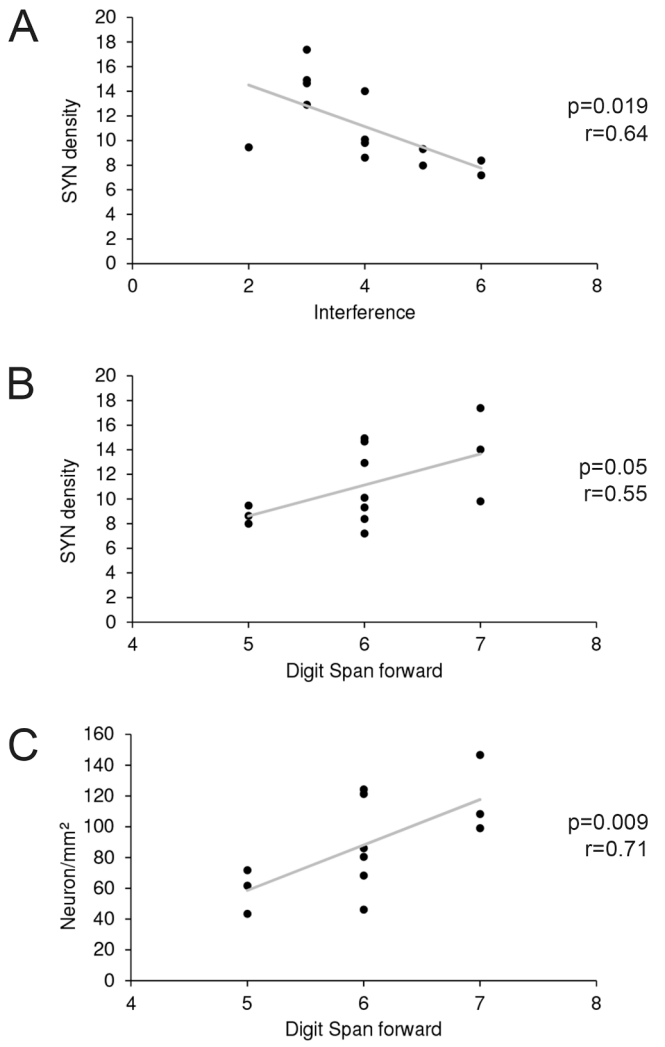

Fig. 6. Linear regression between the optical density of synaptophysin immunoreactivity (A and B), density of WM neurons (C) and the verbal memory scores of the entire TLE patients' population. A. Diagram shows significant negative linear regression between synaptophysin (SYN) density and the interference in AVLT. B. Chart reveals significant positive linear regression between SYN density and scores of digit span forward test. C. Diagram reveals significant positive linear regression between density of WM neurons and scores of digit span forward test.

et al., 2001). Among WM neurons, both excitatory and inhibitory neurons were present (Richter et al., 2016). Regarding the functional significance, as well as the synaptic connections of WM neurons, no clear information was available. In our study, synapses were visualized with immunohistochemistry based on the SYN protein content of the presynaptic axon terminals (Wiedenmann and Francke, 1985). SYN-immunoreactive profiles have been observed in WM of both TLE patients and control samples. Under the light microscope, SYN-immunoreactive terminals were visible as small dots. Using immunoelectron microscopy, we verified the localization of SYN in presynaptic axon terminals, which is in harmony with previous studies (Rehm et al., 1986; Wiedenman and Francke, 1985).

3.1. *Technical considerations and limitation of the study*

In our study, quantification of synapses in the neocortical WM was performed in light microscopic sections and optical density of SYN immunoreactivity was measured. In a previous work, we have compared two different software for optical densitometry of immunoreactive profiles, in which ImageJ, an open source Java-based image processing and analyzer program supported by the NIH USA and AnalySIS software (Olympus Corporation) have been used and compared (Armbruszt et al., 2015). We have found that both programs are suitable for the measurement of optical density on immunostained sections, therefore, in the present study we used ImageJ. Quantification revealed a significantly higher SYN optical density in TLE compared to controls.

Regarding the control samples, we have used the WM of the temporal neocortex of two control groups. Patients who underwent neurosurgery with fast-growing intracranial tumors formed one of our control groups. Resection of neocortical WM in these cases has been performed for strictly therapeutic reasons and has been indicated to guarantee safe and radical removal of the tumor. The resected tissue was put in fixative immediately after removal. Due to the radical resection, WM tissue used in our study did not contain tumor cells. In addition to biopsy samples, autopsy controls with short post-mortem delay were used as well. Probably, due to the longer interval between the time of death and fixation of brain samples, SYN immunoreactivity in autopsy control group was slightly weaker and optical density was slightly lower than that of surgically removed biopsy controls. However, the difference between the two groups was not significant. This indicates that both

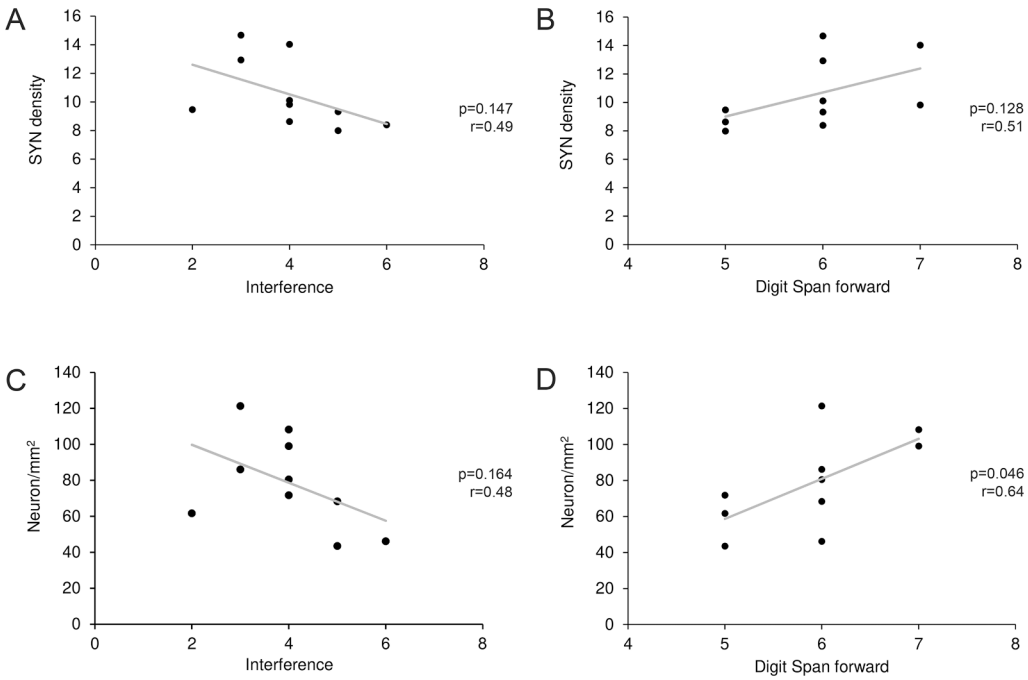

Fig. 7. Linear regression between the optical density of synaptophysin immunoreactivity (A and B), density of WM neurons (C and D) and the verbal memory scores of TLE patients with HS. A. Diagram shows non-significant association between optical density of synaptophysin (SYN) immunostaining and interference in AVLT. B. Non-significant association between SYN density and scores of digit span forward test. C. Non-significant relationship between density of WM neurons and interference in AVLT. D. Diagram reveals significant positive correlation ($p = 0.046$) between density of WM neurons and scores of digit span forward test.

controls can be used as a comparison to the SYN immunodensity of TLE patients' WM samples. Accordingly, we have pooled the optical density values of our control samples, in order to form a single control group that could be used for the comparison of SYN immunodensity in TLE patients.

There are limitations in the present study. First, the sample size is relatively small, which might result in a reduction of statistical power. In the TLE group with HS, we had sections containing NeuN-immunopositive cells and SYN-immunoreactive profiles of 10 patients. The number of samples was especially low regarding the group of TLE patients with MCD ($n = 4$). Despite the small sample size, a significant difference could be found between optical density of SYN-immunoreactivity of controls and TLE patients, and between these parameters of controls and TLE patients with HS. Regarding to the whole cohorts' population, and to HS patients, a significant correlation was found between optical density of SYN-immunoreactive profiles and the density of NeuN-immunopositive cells. In addition, a significant association was observed between the optical density of SYN-immunoreactivity and the postsurgical outcome of TLE patients. A significant correlation was found between NeuN as well as SYN-immunoreactivity and scores of neuropsychological tests measuring verbal memory of patients. It is obvious that a larger sample size would strengthen the statistical power of our study. However, despite the relatively low sample size, our original question that was related to the possible functional role of WM neurons and synapses in TLE, was answered. Based on previous studies, the most frequent form of TLE is HS, and in harmony with these data, the larger group of our epileptic patients was characterized with HS and lower number of patients had MCD (Blümcke et al., 2002; Blümcke, 2009; Howe et al., 2010; Lehericy et al., 1997). Thus, we have to emphasize that the difference observed in the sample size of the examined patients' groups with different etiology correlates with the difference in the prevalence of MR findings in TLE.

Another limitation of our study is the difference between the ages of TLE patients and that of controls. Especially, the ages of autopsy controls were significantly higher than that of TLE patients. We have to emphasize, however, that ages of individuals in the TLE group varied also and significant deviations could be observed in both directions from the average age of the patients. In contrast, no significant difference was found in neuronal and SYN densities in the samples of those TLE patients who were younger than the average age of the whole TLE group when compared with the densities found in those who were older than the average age of the patients. In addition, no correlation could be found between the neuronal or SYN densities and the age of the patients. Although, we cannot exclude that certain level of decrease in neuronal and SYN densities in the WM is associated to aging, the lack of correlation between neuronal or SYN densities and the ages of patients indicates that the significantly higher neuronal and SYN densities observed in TLE patients are rather due to the epilepsy than to the lower ages of the patients.

3.2. *Neurons and synapses in the white matter*

Previous studies have reported the presence of neurons in neocortical WM of patients with TLE, although their impact on generation and maintenance of seizure activity has not been proven (Emery et al., 1997; Hardiman et al., 1988; Kasper et al., 1999; Liu et al., 2014; Richter et al., 2016; Suárez-Solá et al., 2009; Thom et al., 2001). In addition to the higher optical density of SYN immunoreactivity - that we found in TLE patients compared to controls -, we revealed a significant positive linear correlation between the density of WM neurons and the optical density of SYN immunoreactivity. The correlation suggests that WM neurons may be functionally active, integral parts of the neuronal circuitries in the temporal lobe in TLE. Regarding the origin of synaptic terminals in the WM, many options are plausible. The SYN-immunoreactive terminals might originate form cortical and/or subcortical neurons. In addition, the WM neurons might be the source of SYN-immunopositive presynaptic terminals as well.

The functional activity of WM neurons and synapses might be supported by another finding of our study. Correlation of the neuronal numbers and the optical density of SYN immunoreactivity with neuropsychological data of patients revealed a significant association between our histological findings and the verbal memory of TLE patients. Short-term verbal memory significantly correlated with neuronal density as well as with optical density of SYN immunoreactivity in the WM of epilepsy patients. In addition, optical density of SYN immunoreactivity was significantly associated to interference in AVLT. These data clearly indicate the functional importance of WM synapses in the temporal lobe. Interestingly, elimination of SYN in mice induced behavioral changes

including impairments in learning and memory, indicating that SYN is apparently not essential for the synaptic vesicle cycle, but it likely has a function in modulating synaptic strength and efficiency of memory formation (Schmitt et al., 2009).

The existence of functional neural networks within the WM is supported by MRI studies in which the WM appears to display intrinsic functional organizations as interacting networks of functional modules, similarly to the GM (Ding et al., 2016; Peer et al., 2017; Wu et al., 2016). Interestingly, Peer et al. (2017) have shown that these networks extend deeper than the border of the WM and GM, indicating that signals arise from activity within the WM itself, which supports the interpretation of our results.

In TLE, the involvement of the neocortical WM has been confirmed by MRI (Concha et al., 2009; Nagy et al., 2016; Riley et al., 2010). More widespread diffusion abnormalities have been observed in the WM tracts of the temporal lobe in mesial temporal sclerosis than in non-lesional TLE (Liu et al., 2012). Using fMRI, results indicated functional disruption in WM networks in mesial temporal sclerosis and led to the suggestion that deep WM networks are key network nodes that may contribute to massive functional alterations in the GM of TLE patients (Cui et al., 2021). Other studies indicate that heterotopic WM neurons in MCD participate to some degree in normal brain functions (Janszky et al., 2003; Muller et al., 1998; Spreer et al., 2001).

The significance of synapses in the WM of the temporal neocortex is highlighted by the correlation of optical density of SYN immunoreactivity and the postsurgical outcome of TLE patients. For the evaluation of postsurgical outcome, Engel classification was used (Engel et al., 1993). We observed significant negative correlation between postsurgical outcome of TLE patients and the optical density of SYN immunoreactivity showing that the larger optical density of SYN immunoreactivity, the better is the postsurgical outcome of the patients. Despite the low number of patients (n = 4), tendency could be found between the postsurgical outcome of TLE patients with MCD and the optical density of SYN immunoreactivity. A similar trend could be seen when the density of WM neurons and the postsurgical outcome of patients with MCD were correlated. These associations indicate that WM synapses might play a substantial role in the generation and maintenance of epileptic seizures in patients with MCD, and the removal of the area containing WM neurons and synapses largely contributed to the favorable postsurgical outcome of the patients. According to a previous study, the increased density of WM neurons was associated with a worse outcome of TLE patients following surgery (Kasper et al., 1999). However, Hardiman et al. (1988) and Thom et al. (2001) have found an association of the presence of WM neurons with a favorable post-surgical outcome in TLE. Our results are in harmony with their data, and extend it with the finding that in addition to neurons, larger density of synapses in the WM is also associated with a better postoperative outcome.

The exact way and the time of formation of WM synapses on WM neurons, however, are still unclear, and two basic explanations may occur. It may be due to abnormal cortical development that is highlighted by the larger number of neurons in the WM (Hardiman et al., 1988; Kasper et al., 1999; Richter et al., 2016; Thom et al., 2001) and the significant correlation found between the density of WM neurons and synapses. The increased SYN density in the WM found in our study suggests that WM neurons receive synaptic input, although the origin of these axons could not be verified with the histological technique we used. WM synapses may be formed by cortical neurons in normal position and the subcortical origin can also be a possibility. Another option might be that WM neurons terminate on each other and form abnormal subcortical circuitries which may be part of epileptic networks and play a role in the development and maintenance of epilepsy. In an animal model of cortical dysgenesis, complex synaptic responses were observed upon electrical stimulation of the adjacent WM which suggest that heterotopic neurons can form local excitatory and inhibitory synaptic connections and may participate in epileptiform events (Smith et al., 1999). Another explanation for the high density of WM synapses can be synaptic reorganization that is a known feature in epilepsy (Colciaghi et al., 2014; Maglóczky, 2010; Tóth et al., 2010). Axonal sprouting and synapse formation are due to the stimulating effect of trophic factors (Cronin et al., 1992; Peng et al., 2013; Represa and Ben-Ari, 1997). A similar phenomenon is also plausible in the neocortical WM, which may increase the number of synapses.

Our work indicates the functional importance of WM neurons and synapses, although, further research is needed about the exact role of them in TLE. The presence of functional networks within the WM may open new avenues of research in cognitive and clinical neuroscience.

4. Experimental procedures

4.1. Patients

Surgically removed tissue of the middle temporal gyrus of pharmacotherapy-resistant TLE patients (n = 14) were used in this study. In 10 patients HS, in four patients MCD have been verified with magnetic resonance imaging (MRI). In addition, in HS patients, neuropathological examination revealed typical neuronal loss in Ammon's horn and in the hilus of the dentate gyrus. The hippocampal and cortical sections of TLE patients have been partly used in previous studies as well (Ábrahám et al., 2011; Karádi et al., 2012; Richter et al., 2016). Demographics and clinical data of TLE patients used in this study are summarized in Table 1..

TLE patients have been evaluated in the Epilepsy Center of the Department of Neurology of the University of Pécs Medical School (UPMS), and surgery has been performed in the Department of Neurosurgery under general anesthesia, through a standard temporal craniotomy. Fixation of tissue samples has been started immediately after the removal.

4.2. Controls

Neocortical WM of temporal lobe tissues from non-epileptic patients with intracranial tumor (n = 3) and from autopsy (n = 3) were used as controls. Demographics and clinical data of controls are summarized in Table 2.

Neurosurgery of non-epileptic patients (n = 3) has been performed in the Department of Neurosurgery of UPMS, because of rapidly growing brain tumors in the temporal neocortex. Resections have been done for strictly therapeutic reasons and temporal lobectomy has been indicated to guarantee safe and radical removal of the tumor. To ensure this, the tumor was removed with a margin of 2 cm to 3 cm intact temporal neocortex. Fixation of tissue samples has been started immediately after the removal. Histological diagnosis of the tumor was performed in the Department of Pathology at UPMS. In the intact temporal neocortex, histological examinations revealed no peritumoral tissue changes or infiltration of the tumor. The intact peritumoral WM tissues used in this study were examined by Ki-67 immunohistochemistry in our laboratory. All procedures including the surgery were carried out with the adequate understanding and written consent of the patients. Tissue samples were processed and histological evaluation has been carried out according to the institutional regulation (PTE KK RIKEB/5342). In addition, regulations of the Hungarian Ministry of Health and the policy of Declaration of Helsinki has been followed.

Autopsy samples have been received from the Human Brain Research Laboratory of the Institute of Experimental Medicine, Budapest, Hungary. The control subjects were processed for autopsy in the Department of Pathology of Saint Borbála Hospital, Tatabánya, Hungary. Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. Tissue was obtained and used in a manner compliant with the Declaration of Helsinki. All procedures were approved by the Regional and Institutional Committee of Science and Research Ethics of Scientific Council of Health (ETT TUKEB 15032/2019/EKU). Control subjects died from causes unrelated

Table 1
Demographics and clinical data of patients with temporal lobe epilepsy.

Case number	Age (Y)	Gender	Age at onset (Y)	Duration (Y)	Seizure frequency	MR-diagnosis	Affected side	IPI
1	25	M	5	20	6–7/m	HS	L	FS
2	29	M	14	15	4/m	HS	R	FS
3	48	M	8	40	6–8/m	HS	R	FS
4	51	F	22	29	1–4/m	HS	L	–
5	40	M	11	29	1–2/m	HS	L	–
6	27	F	19	8	2–4/w	HS	R	–
7	50	M	4	46	1–2/m	HS	R	–
8	48	F	4	44	6/m	HS	L	FS
9	34	F	23	11	5–8/m	HS	L	–
10	48	F	28	20	2–3/w	HS	L	–
11	48	F	38	10	6–8/m	MCD	L	–
12	33	F	4	29	6–10/m	MCD	L	–
13	32	F	14	18	1–6/d	MCD	R	–
14	19	M	7	12	2–3/d	MCD	L	–

Table 2
Demographics and clinical data of controls.

Tumor patients	Age (Y)	Gender	Tumor location on MRI	Side of tumor	Histopathological diagnosis
1	39	M	Parahippocampal, piriform gyri	R	Glioblastoma, WHO Grade IV
2	74	F	Temporal pole, hippocampus, parahipp. gyrus	R	Astrocytoma, WHO Grade III
3	38	F	Fronto-insulo-temporal cortex, hippocampus, parahipp. gyrus	R	Glioblastoma WHO Grade IV

Autopsy patients	Age (Y)	Gender	Side of the examined cortex	Casue of death
1	77	M	L	Cardiac arrest
2	60	F	R	Respiratory arrest
3	72	M	R	Respiratory arrest

to any brain disease and the clinical data or the autopsy did not show any signs of neurological disorders. The control brains (n = 3) were removed 2–4 h after death, the internal carotid and vertebral arteries were cannulated, and the brains were perfused first with physiological saline (1.5 L in 30 min) containing 5 ml of heparin, followed by a fixative solution containing 4% paraformaldehyde, 0.05% glutaraldehyde and 15% picric acid in phosphate buffer (PB, pH 7.4), (4–5 L in 1.5–2 h). After perfusion, 0.5–1 cm thick blocks were cut from the temporal cortical region Brodmann area 21 and post-fixed in the Zamboni solution without glutaraldehyde overnight (Magloczky et al., 1997; Tóth et al., 2010). Following fixation, blocks were cryo-protected in 30% of sucrose diluted in PB for two days, deep-frozen over liquid nitrogen and stored at −80 °C.

4.3. Tissue processing

The cortical tissues resected from TLE and tumor patients were immediately put in fixative containing 4% paraformaldehyde (PFA) buffered with phosphate buffer (PB, 0.1 M, pH 7.4) and tissues were fixed for 12 h at 4 °C. Autopsy samples were stored following a deep-freezing procedure as described above. Fixation of tissue samples in 4% PFA buffered with PB (0.1 M, pH 7.4) was carried out immediately after melting, then blocks were fixed for an additional 12 h.

Tissue blocks containing neocortical WM and gray matter (GM) were embedded into paraffin and 10 μm thin sections were cut with a sliding-microtome and mounted on gelatin-coated glass slides. Other parts of tissue blocks of surgically removed samples of TLE patients were cut with vibratome at 80 μm, and free-floating sections were processed for immunohistochemistry.

4.3.1. Synaptophysin immunohistochemistry for light microscopy

Following the removal of paraffin, immunohistochemistry was performed according to described earlier (Ábrahám et al., 2001). Briefly, deparaffinization was followed by the washing of sections in Tris-buffer (TRIS 0.1 M, pH 7.4). Antigen retrieval was performed with citrate buffer (pH 6.0) in a microwave oven (800 W), and sections were heated three times for 5 min (min) each. This step was followed by pre-incubation of sections in 10% normal horse serum diluted in TRIS containing 0.4% Triton X-100 for 1 h. Incubation with the primary mouse monoclonal anti-SYN (Novocastra, New Castle upon Tyne, 1:400) antibody diluted in TRIS was carried out in a humid chamber overnight at room temperature. Binding sites were visualized with biotinylated secondary antibody and with the avidin–biotin peroxidase detection system (Universal Vectastain ABC Elite Kit, Vector, Burlingame, CA). The chromogen was 3,3′-diaminobenzidine (DAB).

4.3.2. NeuN immunohistochemistry for light microscopy

Immunohistochemistry detecting WM neurons expressing NeuN pan-neuronal antigen was carried out as previously published (Richter et al., 2016). Briefly, 80 μm thin free-floating sections were pretreated with 1% H_2O_2 diluted in TRIS for 20 min, then pre-incubated in 10% normal horse serum in TRIS containing 0.4% Triton X-100. This step was followed by incubation with monoclonal anti-NeuN (Chemicon, Temecula, CA, USA, 1:500) primary antibody overnight at room temperature. Binding sites were visualized with biotinylated secondary antibody and avidin–biotin peroxidase detection system (Universal Vectastain ABC Elite Kit, Vector, Burlingame, CA) using DAB as chromogen.

4.3.3. Synaptophysin immunoelectron microscopy

In order to visualize synapses with transmission electron microscope (TEM), preparations of neocortical tissues of TLE patients for immuno-electron microscopy have also been done. Therefore, PFA-fixed blocks of temporal lobe were cut with vibratome at 80 μm. Free-floating sections were subjected to „freeze-thaw" process to enhance penetration of antibodies. Sections were cryoprotected in 15%, then in 30% solution of sucrose diluted in PB, and they were „freeze-thawed" by placing them three times above liquid nitrogen. After this pretreatment, indirect immunoreaction was performed using primary anti-SYN antibody (Novocastra, New Castle upon Tyne, 1:400). Binding sites were visualized with biotinylated secondary antibody and avidin–biotin peroxidase detection system (Vector, Burlingame, CA). The immunoreaction was visualized with DAB. Post-fixation of the sections was performed with 2.5% glutaraldehyde and then with 1% osmium-tetroxide diluted in PB. Subsequently, sections were dehydrated with increasing concentrations

of ethyl alcohol, cleared with propylene oxide, then were flat-embedded in Durcupan resin (Sigma-Aldrich, Budapest). Region of interest in the WM was re-embedded in Durcupan resin using gelatin capsules. Ultrathin sections were cut at 65 nm with ultramicrotome (Leica Ultracut, Germany). Then sections were placed on a single-slot grid covered with parlodion membrane. Uranyl-acetate and lead-citrate were used for contrasting the sections which were examined in JEOL 1200 EX-II and JEM-1400Flash transmission electron microscopes (TEM).

4.4. Quantification

4.4.1. Determination of neuronal density

Details of quantification of NeuN-immunoreactive neurons in cortical WM of the temporal neocortex was previously published (Richter et al., 2016). Since the neuronal density differs in the superficial and deep parts of WM, quantification was carried out approximately 500–700 μm below the border between the GM and the WM. In the neocortical WM, NeuN-immunoreactive neurons were counted using an image analyzer system consisting of a Nikon Optiphot 2 microscope equipped with a MicroBrightfield Lucivid, computer-controlled motorized stage attached to a computer running Neurolucida software (Neurolucida 2.0, Microbrightfield Inc., Williston, VT). The number of immunoreactive neurons in the deep WM of the sections of each patient were determined and pooled, then data were averaged, and the density of NeuN-immunoreactive cells was expressed as neurons/mm^2 ± standard deviation (SD).

4.4.2. Determination of synaptophysin immunoreactivity

Determination of the optical density of SYN-immunoreactive profiles was performed in the WM of the temporal neocortex. Measurements were carried out on digital pictures taken with an Olympus BX50 light microscope using 20X magnifying objective lens in the deep neocortical WM, approximately 500 μm below the GM. In black and white (BW) images of the neocortical WM, the density of SYN-immunoreactive profiles was determined using an Image J software (NIH, US-supported image analyzer) that measured the intensity of pixels and expressed it as numerical values without measure unit. Two or three sections per tissue blocks were photographed in each patient. As an average, 10.5 BW photos were captured and analyzed per section.

The density of SYN-immunoreactive profiles and that of the background staining were separately measured. The real optical density of SYN-immunoreactivity was calculated by subtracting intensity values of the SYN from intensity values of the background, the average ± SD was determined.

4.5. Memory tests

Preoperative verbal and visual memory performance of TLE patients were tested. Verbal attention was measured with the forward version of the digit span task. Visual attention was assessed using forward version of the Corsi Block-Tapping task (Lezak et al., 2004). Visual construction ability and memory were assessed using the Rey–Osterrieth Complex Figure (ROCF) test. After copying the ROCF, the patient had to draw it from memory in delayed recall (30 min). In the ROCF test, a standard Taylor's scoring system was administered with a maximum of 36 points over copying and memory versions. Each figure was divided into 18 different blocks. When the subject drew properly placed, correct blocks, 2 points were given. Properly placed and distorted or poorly placed and correct blocks were rated with 1 point. Distorted, poorly placed blocks were scored with half point. In case of absent or unrecognizable blocks, no points were given (Hodges, 1996). Verbal learning and memory were tested using a Hungarian version of the Rey auditory verbal learning test (AVLT). The AVLT measures verbal learning ability using 15 common nouns (A and B lists). Five presentations of the A list were given. After each presentation, the subject had to recall the words from the list. Learning was evaluated over five trials. After the 5th trial, the B list was read, and the subject had to remember this new list (interference trial). In the 7th trial, the subject recalled the A list without auditory presentation. The 8th trial presented the delayed recall after 20 min. After testing, we evaluated the total learning score (TL) by the total number of learned words over the first five trials, short-term retention on the 7th trial, long-term retention on the 8th trial and the interference (remembered number of words of B list).

4.6. Statistical analysis

Optical density of SYN-immunoreactivity in autopsy controls was compared to biopsy controls with Student's *t*-test. Similarly, Student's *t*-test was used for the comparison of optical density of SYN-immunoreactivity in TLE patients and in controls. Optical density of SYN-immunoreactivity and the density of WM neurons, as well as clinical and cognitive data of TLE patients were correlated with Spearmann's correlation and linear regression, respectively. Statistical significance was set at $p \leq 0.05$. Statistical analyses were performed by IBM SPSS software package (version 25. SPSS Inc, MN).

CRediT authorship contribution statement

Noémi Sóki: Investigation, Visualization, Writing – original draft, Formal analysis. **Zsófia Richter:** Investigation, Writing – review & editing. **Kázmér Karádi:** Investigation, Writing – review & editing, Formal analysis. **Katalin Lőrincz:** Investigation. **Réka Horváth:** Resources. **Csilla Gyimesi:** Resources. **Cecília Szekeres-Paraczky:** Resources, Writing – review & editing. **Zsolt Horváth:** Resources. **József Janszky:** Resources, Writing – review & editing. **Tamás Dóczi:** Resources, Writing – review & editing, Funding acquisition. **László Seress:** Writing – review & editing, Supervision. **Hajnalka Ábrahám:** Writing – original draft, Writing – review & editing, Funding acquisition, Supervision.

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