Features of amygdala in patients with mesial temporal lobe epilepsy and hippocampal sclerosis: an MRI volumetric and histopathological study

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Abbreviations:

HS, hippocampal sclerosis; mTLE, mesial temporal epilepsy; ATL+ AH, anterior temporal lobectomies with amygdalo-hippocampectomies; TLEs, mesial temporal epilepsies; sGTC, secondary generalized tonic-clonic seizures; LI, laterality index.

Abstract

Objective: It is well-known that there is a correlation between the neuropathological grade of hippocampal sclerosis (HS) and neuroradiological atrophy of the hippocampus in mesial temporal lobe epilepsy (mTLE) patients. However, there is no strict definition or criterion regarding neuron loss and atrophy of the amygdala neighboring the hippocampus. We examined the relationship between HS and neuronal loss in the amygdala.

Materials and Methods: Nineteen mTLE patients with neuropathological proof of HS were assigned to Group A, while seven mTLE patients without HS were assigned to Group B. We used FreeSurfer software to measure amygdala volume automatically based on pre-operation magnetic resonance images. Neurons observed using Klüver-Barrera (KB) staining in resected amygdala tissue were counted. and the extent of immunostaining with stress marker antibodies was semiquantitatively evaluated.

Results: There was no significant difference in amygdala volume between the two groups (Group A: 1.41 ± 0.24; Group B: 1.41 ± 0.29 cm^3; p= 0.98), nor in the neuron cellularity of resected amygdala specimens (Group A: 3.98 ± 0.97; Group B: 3.67 ± 0.67 $10\times^{-4}$ number of neurons/μm^2; p= 0.40). However, the HSP70 level, representing acute stress against epilepsy, in Group A patients was significantly larger than that in Group B. There was no significant difference in the level of Bcl-2, which is known as a protein that inhibits cell death, between the two groups.

Conclusions: Neuronal loss and volume loss in the amygdala may not necessarily follow hippocampal sclerosis. From the analysis of stress proteins, epileptic attacks are as likely to damage the amygdala as the hippocampus but do not lead to neuronal death in the amygdala.

Keywords:

amygdala, Bcl-2, hippocampal sclerosis, HSP70, mesial temporal epilepsy

1. Introduction

Hippocampal sclerosis (HS) is the most common pathological characteristic identified in patients with mesial temporal lobe epilepsy (mTLE) who undergo surgery. Histopathologically, HS is characterized by segmental pyramidal neuron loss in CA1, CA3, and CA4, whereas neurons in CA2 are relatively seizure-resistant. Some longitudinal magnetic resonance imaging (MRI) studies have provided evidence that chronic seizures result in progressive hippocampal atrophy. A retrospective cross-sectional MRI study suggested a close correlation between hippocampal atrophy and the histopathological grades of the resected hippocampus (Fuerst et al., 2001). On the other hand, there is limited available data regarding neuronal loss in the amygdala associated with HS (Miller et al., 1994; Bernasconi et al., 2003). The involvement of the amygdala in epilepsy has not been explicitly established to date in relation to HS and mTLE. In this study, we investigated whether mTLE patients with and without HS experienced decreases in volume and cellularity of the amygdala and sought to understand the role of the amygdala in epileptic networks in mTLE.

2. Materials and methods

2.1. Subjects

This study investigated 26 patients (14 men, 12 women) who underwent anterior temporal lobectomies with amygdalo-hippocampectomies (ATL+ AH) for intractable drug-resistant mTLE. All patients were preoperatively evaluated by MRI, long-term scalp video-EEG monitoring, and intracranial-EEG if necessary. Interictal and ictal EEG findings and seizure semiology were consistent with temporal lobe epilepsies (TLEs). Neuropsychological tests were performed on all patients and the intracarotid sodium amobarbital procedure (ISAP; the Wada test) was performed as part of the preoperative routine.

Patients were divided into two groups based on whether there was neuropathological

proof of HS (Group A) or not (Group B). Nineteen patients (eleven men, eight women) were assigned to Group A and seven (three men, four women) to Group B.

The mean age of Group A at surgery was 26.9 ± 10.3 (10−42) years, and mean duration of epilepsy was 12.6 ± 8.1 (1−27) years. Seizure frequencies varied from 0.3 to 30 times per month. Specimens were resected from the right hemisphere of 12 of the 19 patients in Group A. The patients in Group B underwent ATL +AH based on a diagnosis of brain tumor or TLE with no neuropathological HS. Three patients in Group B had a tumor (ependymoma, cavernous angioma or dysembryoplastic neuroepithelial tumor), three had TLE without radiological or neuropathological detection of lesions, and the final patient had a history of encephalitis. In the three patients with tumors, the tumors did not involve the amygdala, according to the MRI findings. The mean age at surgery for Group B was 26.4 ± 16.5 (10−52) years, and the mean duration of epilepsy was 6.6 ± 3.8 (1−12) years. Seizure frequencies varied from 0.3 to 120 times per month. Specimens were resected from the right hemisphere of four of the seven patients in Group B.

We do not have autopsy controls. The protocols for this study were approved by the Nishi-Niigata Chuo National Hospital Ethics Committee. Informed consent for research obtained at pre-operation in all cases.

2.2. Clinical features

Descriptions of clinical seizure characteristics were obtained from patients' clinical history charts and long-term video-EEG recordings. Clinical data, including age at surgery, sex, duration from seizure onset, presence of secondary generalized tonic-clonic seizures (sGTC), number of anti-epileptic drugs, and history of febrile seizures were investigated. Postoperative seizure outcomes were evaluated six months after surgery. We classified seizure outcome according to Engel's classification: I, free

from seizure (excluding auras) since surgery; II, seizure frequency up to two times per year; III, reduction in seizure frequency greater than 75%; and IV, reduction in seizure frequency less than 75%. Clinical features of the patients in both groups are summarized in Table 1.

Duration from seizure onset in patients in Group A (12.6 ± 8.1 years) was significantly longer than in Group B (6.6 ± 3.8 years, $p < 0.05$). There were more patients with a history of febrile seizures in Group A (5/19 patients) than in Group B (0/7 patients, $p < 0.05$). The groups did not differ in other clinical features, including age, sex, number of patients with sGTC, number of anti-epileptic drugs at operation, and rates of Engel' s classification I.

2.3. MRI volumetry

To assess the volume of the amygdala, we performed quantitative morphometric analysis of T1-weighted MRI data using automated segmentation and a probabilistic region-of-interest labeling technique (FreeSurfer software version 5.3.0 https://surfer.nmr.mgh.harvard.edu). Image processing included removal of non-brain tissues with a hybrid watershed, surface deformation procedure, automated Talairach transformation, and segmentation of the subcortical white and gray matter (Fig. 1a). To examine the laterality of amygdala volume, the laterality index (LI) was calculated as follows: (operated side − contralateral side)/ (operated side + contralateral side).

2.4. Tissue preparation and counting

The *en bloc* amygdala specimens were fixed with phosphate-buffered 20% formalin and embedded in paraffin wax. Serial sections (4 μm) were then cut and stained with a Klüver-Barrera (KB) stain. Importantly, all sections were processed similarly to ensure standard staining conditions. We examined the sections under a microscope (BX53; Olympus, Tokyo, Japan). The images were captured by a digital camera (DP71;

Olympus). We counted neurons per square micrometer of gray matter within the amygdala. We distinguished neurons from glias or endothelial cells according to size and presence of Nissl bodies (Fig. 2a).

2.5. Immunohistochemistry

We examined the expression of two proteins, heat shock protein 70 (HSP70; Dako, Glostrup, Denmark) and B-cell lymphoma 2 (Bcl-2;Dako), using immunochemistry in the amygdala samples. HSP70 is a useful indicator of stressed neurons in the acute phase of epilepsy, but is not associated with neuronal death, thereby suggesting that HSP70 plays no role in neuroprotection during an epileptogenic state (Yang et al., 2008). In contrast, the Bcl-2 protein family is known to play a specific role in seizure-induced neuronal death and studies have found evidence that cell death controlled by the Bcl-2 protein family is functionally important in epileptogenesis (Henshall and Engel, 2013). To examine the qualitative differences between Groups A and B, we evaluated the proportion of positive neurons against these two stress markers semiquantitatively using a three-grade system: − (absent or almost absent); 1+ (partially positive neurons); and 2+ (mostly positive neurons) (Fig. 3).

2.6. Statistical analysis

Data analysis was carried out using SPSS version 23.0 (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± SD and differences between groups were estimated using unpaired t-test at a significance level of $p < 0.05$.

3. RESULTS

3.1. MRI volumetric analysis

Table 2 displays the amygdala volumes of the two groups. For the operated sides, the amygdala volume of Group A was 1.41 ± 0.24 cm^3 with a range of 0.97 to 1.85 cm^3, Group B was 1.41 ± 0.29 cm^3 with a range of 0.91 to 1.74 cm^3 (Fig. 1b). There was no

significant difference in amygdala volume between the two groups (p = 0.98). The LIs of Groups A and B were -0.03 ± 0.08 and 0.02 ± 0.15, respectively. The differences in LI between the two groups did not reach significance levels (Table 2, p = 0.51). The age at surgery did not correlate with amygdala volume (r = -0.32, p = 0.11).

3.2. Neuron cellularity of amygdala

According to Watson's classification (Watson et al., 1996), no patients in Group A were classified as Grade 1, one patient as Grade 2, nine patients as Grade 3, and nine patients as Grade 4 (Table 1). These findings correspond to the HS International League Against Epilepsy Type I (Blümcke I et al., 2013). Neuron cellularity of resected amygdala specimens in Group A was 3.98 ± 0.97 $10\times^{-4}$ neurons/μm^2 and that in Group B was 3.67 ± 0.67 $10\times^{-4}$ neurons/μm^2 (Fig. 2b). There was no significant difference between the two groups (p = 0.41). The age at surgery did not correlate with neuron cellularity in the amygdala (r = 0.19, p = 0.35).

3.3. Immunohistochemical findings

With respect to HSP70 indicating stress proteins in the amygdala, 11 patients (58%) in Group A were classified as 1+ and eight (42%) as 2+. There were no patients who had a complete or near-complete absence of positive neurons (Table 3). In contrast, two patients (29%) in Group B were classified as −, four (57%) as 1+, and one (14%) as 2+. HSP70 positive neurons were more frequent in Group A than in Group B (r^2 = 0.39, p < 0.05). There was no difference in the levels of Bcl-2 family proteins (known to inhibit cell death) between the two groups (r^2 = 0.05, p = 0.80).

4. Discussion

We compared the amygdala in TLE patients with and without HS radiologically and pathologically in this study. HS was not always associated with neuronal and volume

loss in the amygdala neighboring the hippocampus. However, the results of immunohistochemical studies suggest that amygdala with HS is likely to sustain more damaged than amygdala without HS.

HS based on MRI sometimes causes drug-resistant epilepsy amenable to surgical treatments. HS is characterized in pathological findings by neuronal loss and reactive gliosis in vulnerable segments of hippocampal formation. It represents the most frequent structural abnormalities in TLE (Mathon et al., 2015, Beh et al. 2016). In contrast to HS, there is only sparse information on the clinical relevance of amygdala sclerosis for TLE. The most likely reasons for the inconsistency of data on histopathological alterations in the amygdala is the complicated composition of the individual nucleus with numerous cortical and subcortical subnuclei and interspersed bundles of white matters (McDonald, 1992; Amaral et al., 1992). Additionally, major portions of the amygdala nucleus are sometimes aspirated during ATL+ AH and the small tissue fragments thus obtained are not suitable for detailed histopathological analysis. In this study also, we attempted to acquire the tissue as *en block* as possible, but did not resect the entire amygdala. Therefore, we were unable to identify what parts of the amygdala the neuronal cells we counted came from. Furthermore, this study has some limitations with regard to the heterogeneity of Group B, which consisted of patients with various etiologies (post encephalitis, tumor, or non-significant), although the lesions did not invade the amygdala. There are few opportunities to examine amygdala tissues post-surgery; however, further studies may be needed using samples with similar etiologies.

Earlier histopathological studies have reported that neuronal loss and gliosis in the amygdala have been proven in surgical specimens or autopsy cases (McMillan et al., 1987). Most often, neuronal loss and gliosis have been reported in combination with

hippocampal damage. In a surgical study, amygdala damage was reported in 81/92 (88%) of patients with HS (Bruton, 1988). Several studies in the 1990s reported that there were some patients with amygdala sclerosis in the absence of HS (Hudson et al., 1993; Miller et al., 1994). Some authors reported that there were no consistent correlations in surgical data between lesion patterns within the amygdala and neuropathological changes of the hippocampus (Yilmazer-Hanke et al., 2000). In the present study, we demonstrated that there was no significant difference in the extent of neuronal loss between patients with and without HS, albeit subject to the technical limitations of obtaining amygdala tissues noted above.

Volumetric measurements of the amygdala by MRI provided us with some findings about amygdala damage in vivo. MR volumetric measurements have shown that the reduction in amygdala volume varies from 10% to 30 % in patients with drug-refractory TLE (Cendes et al., 1993a,b,c; Saukkonen et al., 1994; Bronen et al., 1995; Kälviäinen et al., 1997). The most pronounced atrophy of the amygdala (30% volume reduction) was found in drug-refractory patients with TLE who had experienced prolonged febrile convulsions in childhood (Cendes et al., 1993a). In surgical data, amygdala damage as shown in MRI was present in 12% (7/52) of the patients with pathologically proven HS (Bronen et al., 1995). The authors reported some patients in whom amygdala damage was apparent in MR images but hippocampal atrophy was not noted (Cendes et al., 1993a). We found no significant difference in amygdala volume in pre-operative MRIs between HS positive and negative groups. Unlike the majority of manual volumetric assessment of the hippocampus and amygdala, automated methods such as FreeSurfer used in this study provided us with consistent results with repeated iterations on any given dataset.

It is assumed that repeated seizures lead to progressive damage and neuronal loss in vulnerable areas such as the hippocampus (Kälviäninen et al., 1997; Fuerst et al., 2001).

Although the exact molecular events responsible for cell death following seizure remain unknown, experimental studies in animal models of epilepsy and human brain tissues have revealed a role for apoptotic cell death pathways (Henshall and Simon, 2005). Recent studies suggest that HSP70 expression is likely to be associated with cellular stress in hippocampal neurons in patients with mTLE (Ryuhuku et al., 2011). In brain tissues obtained from TLE patients, HSP70 expression was induced in vulnerable neurons in the early phases of epileptogengesis. However, HSP70 expression was not detected in later stages, primarily due to the increased extent of neuronal death (Yang et al., 2008). On the other hand, the Bcl-2 protein family is known to play a specific role in seizure-induced neuronal death. In addition, the neuronal death that is controlled by the Bcl-2 protein family is functionally important in epileptpgeneisis (Henshall and Engel, 2013). The present study is the first to report the results of an investigation of the expression of HSP70 and Bcl-2 in human amygdala tissue from TLE patients. In our results, the Bcl-2 levels did not differ between patients with and without HS, whereas the HSP70 levels in patients with HS were greater than in patients without HS. These findings suggest that epileptic attacks lead to neuronal damage in both the amygdala and the hippocampus. Consequently, neuronal loss occurs in the hippocampus but not in the amygdala. In our study, patients in Group A had longer histories of epilepsy and more febrile seizures than those in Group B. The neuronal loss in the hippocampus and neuronal damage in the amygdala in patients with HS may be caused by an initial precipitating insult with prolonged febrile seizures and epileptic attacks during longer periods (Mathern et al., 2002; Henshall and Meldrum, 2012).

It is unclear why damage to the hippocampus is not parallel to damage to the amygdala. Recent studies have shown that selective alteration of each GABA receptor subtype exists in human TLE patients (Loup, et al., 2000). Differential rearrangements of $GABA_A$ receptor subtypes were seen in CA2, where neurons were relatively stable compared with CA1, CA3 and CA4. There may be a different GABA receptor subtypes

in the hippocampus and the amygdala. Other authors have demonstrated that some patients with imaging-negative TLE had significantly larger amygdala than those with HS based on MRI. The hippocampus and amygdala are likely to have different spreading pathways of epileptoform activity (Bower et al. 2003, Beh et al.2016). Further investigation is needed to clarify the relationship of the hippocampus and amygdala with epileptiform activity.

5. Conclusions

Our present findings indicate neuronal loss and volume loss in the amygdala may not necessarily follow HS. The analysis of stress proteins indicates the possibility that amygdala neurons do not lead to neuronal death; nevertheless, epilepsy attacks may damage the amygdala as well as the hippocampus. Though small sample size limits the generalizability of the findings, this information may suggest fruitful areas for future research to understand the relationship that underlies mTLE and the amygdala.

Conflicts of interest: none

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Figure captions

Fig. 1 Volumetry of the amygdala

a: An example of volumetry on the basis of preoperative magnetic resonance imaging. The red cross marks the amygdala on the right side.

b: Comparison of amygdala volume between Groups A and B.

A closed rhombus depicts individual volume of the amygdala. No significant differences in amygdala volume between two groups ($p = 0.99$) .

Fig. 2 Neuron counts in the amygdala

a: An example of neuron counts. Neurons counted manually in the area surrounded by the orange line.

b: Comparison of amygdala neuron cellularity between Groups A and B. No significant differences between the two groups ($p = 0.41$).

Fig. 3 Examples of HSP70 and Bcl-2 expression in the amygdala

We undertook semiquantitative analysis of positive neurons in HSP70 (upper row of Fig. 3). Positive neurons were absent or almost absent (-, Fig. 3a); partially present (1+, Fig. 3b); or mostly present (2+, Fig. 3c). An example of a neuron positive for HSP is indicated in the small box on the right-hand side of Fig. 3c. We undertook semiquantitative analysis of positive neurons in Bcl-2 (lower row of Fig. 3). Positive neurons were absent or almost absent (-, Fig. 3d); partially present (1+, Fig. 3e); or mostly present (2+, Fig. 3f). An example of a neuron positive for Bcl-2 is indicated in the small box on the right-hand side of Fig. 3f.

Table 1. **Clinical profiles of 26 patients with temporal lobe epilepsy**

No.	Age (y) /sex	Epilepsy duration (y)	Side	sGTC (+/-)	AEDs	FC (+/-)	MRI Lesion	SE (+/-)	Engel's classification	histopathology (Watson's grade)
1	10/M	6	Rt.	-	2	+	Rt.HHI	-	I	HS grade 4
2	14/M	4	Lt.	-	3	-	Lt. HHI	-	I	HS grade 3
3	14/F	1	Rt.	-	3	+	Rt. HA, HHI	-	I	HS grade 3
4	15/M	9	Rt.	-	3	+	Rt. HHI	-	I	HS grade 4
5	15/M	14	Lt.	-	3	-	Lt. HA, HHI	-	I	HS grade 4
6	20/F	9	Rt.	+	2	-	Bil. HHI	-	I	HS grade 3
7	23/F	6	Rt.	-	4	+	Rt. HA, HHI	-	I	HS grade 3
8	23/M	20	Rt.	+	2	-	Rt. HHI	-	I	HS grade 3
9	24/F	10	Rt.	+	3	-	Rt. HA	+	III	HS grade 3
10	27/M	24	Rt.	+	4	-	Rt. HA	-	I	HS grade 4
11	28/M	19	Rt.	-	2	-	Rt. HA, HHI	-	I	HS grade 3
12	32/F	5	Lt.	-	3	-	Lt. HA, HHI	-	I	HS grade 4
13	32/F	15	Rt.	-	2	-	Bil. HHI	+	I	HS grade 3
14	34/M	24	Rt.	+	2	-	Bil. HHI	+	I	HS grade 2
15	36/F	7	Rt.	+	2	+	Rt. HA, HHI	-	I	HS grade 4
16	39/M	24	Lt.	+	1	-	Lt. HA, HHI	-	I	HS grade 4
17	42/M	27	Lt.	+	2	-	not particular	+	I	HS grade 3
18	42/M	2	Lt.	-	2	-	Bil. HHI	-	II	HS grade 4
19	42/F	14	Lt.	-	3	-	Lt. HA, HHI	-	II	HS grade 4
20	10/F	1	Rt.	-	1	-	DNT	+	I	DNT
21	12/F	8	Lt.	-	3	-	not particular	+	I	no significant
22	15/M	8	Lt.	-	4	-	not particular	+	I	no significant
23	20/M	1	Rt.	-	3	-	Tumor	-	I	Ependymoma
24	25/M	8	Rt.	-	3	-	not particular	+	I	no significant
25	51/F	8	Lt.	+	4	-	Lt. HHI	+	III	post encephalitis
26	52/F	12	Rt.	-	3	-	CA	-	I	CA

Group A: patients 1–19; Group B: patients 20–26

Abbreviations: sGTC, secondary generalized tonic-clonic seizures; AEDs, anti-epileptic drugs; FC, febrile convulsion; SE, subdural electrodes; HHI, hippocampal hyper-intensity; HA, hippocampal atrophy; HS, hippocampal sclerosis; DNT, Dysembryoplastic neuroepithelial tumor; CA, cavernous angioma.

Table 2. **Volumetry and histological findings in the amygdala**

	MRI volumetry			Histological evaluation		
	amygdala volume (cm^3)			amygdala cellularity	immunohistochemistry	
No.	operation side	contralateral side	laterality index	($10\times^{-4}$ neurons/μm^2)	HSP70	Bcl-2
1	1.73	1.62	0.034	3.76	1+	-
2	1.42	1.39	0.009	4.83	2+	1+
3	1.23	1.73	-0.167	3.49	1+	1+
4	1.44	1.66	-0.073	4.58	1+	1+
5	1.85	1.47	0.114	2.62	2+	1+
6	0.99	1.15	-0.076	4.33	1+	-
7	1.54	1.53	0.0050	2.56	1+	1+
8	1.27	1.46	-0.070	4.10	2+	1+
9	1.69	1.53	0.051	4.06	1+	1+
10	1.36	1.35	0.005	3.15	2+	1+
11	1.73	1.53	0.063	2.53	1+	1+
12	0.98	1.62	-0.246	3.32	2+	1+
13	1.55	1.60	-0.016	3.04	1+	1+
14	1.38	1.28	0.037	5.62	1+	1+
15	1.38	1.40	-0.006	4.42	2+	1+
16	1.46	1.54	-0.027	4.98	2+	1+
17	1.35	1.40	-0.016	5.93	2+	1+
18	1.50	1.65	-0.048	3.60	1+	1+
19	0.97	1.22	-0.114	4.68	1+	1+
20	1.54	1.28	0.091	4.19	1+	-
21	1.62	1.64	-0.006	3.67	-	1+
22	0.91	1.70	-0.304	3.06	1+	1+
23	1.67	1.50	0.055	3.35	1+	1+
24	1.74	1.64	0.030	4.42	2+	1+
25	1.13	0.69	0.246	2.57	1+	1+
26	1.25	1.23	0.008	4.45	-	1+

Group A: patients 1−19; Group B, patients 20−26

Table 3. **Results of immunostaining with stress markers**

marker	Group	- n(%)	+ n(%)	++ n(%)	p-Value
HSP70	**A**	0(0)	11(57.9)	8(42.1)	<0.05
	B	2(28.6)	4(57.1)	1(14.3)	
Bcl-2	**A**	2(10.5)	17(89.5)	0(0)	0.80
	B	1(14.3)	6(85.7)	0(0)	