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Granular cell dispersion and bilamination: two distinct histopathological patterns in epileptic hippocampi?

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ABSTRACT – Cytoarchitectural modifications of the dentate gyrus are among the most obvious abnormalities observed in the hippocampal sclerosis associated with refractory epilepsy. Here, we examined the morphological changes of granular cells (dispersion, bilamination and cell loss) in sclerotic hippocampi from nine TLE patients, comparing abnormal and preserved areas. A total of 2 577 granular cells were analyzed with respect to four different histopathological patterns: areas with bilamination (n = 936), areas with dispersion (n = 905), areas with neuronal loss (n = 279), and preserved areas (n = 457). Quantitative parameters included somatic perimeter *(P)*, area *(A)* and form factor *(ff)*.Although different patterns were often observed in the same patient, highly significant differences were observed ($p < 0.0001$) when patterns were compared to one another. Since granular cell dispersion and bilamination have different morphological aspects in sclerotic hippocampi from TLE patients, we suggest that these patterns should be considered separately. Future studies are needed to determine the frequency with which these patterns occur in the general population and whether each one can interfere with seizure susceptibility.

Key words: hippocampal sclerosis, refractory epilepsy, immunocytochemistry, morphometry, temporal lobe

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The dentate gyrus is believed to play a key role in the pathogenesis of temporal lobe epilepsy (TLE) associated with hippocampal sclerosis (HS). Lesions in this anatomical structure range from granule cell dispersion to severe cell loss accompanied by reactive cellular and fibrilary gliosis. Severe granule cell dispersion has been observed in 40% of HS cases, in 10% of the cases combined with a focal or extensive "bilaminar arrangement" of neurons (Thom *et al.* 2002). Although this particular arrangement of granular neurons has been recognized by many investigators, it is generally conside-

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red to be a mere variation of the classic granular cell dispersion.
In the present investigation, we performed a morphological analysis of HS specimens from refractory TLE patients, comparing different histopathological patterns of the granular cell layer.

Methods

Surgical specimens from TLE patients with mesial temporal sclerosis (MTS) diagnosed on pre-surgical MRI and routine histopathological examination were studied. Only patients in which all the subfields of the hippocampus were present and adequately oriented were included in the present investigation. All included patients (n = 9) underwent detailed anamnesis, video-EEG recordings and MRI studies. Clinical data included patient's age at epilepsy-onset, age at surgery, seizure frequency, epilepsy duration and surgical outcome according to Engel's classification one year after surgery *(table 1)*. Histological processing followed standard protocols. Briefly, hippocampi were fixed in 4% paraformaldehyde for 36-48 hours at 4°C. Each hippocampus was carefully oriented and cut into 4-5 slabs, which were vibratome-sectioned. Because sections cut tangentially to the principal cell layers or at inconsistent angles from the longitudinal axis of the hippocampus can produce unusual histological features, considerable care was taken to ensure that all hippocampal slabs were cut along a plane strictly perpendicular to the longitudinal axis of the hippocampus. Only hippocampi presenting all subfields and which were adequately oriented were included in the present investigation. Fifty-micron sections were processed alternatively for cresyl violet staining or immunocytochemistry against monoclonal Neu-N to reveal the cytoarchitectural organization of the tissue and granular cell profiles. Free-floating sections were treated with 3% H_2O_2 for 10 minutes, washed in phosphate-buffered saline (PBS), pre-incubated with 10% normal horse serum (NHS) in 0.4% Triton X-100 PBS for 45 minutes and then incubated overnight at 4°C with monoclonal primary antibody anti-NeuN (1:1000, Chemicon). Sections were then washed in PBS, incubated at room temperature with biotinylated secondary anti-mouse IgG (1:200 in NHS 1%, Vector) for one hour, washed in PBS, incubated in avidin-biotin complex peroxidase (ABC, Vectastain, Vector) for one hour, washed several times in PBS and incubated with 0.075% diaminobenzidine (DAB, Sigma) in 0.002% H_2O_2. Sections were finally washed in PBS, mounted in gelatin-coated slides, dehydrated, covered and observed with light microscopy using a 40X magnification objective.

Morphological analysis

The morphology of the granular cells was quantitatively analyzed using the Neurolucida system (MicroBrightField, USA). This consists of a microscope equipped with a high resolution display monitor coupled to the drawing tube and a high resolution motorized stage, allowing for perfect merging and a continuous alignment of the image of the histological section with the digital drawing, as the latter proceeds across the section. Only granular cells with clearly identified contours were analyzed. The morphological parameters included: perimeter of the cellular body *(P)*, somatic area *(A)*, and somatic form factor *(ff)*, estimated as the ratio between largest and smallest somatic axes. A total of 2 577 granular cells were analyzed respective to the four different histopathological patterns *(figure 1)*: areas with bilamination (n = 936), areas with dispersion (n = 905), areas with neuronal loss (n = 279), and preserved areas (n = 457). Statistical analysis was performed using the Kruskal-Wallis (KW) test and Dunn's test for multiple comparisons.

Table 1. Pre-surgical and post-surgical data of the patients.

Case	*Age at epilepsy onset (years)*	*Epilepsy duration (years)*	*Seizure frequency (per month)*	*MR diagnosis*	*Volume of the affected hippocampus (cm^3)*	*Age at surgery (years)*	*Pathological-diagnosis*	*GC bilamination*	*GC dispersion*	*GC neuronal loss*	*Engel's classification (1 year)*
1	15	18	~ 4	B MTS	1.13	33	R AHS	+	+	+	IB
2	12	38	~ 5	L MTS	0.73	50	L AHS	+	-	++	IB
3	14	23	~ 4	L MTS	1.36	37	L AHS	+	+	+	IB
4	35	14	~ 3	R MTS	0.82	49	R AHS	+	+	+	IA
5	14	15	~ 5	L MTS	0.78	29	L AHS	+	+	+	IA
6	23	28	~ 3	R MTS	1.32	51	R AHS	+	+	++	IA
7	12	9	~ 2	L MTS	1.46	21	L AHS	+	+	-	IA
8	6	23	~ 3	R MTS	0.70	29	R AHS	+	+	+	IB
9	8	26	~ 6	L MTS	-	34	L AHS	+	+	++	II

MR: magnetic resonance; R: right; L: left; B: bilateral; MTS: mesial temporal sclerosis; AHS: Ammon's horn sclerosis; GC: granular cell. GC bilamination and GC dispersion: (+) present, (-) absent. GC neuronal loss: (++) severe, (+) mild, (-) absent.

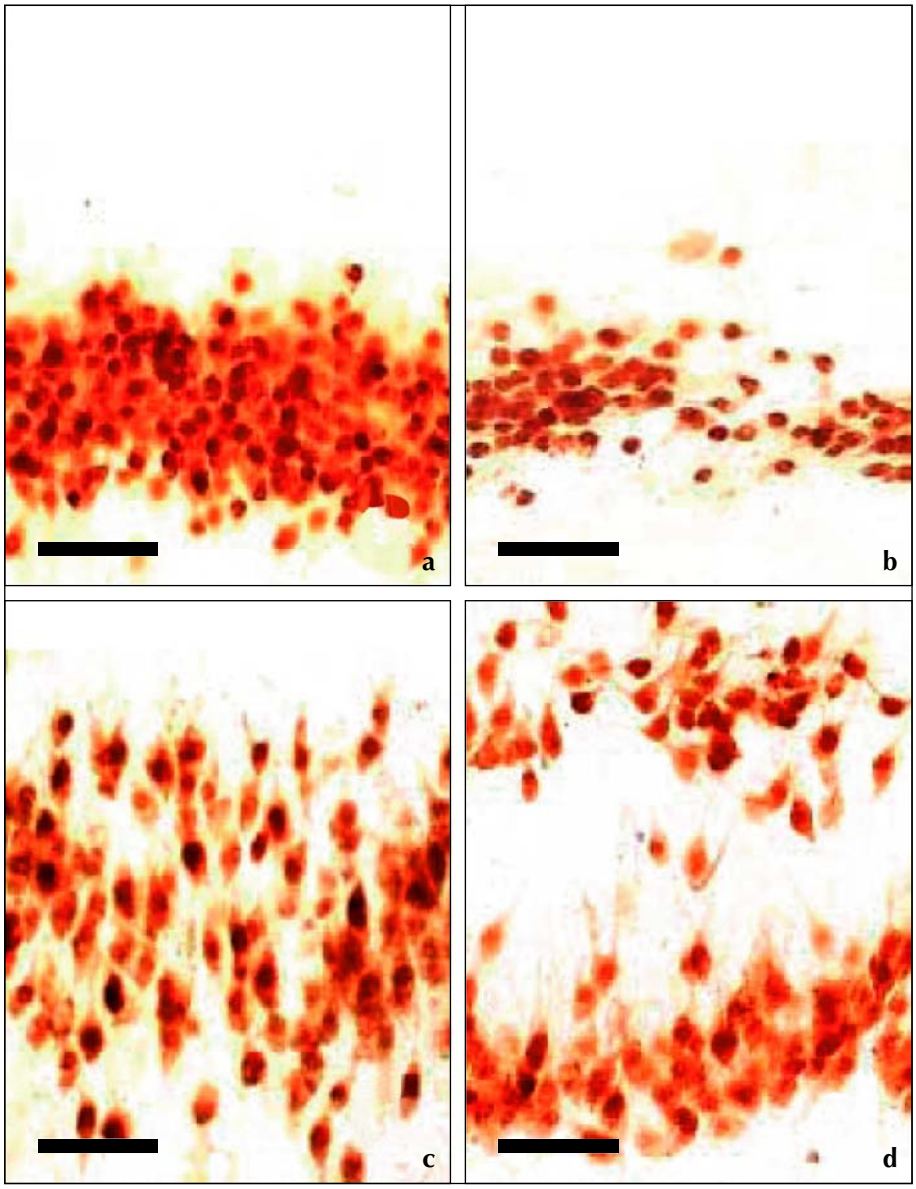

Figure 1. Different histopathological patterns observed in granular cell layer using anti-NeuN immunostaining.
A) Preserved areas, **B**) areas with neuronal loss, **C**) areas with dispersion, **D**) areas with bilamination. Scale bar = 50 µm.

Results

Significant differences between studied areas were observed, particularly considering granule cell perimeter *(P)* and somatic form factor *(ff)* (KW test, $p < 0.0001$) *(tables 2* and *3)*. Granular cellsin dispersion areas differed from those in preserved areas with respect to size *(P)* and shape *(ff)* (Dunn's test, $p < 0.001$). Somatic profiles in these regions were smaller and thinner when compared with preserved areas (*table 2*, *figure 2*). On the other hand, granular cells in bilamination areas differed from those in preserved areas in shape (Dunn's test, $p < 0.001$), but not in size (Dunn's test, $p > 0.05$). In bilamination areas, granular cells showed a rounder profile when compared to the elliptic profile observed in preserved areas *(table 2*, *figure 2)*. Correspondingly, quantitative analysis showed that dispersion and bilamination areas differed from each other in size (Dunn's test, $p < 0.05$) and shape of somatic profiles (Dunn's test, $p < 0.001$) (*table 3*). Granular cell morphology in areas with neuronal loss was similar to that observed in preserved areas *(table 2*, *figure 2)*.

Table 2. Comparison of granular cell morphology in regions with different cytoarchitectural patterns (Kruskal-Wallis test/non parametric ANOVA).

	Normal (n = 457)	Bilamination (n = 936)	Cell loss (n = 279)	Dispersion (n = 905)	p value
Perimeter	51.8 ± 9.7 µm	51.0 ± 10 µm	52.7 ± 10 µm	49.7 ± 9.7 µm	p < 0.0001 (*)
Area	291 ± 260 µm^2	249.1 ± 219 µm^2	263 ± 279 µm^2	345.4 ± 317 µm^2	p > 0.05 (ns)
Form Factor	0.71 ± 0.13	0.76 ± 0.13	0.71 ± 0.14	0.65 ± 0.13	p < 0.0001 (*)

(*): significant, (ns): non-significant.

Discussion

The dentate gyrus granule cell population displays pathological alterations in almost 50% of TLE patients with hippocampal sclerosis (HS), but the mechanisms that underlie these alterations remain little understood. For instance, some authors observed that granule cell dispersion (GCD) is associated with early seizure onset or status epilepticus at an initial stage of the disease (Sagar and Oxbury 1987, Houser *et al.* 1992). Conversely, other studies showed that the severity of GCD does not correlate with the age-at-first seizure nor with the history of a precipitating event for epilepsy, but correlates closely with the degree of hippocampal neuronal loss (Thom *et al.* 2002, Blümcke *et al.* 2002). In a recent *post-mortem* study, obvious GCD was noted in 86% of the HS cases (n = 8) (Thom *et al.* 2005). Nevertheless, thickening of the granule cell layer or focal GCD was also observed in 43% of the non-HS cases and 30% of the controls. The authors also noted that GCD is often seen bilaterally in TLE patients, even in those with unilateral hippocampal sclerosis (Thom *et al.* 2005). Although GCD is more severe in HS cases, these data suggest that GCD could be generated by a mechanism not exclusively related to HS. Moreover, the observation of GCD in patients with widespread cortical malformations but no hippocampal cell loss, suggests the possibility that a developmental malformation might cause the dentate disorganization (Harding and Thom, 2001).

While GCD is widely discussed in the literature, much less attention has been given to granular cell bilamination (GCB). GCB has been described as a focal or complete duplication of the granular cell layer, in which an additional band of granular cells can be observed above the ordinary granular layer, with a thin stripe of white matter in between. Rougier and colleagues recently reported a complete GCB in a four-year-old infant undergoing temporal lobectomy for the treatment of refractory TLE associated with hippocampal sclerosis (Rougier *et al.* 2003). In a retrospective analysis of sclerotic hippocampi from different epilepsy centers, Blümcke and colleagues observed a "bilaminar arrangement" of the granular cells in 10% of cases showing severe GCD (Blümcke *et al.* 2002).

There is a normal tendency to assume that the structural features of the hippocampus will be relatively uniform throughout its length. Given this assumption, investigators often do not carry out a detailed examination throughout all sections, nor an extensive sampling spanning from the head to the tail of every hippocampal specimen. Therefore, it is possible that the analysis of a limited number of hippocampal sections during routine histopathological examination contributes to an underestimation of the occurrence of focal GCB. Moreover, since hippocampal sections cut tangentially to the principal cell layers might produce unusual histological features, GCB may have been noted by many investigators but dismissed as artifacts of the sectioning process.

In the present investigation, we observed that GCD and GCB have different morphological aspects in sclerotic hippocampi from TLE patients. In areas of bilamination, granular cells present normal dimensions with a rounder morphology, while in areas with dispersion, granular cells are smaller and thinner. Therefore, we suggest that these patterns should be considered separately. Future studies

Table 3. Comparison of cell size and form among different cytoarchitectural patterns (Dunn's test for multiple comparisons).

	Perimeter	Form factor
Normal *versus* bilamination	p > 0.05 (ns)	p < 0.001 (*)
Normal *versus* dispersion	p < 0.001 (*)	p < 0.001 (*)
Normal *versus* cell loss	p > 0.05 (ns)	p > 0.05 (ns)
Bilamination *versus* dispersion	p < 0.05 (*)	p < 0.001 (*)
Bilamination *versus* cell loss	p > 0.05 (ns)	p < 0.001 (*)
Dispersion *versus* cell loss	p < 0.001 (*)	p < 0.001 (*)

(*): significant, (ns): non-significant differences.

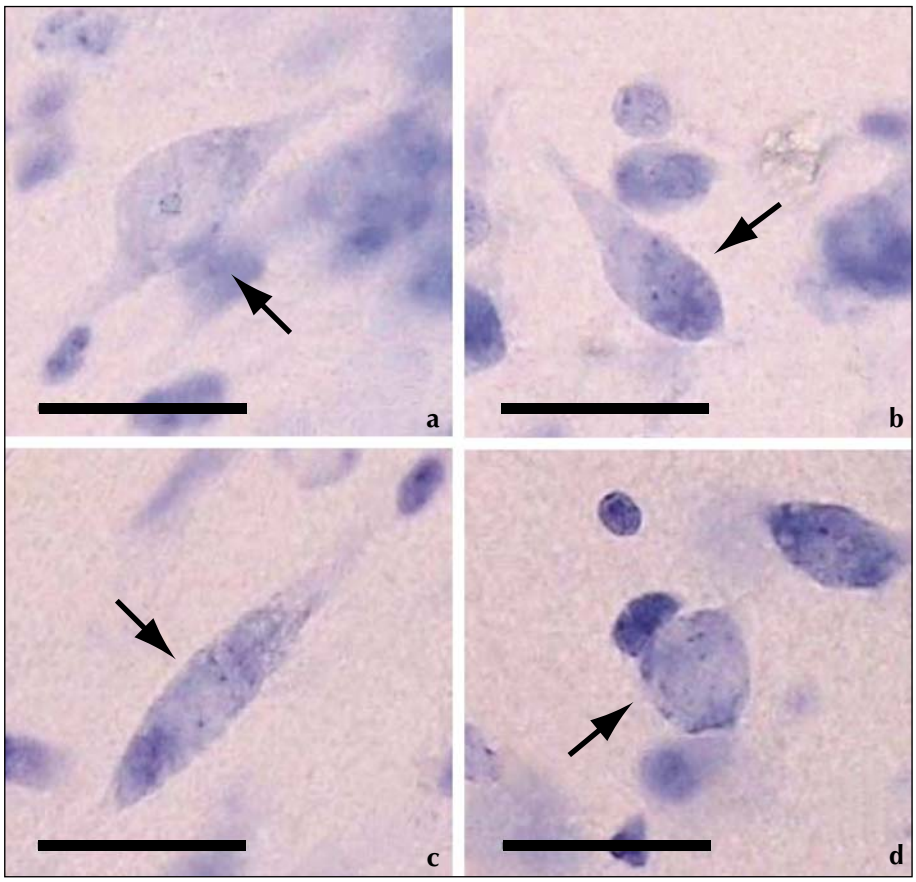

Figure 2. Nissl-stained granular cells in areas with different histopathological patterns.
A) Preserved areas, **B**) areas with neuronal loss, **C**) areas with dispersion, **D**) areas with bilamination. Scale bar = 25 µm.

are needed to determine the rate at which GCB occurs in the general population and in patients with epilepsy, and whether this abnormality can interfere with seizure susceptibility and establishment of hippocampal sclerosis. □

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