FULL-LENGTH ORIGINAL RESEARCH

*Epilepsia*

# Drebrin expression patterns in patients with refractory temporal lobe epilepsy and hippocampal sclerosis

Thaís Caroline Dallabona Dombroski[1] | Jose Eduardo Peixoto-Santos[2] | Karina Maciel[3] | Munira Muhammad Abdel Baqui[4] | Tonicarlo Rodrigues Velasco[5] | Americo Ceiki Sakamoto[5] | João Alberto Assirati[6] | Carlos Gilberto Carlotti[6] | Hélio Rubens Machado[6] | Gleice Kelly de Sousa[7] | Kenji Hanamura[8] | João Pereira Leite[9] | Jaderson Costa da Costa[10] | André Luiz Palmini[10] | Eliseu Paglioli[11] | Luciano Neder[12] | Roberto Spreafico[13] | Tomoaki Shirao[8] | Rita Garbelli[13] | Antonio Roberto Martins[3,14]

[1]Discipline of Physiology, Federal University of Triângulo Mineiro, Uberaba, Brazil

[2]Discipline of Neuroscience, Department of Neurology and Neurosurgery, Paulista Medical School, UNIFESP, São Paulo, Brazil

[3]Department of Pharmacology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil

[4]Department of Cellular and Molecular Biology and Pathogenic Bioagents, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil

[5]Ribeirao Preto Epilepsy Surgery Center, Clinics Hospital, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil

[6]Department of Surgery, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil

[7]Graduate Program of Health Sciences, Federal University of Triângulo Mineiro, Uberaba, Brazil

[8]Department of Neurobiology and Behavior, Gunma University Graduate School of Medicine, Maebashi, Japan

[9]Department of Neurosciences and Behavioral Sciences, Ribeirão Preto

## Abstract

**Objective:** Drebrins are crucial for synaptic function and dendritic spine development, remodeling, and maintenance. In temporal lobe epilepsy (TLE) patients, a significant hippocampal synaptic reorganization occurs, and synaptic reorganization has been associated with hippocampal hyperexcitability. This study aimed to evaluate, in TLE patients, the hippocampal expression of drebrin using immunohistochemistry with DAS2 or M2F6 antibodies that recognize adult (drebrin A) or adult and embryonic (pan-drebrin) isoforms, respectively.

**Methods:** Hippocampal sections from drug-resistant TLE patients with hippocampal sclerosis (HS; TLE, n = 33), of whom 31 presented with type 1 HS and two with type 2 HS, and autopsy control cases (n = 20) were assayed by immunohistochemistry and evaluated for neuron density, and drebrin A and pan-drebrin expression. Double-labeling immunofluorescences were performed to localize drebrin A–positive spines in dendrites (MAP2), and to evaluate whether drebrin colocalizes with inhibitory (GAD65) and excitatory (VGlut1) presynaptic markers.

**Results:** Compared to controls, TLE patients had increased pan-drebrin in all hippocampal subfields and increased drebrin A–immunopositive area in all hippocampal subfields but CA1. Drebrin-positive spine density followed the same pattern as total drebrin quantification. Confocal microscopy indicated juxtaposition of drebrin-positive spines with VGlut1-positive puncta, but not with GAD65-positive puncta. Drebrin expression in the dentate gyrus of TLE cases was associated negatively with seizure frequency and positively with verbal memory. TLE patients with lower

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Medical School, University of São Paulo, Ribeirão Preto, Brazil

[10]Department of Internal Medicine, School of Medicine, Epilepsy Surgery Program and Brain Institute, Pontifical Catholic University of Rio Grande do Sul, Porto Alegre, Brazil

[11]Department of Surgery, School of Medicine, Pontifical Catholic University of Rio Grande do Sul, Porto Alegre, Brazil

[12]Department of Pathology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil

[13]Clinical Epileptology and Experimental Neurophysiology Unit, Scientific Institute for Research and Health Care Foundation Carlo Besta Neurological Institute, Milan, Italy

[14]Institute for Neuroscience and Behavior, Ribeirão Preto, Brazil

**Correspondence**
Thais Caroline Dallabona Dombroski, Centro Universitário de Várzea Grande, Av Dom Orlando Chaves, 2655, 78118-000 - Várzea Grande, MT, Brazil.
Email: thais.dombroski@univag.edu.br

Jose Eduardo Peixoto-Santos, Department of Neurology and Neurosurgery, Paulista Medical School, Unifesp, Rua Pedro de Toledo, 669, 04039-032 - São Paulo, SP, Brazil.
Email: peixoto.santos@unifesp.br

drebrin-immunopositive area in inner molecular layer (IML) than in outer molecular layer (OML) had a lower seizure frequency than those with higher or comparable drebrin-immunopositive area in IML compared with OML.

**Significance:** Our results suggest that changes in drebrin-positive spines and drebrin expression in the dentate gyrus of TLE patients are associated with lower seizure frequency, more preserved verbal memory, and a better postsurgical outcome.

**KEYWORDS**
dendritic spines, memory deficit, seizure frequency, surgery outcome, synaptic plasticity

# 1 | INTRODUCTION

Up to 30% of temporal lobe epilepsy (TLE) patients have drug-resistant seizures with variable frequency and significant cognitive and memory deficits.[1,2] For drug-resistant TLE cases, surgical resection of temporal lobe structures is recommended for seizure control.[3,4] During epileptogenesis (ie, between the initial injury and seizure recurrence), several plastic changes occur in the hippocampal formation, culminating with the establishment of hippocampal sclerosis (HS).[5] HS is characterized by neuron loss, often severe in

> **Key Points**
> - Drebrin expression is increased in the hippocampus of TLE and presents differential patterns of expression in the molecular layers
> - Increased drebrin in the inner molecular layer is associated with lower seizure frequency and better verbal memory in HS 1 cases
> - Drebrin patterns in the molecular layers are associated with seizure frequency and postsurgical outcome

CA1 and CA4, moderate loss in the granule cell layer (GCL), and preservation of the subiculum (SUB) density.[6] With the loss of CA4 neurons during the silent period, the glutamatergic mossy fibers (ie, axons from GCL) undergo synaptic reorganization from the CA4, mainly to the inner molecular layer (IML) of the dentate gyrus.[7] It has been suggested that synaptic reorganization contributes to neuron synchronization and seizure generation.[8]

Several molecules are involved in brain plasticity, among them developmentally regulated brain protein or drebrin,[9,10] which occur in humans as two isoforms, embryonic (E) and adult (A).[11] Drebrin E is expressed during development, being gradually replaced by drebrin A. In adults, drebrin A occurs in the cerebral cortex, amygdala, and hippocampus, is neuron-specific, and predominates in dendritic spines.[12] Drebrin participates in the maintenance of dendritic spines under α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated excitatory input.[13]

Drebrin A forms a complex with filamentous actin, among other proteins,[14] and participates in spine morphogenesis and plasticity.[15] Drebrin A participates in higher brain functions, which include learning and cognition,[16] and in diseases such as Alzheimer disease[17] and its animal models,[18] Down syndrome,[19] amyotrophic lateral sclerosis,[20] and animal models of chronic epilepsy.[21]

Drebrin A plays a pivotal role in the structure-based plasticity of dendritic spines in excitatory synapses,[10] and synaptic reorganization has important implications in seizure generation and cognitive deficits observed in TLE patients.[7] Thus, the objective of the present work was to evaluate the expression of drebrin A in the hippocampus of drug-resistant TLE patients, and its possible consequences.

## 2 | MATERIALS AND METHODS

### 2.1 | Patients and clinical data

Drug-resistant TLE (n = 33) patients were evaluated at the Ribeirao Preto Epilepsy Surgery Program of the Ribeirão Preto Medical School, University of São Paulo and the Clinical Epileptology and Experimental Neurophysiology Unit, Carlo Besta Neurological Institute, Milan, Italy, using standardized protocols approved by the ethics committees of our institutions, which evaluated and approved the use of biopsies and publication of the study results, on the basis of the informed, written consent given by the patients. Cases for this study were from patients with drug-resistant epilepsy who underwent surgical resection of the epileptogenic region for treatment and presented with HS in the pathological assessment. A control group (control, n = 20) consisted of autopsy cases without a history of neurological or psychiatric diseases, and without evidence of brain pathology at systematic

autopsy evaluation. Autopsy cases had time between death and tissue collection < 12 hours, and causes of death were pulmonary disease (n = 7), acute myocardial infarction (n = 4), cancer (n = 4), multiple organ failure (n = 2), and acute mesenteric ischemia, pancreatitis, and sepsis (n = 1 each). Patients' clinical data were collected from their medical records. The clinical variables assessed comprised age at death (control) or at surgery (TLE), cause of death (control), epilepsy duration, age at seizure recurrence, seizure frequency, initial precipitating injury (IPI) presence, IPI type, age at IPI, memory, intelligence quotient (IQ), and surgical outcome (TLE). Surgical outcome was defined following the new International League Against Epilepsy (ILAE) classification.[22] Presurgical memory was evaluated in TLE patients with the following tests: logic memory from Wechsler Memory Scale–Revised (WMS-R) and Rey Auditory Verbal Learning Test for the evaluation of verbal memory, and visual reproduction (WMS-R) and Rey Visual Design Learning Test for nonverbal memory. The patient's score on each test was classified according to age- and sex-matched population results, and the patients were classified in the classes "below average" or "average or above" for verbal and nonverbal results after averaging the results of the tests. IQ was defined according to Wechsler Adult Intelligence Scale III.

### 2.2 | Tissue processing and immunohistochemistry/ histochemistry protocols

Coronal hippocampal slices were fixed in 4% (weight/volume) buffered formaldehyde overnight and paraffin embedded. Five-micrometer-thick sections were submitted to immunohistochemistry for the detection of drebrin with mouse monoclonal anti–pan-drebrin antibody (clone M2F6), which recognizes both adult and embryonic drebrin isoforms,[9] and rabbit polyclonal anti–drebrin A antibody (clone DAS2), which recognizes only drebrin A (Immuno-Biological Laboratories), following a published protocol.[23] Pan-drebrin, as used here, refers to drebrin epitope(s) detected by the M2F6 antibody in immunohistochemistry experiments. Briefly, sections were microwave-treated in 50 mmol·L$^{-1}$ Tris-HCl buffer, pH 9.0, incubated with endogenous peroxidase block solution, followed by overnight incubation with primary antibodies in blocking buffer, which consisted of 50 mmol·L$^{-1}$ Tris-HCl buffer, pH 7.5 with 0.03% Triton X-100 (New England Nuclear), 15% normal goat serum, and 3% bovine serum albumin (Sigma 0643). Primary antibody detection was performed with secondary biotinylated antibodies (swine antirabbit IgG for DAS2 [Dako code E0353] and rabbit antimouse IgG for M2F6 [Dako code E0354]), ABC Elite Kit (Vector PK-6101), and 3,3'-diaminobenzidine tetrahydrochloride (Pierce 34001) as the chromogen. As controls for nonspecific staining, some sections

were incubated with the omission of the primary antibodies. Moreover, every incubation experiment contained one or two sections that were included in all experiments, to provide for inter- and intra-assay controls. Adjacent hippocampal sections were submitted to hematoxylin-eosin staining for the evaluation of neuron density.

## 2.3 | Confocal microscopy

Immediately after resection, a small hippocampus fragment was cut in the coronal plane, embedded in Tissue-Tek compound (Sakura Finetek), submerged for 20-25 seconds in 2-methylbutane cooled with dry ice, removed, and stored at −80°C until use. Frozen fragments were sectioned at 20 μm, using a cryostat (Leica model CM3050 S), and placed on gelatin-coated glass slides (Ted Pella). Immunofluorescence localization of drebrin was performed with a protocol similar to that used for immunohistochemistry, except for the omission of peroxidase block and detection steps. DAS2 polyclonal antibody against drebrin A was incubated with monoclonal antibodies against microtubule-associated protein (MAP2; clone AP20, code MAB3418, Millipore), a marker of dendritic branches; vesicular glutamate transporter 1 (VGlut1; clone N28/9, code 73-066, University of California, Davis/National Institutes of Health NeuroMab Facility), a presynaptic marker of excitatory synapses; or glutamic acid decarboxylase 65-kDa isoform (GAD65; mouse anti-GAD65, clone N-GAD65, Sigma-Aldrich), a marker of inhibitory synapses. Secondary antibodies used for the visualization were goat anti-mouse IgG conjugated with Alexa 594 and goat anti-rabbit IgG conjugated with Alexa 488 (both from Molecular Probes). Slices were examined by a Leica TCS-SP5 confocal microscope (Multiuser Laboratory of Confocal Microscopy [LMMC], Medical School of Ribeirão Preto, University of São Paulo) using argon (488-nm excitation) and helium/neon (594-nm excitation) excitation laser lines. Images were acquired at 1024 × 1024-pixel resolution, collecting z-optical sections at a 0.1-0.3 μm interval with the pinhole set at 1 Airy. Specificity of immunostaining was confirmed by control experiments with the omission of the primary antibody. The colocalization rate (%) of a selected area was done with the confocal Leica SP5 program (LMMC), and the same program calculated the Pearson correlation coefficient. Pictures were exported in TIFF format and processed with Adobe Photoshop CS5 for brightness and contrast adjustments.

## 2.4 | Immunohistochemistry/histochemistry evaluation

Images from the regions of interest were collected with an AxioCamMR5 attached to an AxioImager M1 microscope and fed to AxioVision 4.8.1 software (Zeiss). All images were obtained with 200× magnification and constant illumination. Image analysis was performed with ImageJ 1.49 software (National Institutes of Health). Semiquantitative analysis of drebrin-immunopositive area fraction (ie, the percentage of immunopositive area within the regions of interest) was obtained using a threshold tool, according to published protocols.[24,25] Briefly, all pixels within the region of interest whose intensities were equal to or higher than the threshold were considered positive, whereas those below the threshold were considered negative. The results were presented as the percentage of positive pixels within the region of interest. The thresholds used were 120 for drebrin A and 130 for pan-drebrin. The hippocampal regions evaluated for drebrin area fraction were outer molecular layer (OML), IML, GCL, subgranular zone (SGZ), CA4, CA3, CA2, CA1, prosubiculum (PRO), and SUB. Similarly to previous studies,[7,26] we subdivided the molecular layer into three regions of equal thickness, and considered to be IML the third closest to the granule cell layer, whereas the middle and outer thirds were considered OML. Additionally, patients were classified according to differences in immunopositive area in the IML and OML. For this, the drebrin-immunopositive area of the IML was divided by the immunopositive area of the OML, and patients were classified as those with lower IML staining (values < 0.95) and those with IML staining equal to or higher than the OML (values ≥ 0.95). The abovementioned classification was done independently for drebrin A and pan-drebrin, because the staining pattern seen with the DAS2 antibody was not always equal to the M2F6 antibody.

Additionally, the quantification of dendritic spines positive for drebrin A and pan-drebrin was performed in the OML, IML, GCL, SGZ, and the dendritic and pyramidal layers of the SUB using the threshold and analyze particles tool. Figure S1 shows a representative image of drebrin-positive puncta (ie, drebrin-positive dendritic spines) count. The neuronal population was evaluated in hippocampal sections stained with Harris' Hematoxylin (Merck) and Eosin Yellow-Phloxine B (Merck) by an experienced neuroscientist (J.E.P.-S.). Neurons were counted, and neuron density was estimated according to Abercrombie's method[27] in the GCL, CA4, CA3, CA2, CA1, PRO, and SUB. All hippocampi from TLE patients were classified according to the ILAE's HS types.[6]

## 2.5 | Statistics

For parametric variables, Student $t$ test was performed, whereas Mann-Whitney was used for nonparametric data. Categorical data were evaluated with $\chi^2$ or Fisher exact test, according to the number of observations in each category of the contingency table. Pearson correlation coefficient was

used to evaluate correlations of neuron density and clinical variables with drebrin expression.

# 3 | RESULTS

## 3.1 | Clinical data

Control cases were significantly older than TLE patients (64 ± 19 years vs 37 ± 10 years, Mann-Whitney, $P < .001$). Sex was balanced between groups (45% female in controls vs 55% in TLE, $\chi^2$, $P = .696$). Twenty of the 33 TLE patients had IPI early in life (26 ± 15 months of age), the most frequent being febrile and afebrile seizures (nine patients each), followed by meningitis and traumatic brain injury (one each). Recurrent seizures started at 12 ± 7 years of age, with a frequency of 9 ± 8 seizures per month. Pathology evaluation revealed HS type 1 in 31 patients and HS type 2 in two. Verbal memory was below average in 61% of the patients, and nonverbal memory was below average in 66%. Postsurgical follow-up was of 8 ± 7 years, and the outcome was ILAE 1 in 11 patients, ILAE 2 in seven, ILAE 3 in three, ILAE 4 in four, ILAE 5 in six, and ILAE 6 in two.

## 3.2 | Immunohistochemical localization of drebrin in the hippocampus of TLE patients

Moderate drebrin A staining in TLE was seen in the dentate gyrus, whereas the remaining hippocampal subfields presented weak staining (Figure 1A). In contrast, TLE cases showed a strong pan-drebrin expression in the dentate gyrus, CA4, CA3, CA2, and SUB, whereas CA1 was weakly stained (Figure 1B). Strong dendritic staining was evident in the molecular layers, CA2, and SUB (Figure 1B). In summary, pan-drebrin showed a stronger expression pattern when compared to drebrin A.

## 3.3 | Confocal analysis of drebrin A expression in the dentate gyrus of TLE patients

Double labeling of hippocampal sections from TLE-HS patients with DAS2 (green) and anti-MAP2 (red) antibodies (Figure 2A-M) showed a conspicuous expression of drebrin A in the GCL and in the molecular layers (Figure 2A-C) that colocalized ($r = 0.6727$, Pearson) with MAP2 (Figure 2D and insert). High-magnification images (Figure 2E-G of the region indicated in C) showed drebrin A expression in dendritic spines that emerged from MAP2-positive dendritic branches (arrowheads in Figure 2H, J, which are higher magnifications of the region in the small square of G). Dendrites exhibited degeneration signs such as vacuoles (arrows in Figure 2I) and "beads" (groups of arrowheads in I and L).

The second set of experiments confirmed that drebrin A puncta spanned the whole molecular layers (Figure 3A, C, G, I). Higher magnification (Figure 3D-F) showed that drebrin puncta were juxtaposed to VGlut1 puncta (arrows in Figure 3F), a marker of excitatory synapses, but they were not colocalized ($r = 0.029$). The puncta of drebrin A and GAD65, a marker of presynaptic inhibitory synapses (Figure 2G-L), were not juxtaposed or colocalized ($r = 0.001$). Both VGlut1 (Figure 3B) and GAD65 (Figure 3H) puncta spanned the whole molecular layer, but VGlut1 fluorescence was more intense in IML, whereas GAD65 was more intense in OML and SGZ. The above results indicate that drebrin A in TLE–HS patients was mainly expressed in dendritic spines of excitatory synapses.
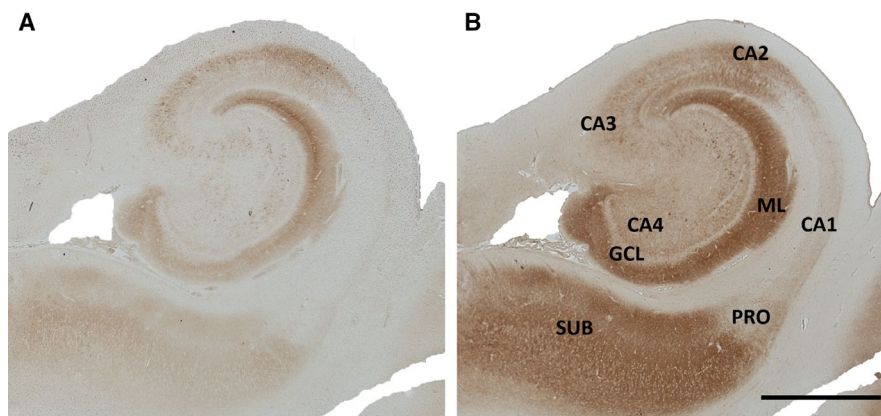


**FIGURE 1** Expression of drebrin in representative hippocampal sections from a temporal lobe epilepsy patient . Sections of the hippocampus were probed with DAS2 (A ) and M2F6 (B) antibody. Although DAS2 shows a weaker staining than M2F6, the pattern on both are matched , with more intense staining in CA4, dentate gyrus, CA3, CA2, and subiculum (SUB), and faint staining in CA1. The bar in B indicates 2 mm. GCL, granule cell layer; ML, molecular layer; PRO, prosubiculum
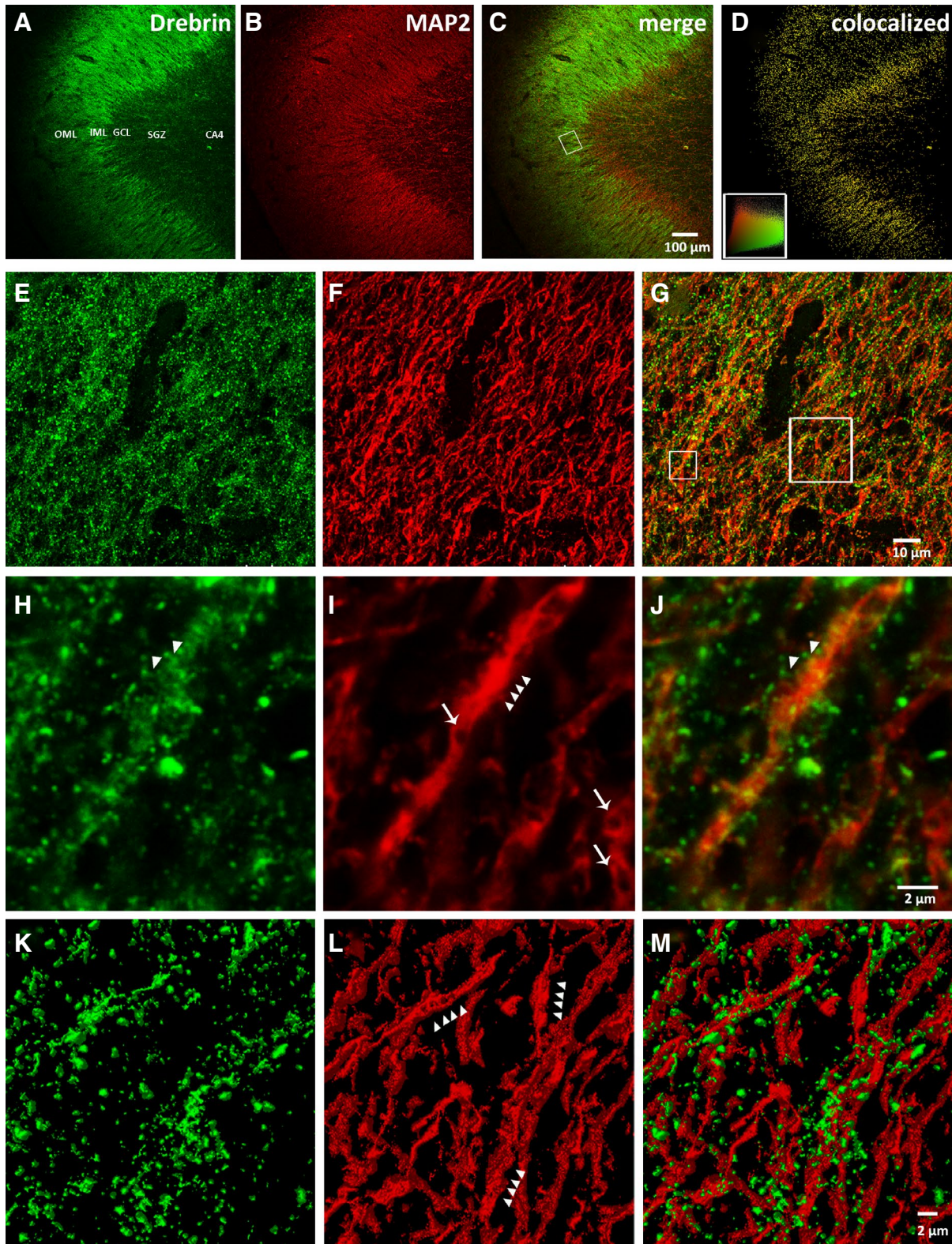
**FIGURE 2** Drebrin A is localized in dendritic spines in the dentate gyrus of temporal lobe epilepsy patients. A-D, Confocal microscopy showed that drebrin A (A, green) and MAP2 (B, red) were highly expressed in the dentate gyrus, and the colocalization (C); the areas of colocalization are shown in D (yellow pixels), and the inset represents the distribution spectrum of image pixels; bar = 100 µm. E-G, Enlarged area of the dentate gyrus (boxed area in C) evidencing colocalization (G); bar = 10 µm. H-J, Higher magnification of the small boxed area in G showing dendritic spines as puncta (arrowheads in H and J) attached to dendritic branches. Arrows indicate vacuoles in dendrites; bar = 2 µm. K-M, Three-dimensional maximum intensity projection reconstruction of dendritic spine images of the large boxed area in G. Bar = 2 µm. Groups of arrowheads indicate beaded dendrites (I, L). GCL, granule cell layer; IML, inner molecular layer; OML, outer molecular layer; SGZ, subgranular zone
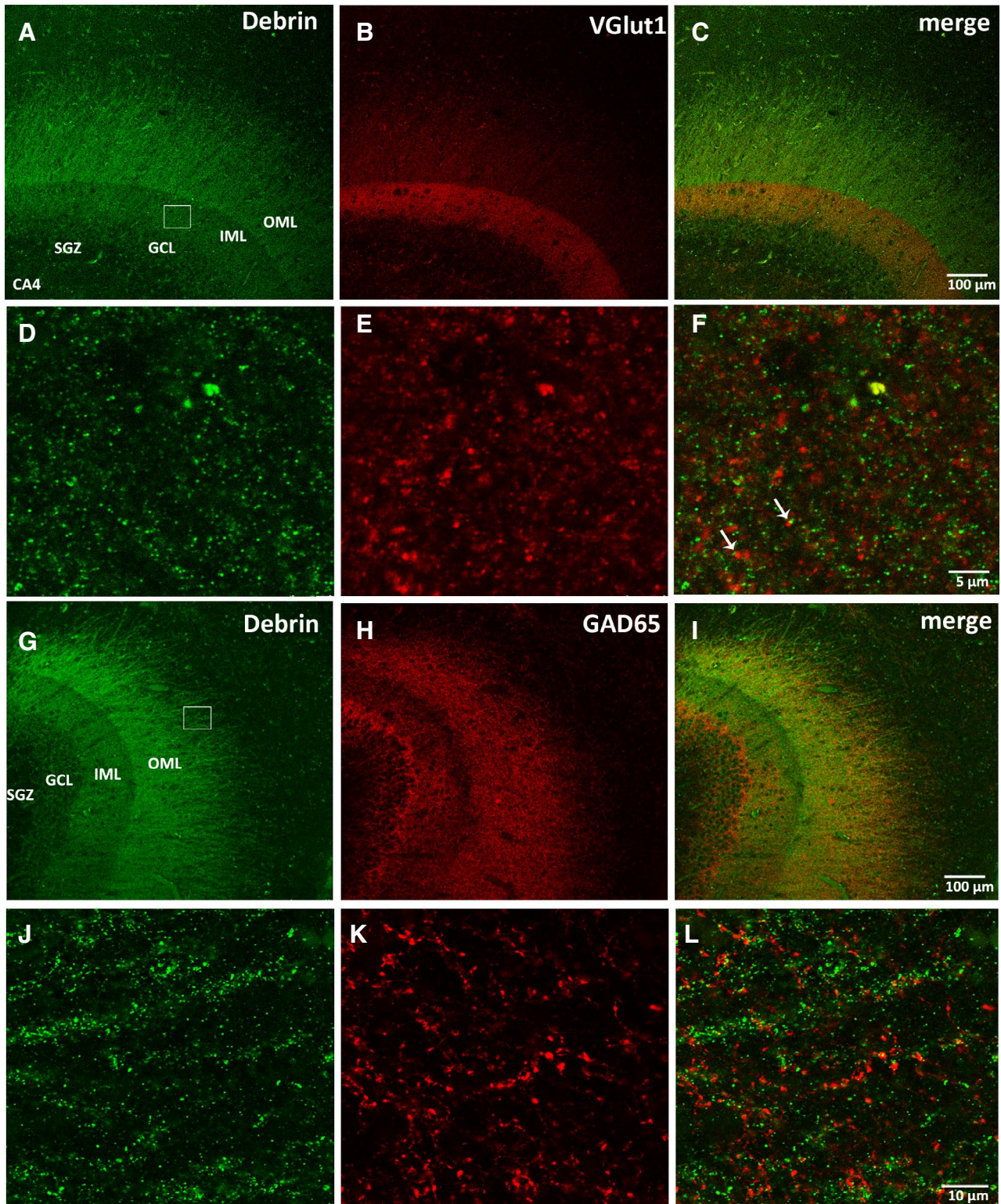
**FIGURE 3** In temporal lobe epilepsy patients, drebrin A–positive puncta were juxtaposed to VGlut1-positive puncta in the dentate gyrus, but not to GAD65-positive puncta analyzed by confocal microscopy. A-C, Double labeling of drebrin A (green) and VGlut1 (red) showed that VGlut1 was expressed in the granular and inner molecular layers and drebrin A was localized in the molecular layers. Bar = 100 μm. D-F, Higher magnification from the boxed area in A showed that drebrin A distribution was juxtaposed to VGlut1 (arrows in F), but did not colocalize. Bar = 5 μm. G-I, Distribution of drebrin A (green) and GAD65 (red) in the dentate gyrus; drebrin puncta spanned the whole dentate gyrus (G), and GAD65 expression was higher in the outer than in the inner molecular layer (H). Bar = 100 μm. J-L, Enlarged area from the boxed region in G showed that drebrin A and GAD65 were not juxtaposed or colocalized (L). Bar = 10 μm. GCL, granule cell layer; IML, inner molecular layer; OML, outer molecular layer; SGZ, subgranular zone
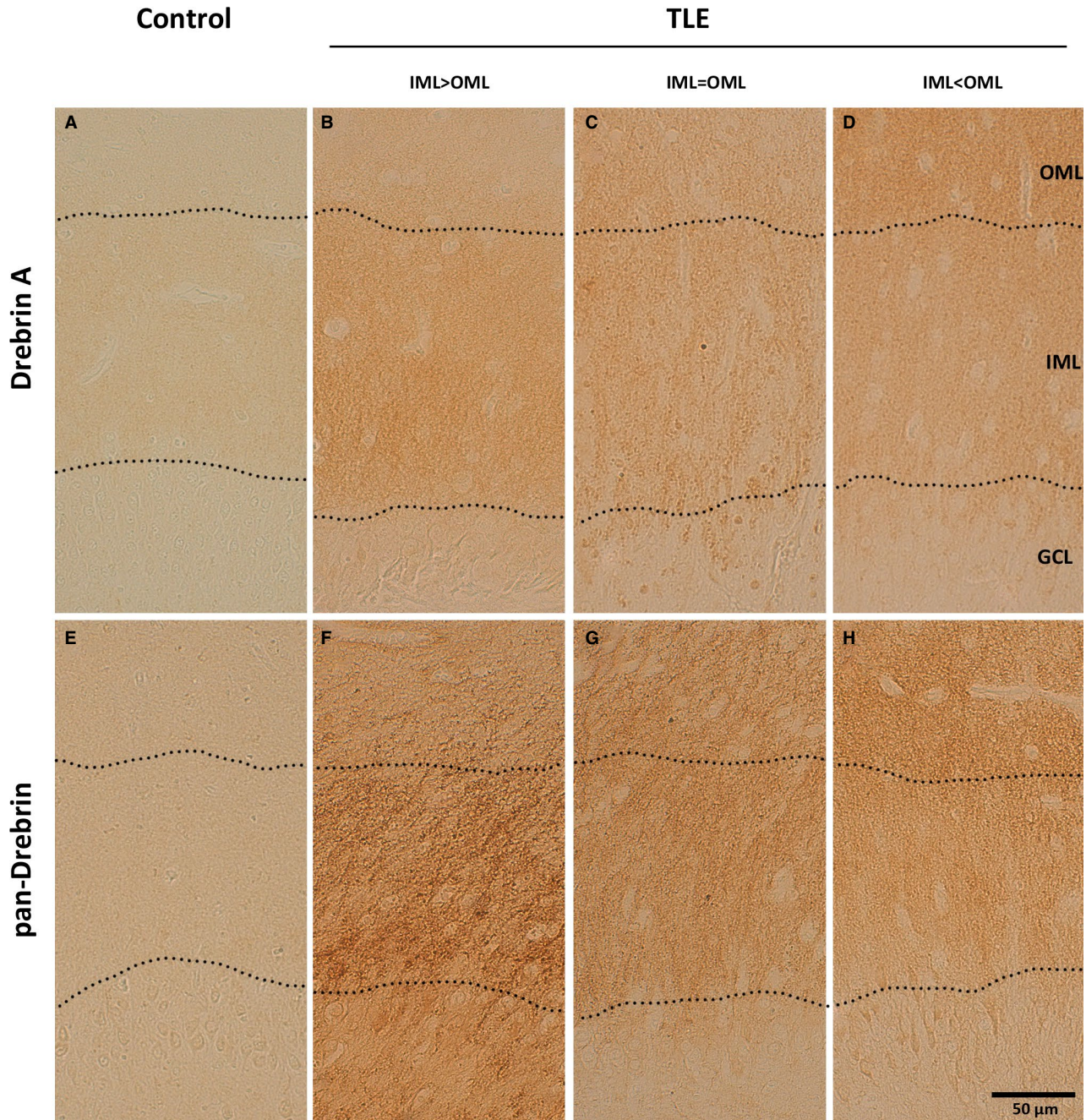
**FIGURE 4** Representative micrographs depicting drebrin A (A-D) and pan-drebrin (E-H) expression in the dentate gyrus of control cases (A and E) and temporal lobe epilepsy (TLE) cases (B-D and F-H). Compared to control cases (A and E), TLE patients presented increased immunopositive area for drebrin A and pan-drebrin in the dentate gyrus, but with different expression patterns. Staining patterns were first, higher staining intensity in inner molecular layer (IML; B, F); second, similar staining intensity in both IML and outer molecular layer (OML; C, G); or third, higher staining intensity in OML (D, H). Dotted lines separate OML (top of the micrographs), IML (middle), and granule cell layer (GCL; bottom) from each other. Bar = 50 μm for all panels

## 3.4 | Differential immunohistochemical expression of drebrin in the dentate gyrus

In TLE cases, drebrin expression detected by DAS2 and M2F6 antibodies was observed as strong immunopositive puncta, which were predominantly seen in the molecular layers of the dentate gyrus (Figure 2A; Figure 3A,G; Figure 4B-D and F-H). Staining by the M2F6 antibody was more intense than that by DAS2 antibody in both autopsy controls (Figure 4A,E) and in TLE patients (Figure 4B-D and F-H). There were three patterns of drebrin expression in the molecular layers among TLE patients: first, higher drebrin expression in the IML than in the OML (Figure 4B,F); second, no difference in drebrin expression intensity between
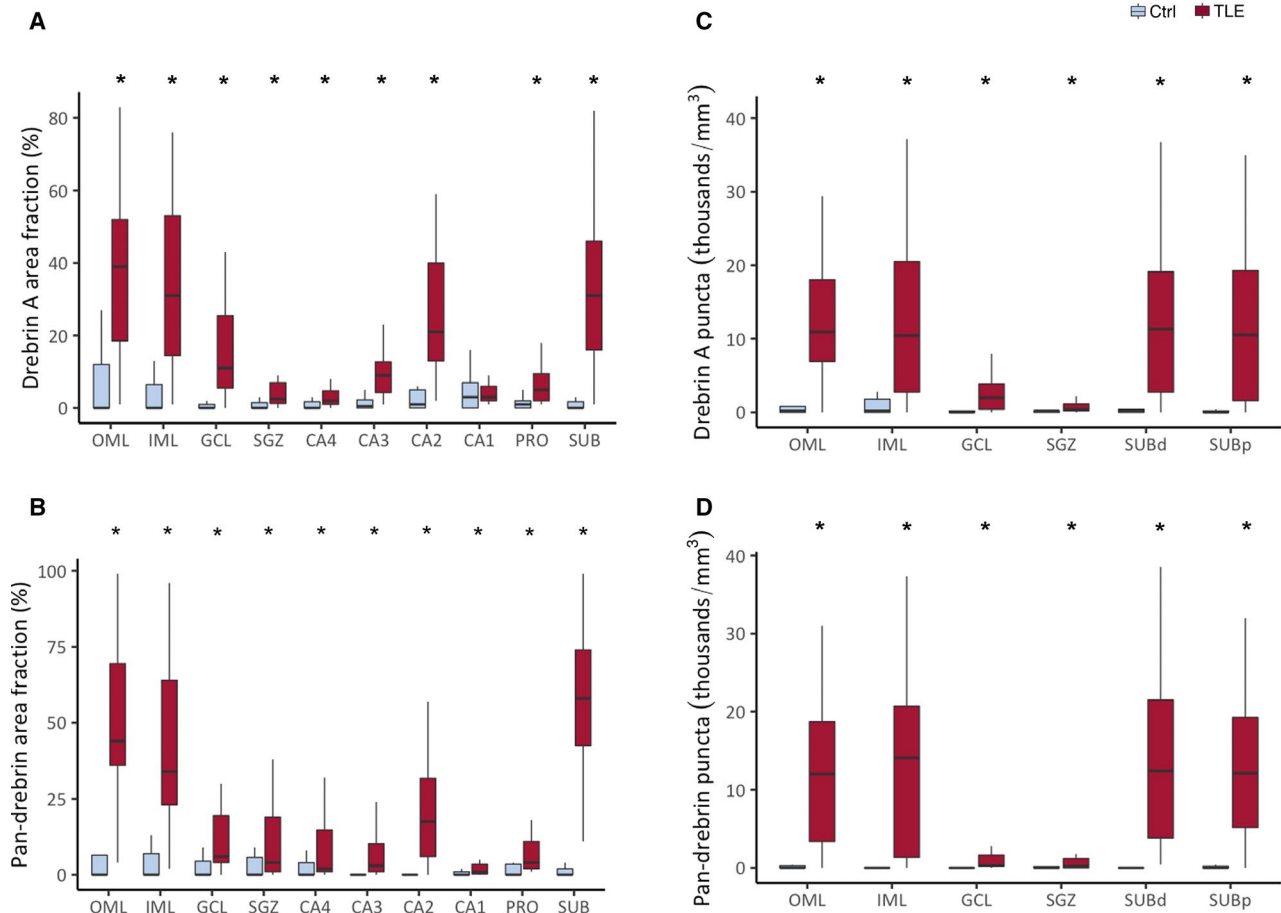
**FIGURE 5** Immunopositive areas for drebrin A (A) and pan-drebrin (B), and density of dendritic spines positive to drebrin A (C) and to pan-drebrin (D) in hippocampal sections of controls (blue boxplots) and temporal lobe epilepsy (TLE) patients (red boxplots). A, TLE cases had increased drebrin A–immunopositive area compared to control cases in all hippocampal subfields ($P < .001$) except for CA1 ($P = .225$). B, TLE patients also had increased pan-drebrin area fraction compared to control cases in all hippocampal subfields ($P \leq .015$). C, D, TLE cases had increased drebrin A (C) and pan-drebrin–positive dendritic spine density (D) when compared to control cases in the molecular layers, granule cell layer (GCL), and subiculum (SUB). Asterisks indicate difference from the control group. IML, inner molecular layer; OML, outer molecular layer; PRO, prosubiculum; SGZ, subgranular zone; SUBd, dendritic layer of SUB; SUBp, pyramidal layer of SUB

the OML and IML (Figure 4C,G); and third, lower expression in the IML than in the OML (Figure 4D,H). Although the IML-OML patterns seen with DAS2 antibody matched M2F6 in most cases, 10 patients presented with nonmatching DAS2-M2F6 (nine had higher OML staining in M2F6 but equal in DAS2, and one had higher OML in DAS2 but equal OML-IML in M2F6).

## 3.5 | Neuron density

In comparison to control, TLE patients had reduced neuron density in GCL (Student, $P < .001$), CA4 (Mann-Whitney, $P < .001$), CA3 (Student, $P < .001$), CA2 (Mann-Whitney, $P < .001$), CA1 (Mann-Whitney, $P < .001$), and PRO (Mann-Whitney, $P < .001$). No significant difference in neuron density was observed in SUB (Student, $P = .247$).

## 3.6 | Semiquantitative evaluation of drebrin expression

Increased drebrin A immunopositive area was seen in OML, IML, GCL, SGZ, CA4, CA3, CA2, PRO, and SUB (Mann-Whitney, $P < .001$) of TLE patients, as compared to control (Figure 5A). There was no significant difference between TLE and control in CA1 (Mann-Whitney, $P = .225$; Figure 5A). All TLE patients had increased pan-drebrin–immunopositive area, when compared to control, in OML, IML, GCL, SGZ, CA4, CA3, CA2, CA1, PRO, and SUB (Mann-Whitney, $P = .004$ for SGZ and PRO, $P = .015$ for CA4, $P = .001$ for CA3, $P = .012$ for CA1, and $P < .001$ for the remaining subfields; Figure 5B).

The density of drebrin-positive dendritic spines was evaluated in dentate gyrus and SUB of TLE and controls. Increased drebrin A–positive dendritic spine density was

seen in OML, IML, GCL, SGZ, and the pyramidal and dendritic layers of the SUB of TLE, when compared to control (Mann-Whitney, $P = .04$ for SGZ and $P < .001$ for the remaining subfields; Figure 5C). TLE patients also had an increased density of pan-drebrin–positive dendritic spines than controls in OML, IML, GCL, SGZ, and the dendritic and pyramidal regions of SUB (Mann-Whitney, $P = .035$ for SGZ and $P < .001$ for the remaining subfields; Figure 5D).

### 3.7 | Drebrin, clinical variables, and neuron density in TLE patients

Seizure frequency had negative correlation with drebrin A–immunopositive area in IML ($r = -0.479$, $P = .016$) and GCL ($r = -0.410$, $P = .042$), and also a trend toward negative correlation in the OML ($r = -0.376$, $P = .064$; Figure S2). An increased pan-drebrin–positive dendritic spine density in the IML was associated with better verbal memory in TLE-HS type 1 (average or above = $18.1 \pm 7.7$ thousand spines/mm³ vs below average = $9.2 \pm 11.8$ thousand spines/mm³; Mann-Whitney, $P = .034$). An increased pan-drebrin–immunopositive area in the OML had a trend toward better verbal memory in TLE-HS type 1 (average or above = $62.5\% \pm 22.0\%$ immunopositive area vs below average = $42.4\% \pm 25.7\%$ immunopositive area; Student, $P = .072$). There was no correlation between the immunopositive area or dendritic spine density of drebrin A or pan-drebrin and the remaining clinical variables. Drebrin expression showed no correlation with age or postmortem interval in the control cases. An example of different drebrin expression in controls with similar postmortem intervals in shown in Figure S3.

Neuron density and drebrin A–immunopositive area had positive correlation in the CA4 ($r = 0.417$, $P = .0339$), CA2 ($r = 0.624$, $P = .002$), CA1 ($r = 0.420$, $P = .037$), and the SUB ($r = 0.425$, $P = .034$). Neuron density in the PRO correlated negatively with pan-drebrin immunopositive area in the PRO ($r = -0.455$, $P = .038$). Drebrin A and pan-drebrin spine density had no correlation with neuron density.

Neuron density had no correlation with any of the clinical characteristics.

### 3.8 | Drebrin expression patterns in the dentate gyrus and clinical variables in TLE

There was a higher percentage of TLE cases with lower pan-drebrin staining in the IML than in the OML, when compared to controls (78% vs 19%, $\chi^2$ test, $P < .001$; Table 1), and a similar trend regarding drebrin A staining (44% vs 22%, $\chi^2$ test, $P = .09$).

TLE patients with lower pan-drebrin–positive area in IML than OML had a trend toward better postsurgical outcome when compared to those whose pan-drebrin–positive area fraction in IML was higher than or equal to OML (76% ILAE 1-3 for lower in IML vs 33% ILAE 1-3 for equal or higher in IML, exact test, $P = .073$). Patients with lower pan-drebrin–positive area fraction in IML had lower seizure frequency than patients with drebrin staining in IML equal to or higher than those in OML (five seizures/month for patients with lower IML area vs eight seizures/month for those with equal or higher IML area, Mann-Whitney, $P = .043$).

## 4 | DISCUSSION

Drebrin expression, analyzed with both drebrin A–specific and pan-drebrin antibodies, was higher in the hippocampus of TLE patients, as compared to autopsy controls. Drebrins presented a differential expression pattern in the molecular layers of the dentate gyrus. Patients whose IML had a lower pan-drebrin–immunopositive area as compared to OML had a lower seizure frequency and a trend toward better postsurgical outcome than those whose IML staining was equal to or higher than OML staining. Drebrin A expression correlated positively with neuron survival, and negatively with seizure frequency. Finally, patients with preserved verbal memory had higher pan-drebrin in the molecular layers than those with verbal memory deficits.

| | | IML < OML[a] | IML ≥ OML[a] | $P$[b] |
|---|---|---|---|---|
| Drebrin A | Control | 4 (22) | 14 (78) | .090 |
| | TLE-HS | 12 (44) | 16 (48) | |
| Pan-drebrin | Control | 3 (19) | 13 (81) | <.001 |
| | TLE-HS | 21 (78) | 6 (22) | |

**TABLE 1** Comparison between the number of TLE cases and staining intensity pattern of molecular layers

Abbreviations: HS, hippocampal sclerosis; IML, inner molecular layer; OML, outer molecular layer; TLE, temporal lobe epilepsy.

[a]Number of cases (percentage of cases) in the group.

[b]$\chi^2$ test.

Although all hippocampal regions of TLE patients expressed drebrin, the most striking changes in drebrin expression were seen in the molecular layers of the dentate gyrus, where a differential pattern of expression between inner and outer molecular layers occurs. Drebrin A expression in TLE patients with HS was seen in dendritic spines emerging from MAP2-positive dendritic branches. Drebrin A in spines was juxtaposed with the presynaptic marker VGlut1 and did not colocalize with VGlut1 or GAD65. The close apposition of drebrin A–positive puncta to VGlut1-positive puncta suggests that drebrin A localized to functional excitatory synapses. Takahashi et al[13] showed that drebrin A clustering in dendritic spines is regulated by AMPA receptors, and higher drebrin A expression is shown to drive a shift in favor of excitatory synapses,[28] thus pointing to its close link to the postsynaptic membrane of excitatory synapses. Drebrin was also observed in dendritic branches and cytoplasm of some hippocampal neurons of TLE patients, an unusual localization in adulthood. This cytoplasmic presence may be linked to the hyperexcitability of the epileptogenic zone, as N-methyl-D-aspartate receptor stimulation can promote a shift of drebrin from dendritic spines to dendritic shafts and neuron cytoplasm.[29]

Changes of spine number and shape appear to correlate with memory and learning.[30,31] In this context, drebrin levels are crucial for proper synaptic and cognitive functions, as they participate in the formation and stabilization of synapses.[13] Drebrin knockdown induces spine maturation in developing neurons,[32] and drebrin knockout reduces spine density and inhibits memory-related hippocampal synaptic plasticity.[33] Reinforcing the role of drebrin in memory, mice with double-knockout for amyloid precursor protein and presenilin-1, a model of familial Alzheimer disease, have a decrease of drebrin A in spines that precedes Alzheimer cognitive deficits.[18] In humans, low drebrin was seen in several cortical regions of patients with Alzheimer disease or mild cognitive impairment.[17,19] In the present study, higher pan-drebrin spine density and immunopositive area in the molecular layers were associated with better verbal memory in the TLE patients that had HS type 1, the HS subtype more frequent in our cases (86%). TLE patients with HS types 1 and 3 present verbal memory deficits more frequently than those with HS type 2.[1,2] Because drebrin is crucial to synaptic stabilization and formation, a higher drebrin level may indicate better preservation of the remaining hippocampal circuitry, thus explaining the better verbal memory in HS type 1 cases with higher drebrin.

Seizures had negative correlation with drebrin A–immunopositive area fraction in the IML. However, patients in whom OML-immunopositive area was greater than IML-immunopositive area, seizures were less frequent. Given the importance of drebrin for synaptic maturation and maintenance,[12,13] the differential drebrin expression pattern in the molecular layers could be related to the degree of the synaptic reorganization, and imply a differential excitation-inhibition balance in some TLE patients, thus explaining these seeming discrepancies in seizure frequency. Several studies have shown differences between IML and OML. For instance, IML and OML receive input from different brain regions[8,26] and have differential neurotransmitter receptors.[34–36] Besides these differences, the reorganization of GCL mossy fibers from CA4 to the IML is an important plastic change seen in TLE patients and animal models of epilepsy.[7,8] Although mossy fiber sprouting is not a precondition for spontaneous seizure occurrence,[37] the intensity of the reorganization in the IML has been linked to seizure frequency.[38–40] Thus, differential expression of drebrin in IML and OML could reflect differences in the weights of input between these two regions, which could reflect overall differences in excitability and, as a consequence, seizure frequency.

Besides seizure frequency, cases with lower pan-drebrin expression in the IML presented a trend toward better postsurgical outcome. Although magnetic resonance findings and electrographic patterns have been linked to surgical outcome, the best prognostic finding associated with seizure control after temporal lobectomy is the presence of HS type 1 in the pathological evaluation.[6,41,42] Because drebrin expression closely follows the synaptic reorganization in the dentate gyrus,[21] the increased drebrin expression pattern in the molecular layers of TLE patients with HS may be a surrogate marker for the circuitry changes associated with neuronal hypersynchronization and seizure spread, and could indicate subtypes of HS 1 with a better outcome.

The present study has some limitations. First, the lack of a specific antibody to immunolocalize drebrin E prevents us from determining whether the associations between clinical data and pan-drebrin seen here are directly linked to the expression of drebrin E in adults. It would also be important to evaluate drebrin in other types of HS, as well as non-HS TLE cases. Another significant limitation is the difference in drebrin expression between patients and controls. It is known that drebrin expression reduces with age,[43] and our controls were significantly older than TLE cases. However, we saw no correlation between age and drebrin in control cases. The time between death and fixation could also impact drebrin levels in controls. It is known that postmortem intervals affect levels of several mRNAs,[44] but protein levels tend to be more stable several hours postmortem.[45] In agreement, we saw no significant difference between drebrin expression and the postmortem interval. Notwithstanding, a better control to define differences in drebrin expression between non-TLE and TLE cases would be surgical hippocampal samples from patients with temporal lobe tumors without a history of seizures. Although we had no such samples, the more critical results from the present study are the associations between

clinical characteristics and drebrin expression among the TLE cases. Regarding tissue fixation, TLE and control samples were fixed for the same time, so fixation itself had no impact on masking epitopes differentially between groups. Finally, the differential drebrin patterns in the molecular layer could match the degree of mossy fiber sprouting, a common finding in TLE models and patients.[7,45,46] However, neo-Timm staining, a specific histochemistry for the zinc-enriched mossy terminals, requires a special fixation process with sodium sulfide and glutaraldehyde. None of our cases was fixed with the proper mixture, so we were unable to evaluate mossy fiber sprouting in the present cases. Although immunohistochemistry for dynorphin or doublecortin proteins can be used to evaluate sprouting, we tried several dynorphin antibodies in past studies without good results. Further studies should evaluate the association between mossy fiber sprouting and drebrin patterns in the molecular layers.

# 5 | CONCLUSIONS

We report here for the first time that changes in drebrin-positive spines and overall drebrin expression in the dentate gyrus are associated with lower seizure frequency, more preserved verbal memory, and a better postsurgical outcome. Our results suggest that the expression patterns and levels of drebrin can indicate hippocampal reorganization of excitatory synaptic circuitry and surgical outcome in TLE patients.

## CONFLICT OF INTEREST
None of the authors has any conflict of interest to disclose. The funding agencies took no part in the study design or analysis. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

## ORCID
*Thaís Caroline Dallabona Dombroski* https://orcid.org/0000-0003-2976-8614
*Jose Eduardo Peixoto-Santos* https://orcid.org/0000-0001-7461-1902
*Munira Muhammad Abdel Baqui* https://orcid.org/0000-0002-7945-2899
*Tonicarlo Rodrigues Velasco* https://orcid.org/0000-0001-7756-8882
*Carlos Gilberto Carlotti* https://orcid.org/0000-0002-7055-0564
*Kenji Hanamura* https://orcid.org/0000-0002-2244-648X
*João Pereira Leite* https://orcid.org/0000-0003-0558-3519
*Jaderson Costa da Costa* https://orcid.org/0000-0001-6776-1515
*André Luiz Palmini* https://orcid.org/0000-0002-4163-6924
*Rita Garbelli* https://orcid.org/0000-0002-6475-4974
*Antonio Roberto Martins* https://orcid.org/0000-0002-9560-280X

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## SUPPORTING INFORMATION

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