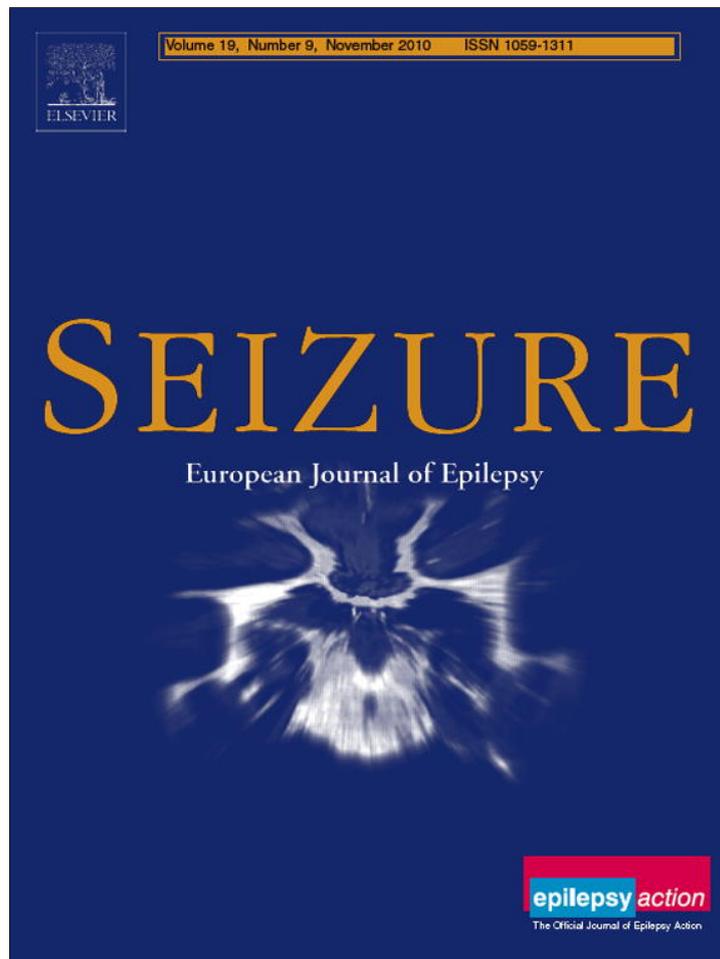


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Doublecortin (DCX) immunoreactivity in hippocampus of chronic refractory temporal lobe epilepsy patients with hippocampal sclerosis

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ABSTRACT

Introduction: Status epilepticus increases the production of new neurons (hippocampal neurogenesis) and promotes aberrant migration. However chronic experimental models of epilepsy and studies performed in human epilepsy showed controversial results suggesting a reduction in hippocampal neurogenesis in late stages of the disease. Doublecortin (DCX) has been validated to determine alterations in the production of new neurons in the human hippocampus.

Objectives: Determine DCX expression in human hippocampal sclerosis (HS) from patients who underwent epilepsy surgery for refractory temporal lobe epilepsy (TLE).

Methods: Hippocampal sections of 9 patients with HS and TLE who underwent surgery, were processed using immunoperoxidase for DCX. Archival material from 5 normal post-mortem hippocampus were simultaneously processed.

Results: Significantly lower staining intensity was observed in DCX-positive neurons localized in dentate gyrus (DG) and in CA1 of epileptic hippocampus; lower DCX reactive area was observed in pyramidal layers of CA1; and a reduced in the mean number of DCX-positive neurons were determined in DG compared to normal hippocampus ($p < 0.05$).

Conclusions: This study found a decrease in DCX expression in hippocampus of patients with HS and chronic and refractory TLE suggesting alterations in NG and hippocampal synaptogenesis with potential cognitive and emotional repercussion.

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1. Introduction

In the last years, altered hippocampal neurogenesis (NG) has emerged as another hallmark of temporal lobe epilepsy (TLE) with hippocampal sclerosis (HS)¹. It has been demonstrated that epileptic discharges lead to a significant increase of mitotic activity in the hippocampal dentate gyrus^{2–5}. Proliferative response is mediated by glia-like neuroprogenitor cells, and is followed by an increase of 5'- bromodeoxyuridine (BrdU) positive neuroblasts and markers of immature neurons such as doublecortin (DCX)^{5,6}.

Doublecortin is a microtubule-associated phosphoprotein, and regulates neuronal migration during development⁷. In adult brain, DCX expression is associated with neurite and axon elongation, and synaptogenesis^{8–10}. Furthermore, DCX has been considered a

marker of newly born neurons and is used to determine the levels of neurogenesis in the adult DG and it has been validated to determine alterations in the production of new neurons as a function of ageing or different neurological diseases in the human hippocampus^{10,11}.

Experimental evidence demonstrated status epilepticus increases the production of new neurons and promotes aberrant migration into the dentate hilus and into the molecular layer in early stages of the disease^{3,12,13}. However, chronic experimental models of epilepsy and a few studies performed in human epileptic tissue, showed controversial results suggesting a reduction in hippocampal neurogenesis in late stages of the disease^{14–19}. In addition, decreased hippocampal neurogenesis has been associated with impairments in hippocampal-dependent learning, memory and emotional functions such as depression, observed in TLE patients^{1,20}.

The aim of this study was to determine if chronic seizures of intractable TLE modify DCX immunoreactivity in the hippocampus of patients with TLE and HS, who underwent epilepsy surgery for chronic intractable epilepsy.

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2. Methods

2.1. Patients and samples

Hippocampal sections of 9 patients; 6 women and 3 men; mean age 40.1 ± 6 years; with hippocampal sclerosis (HS) and refractory TLE (temporal lobe epilepsy), who underwent therapeutic surgery, were processed using immunoperoxidase for DCX.

All patients underwent a thorough clinical, electrophysiological (Video-EEG), imaging evaluation (magnetic resonance image or MRI), neuropsychological and psychiatric assessment prior to surgery^{21–23}.

All patients included in this study had a diagnosis of HS according MRI. This method was performed using optimized imaging for the hippocampus and temporal lobe structures according to the following protocol: Sagittal T1-weighted; inversion–recovery, fluid-attenuated inversion recovery (FLAIR) T1 FFE 3D acquisition, perpendicular to the long axis of the hippocampus and T2-weighted axial, parallel to the long axis of the hippocampus. Diagnostic Criteria for Hippocampal Sclerosis by MRI was atrophy and signal change of the hippocampus: hypointense in T1W and IR, hyperintense in T2W and Flair and alteration of the internal structure of the hippocampus^{24,25}.

After surgery, all the samples were studied by a neuropathologist to confirm the diagnosis of HS and the degree of it. For this, coronal sections of the hippocampus nucleus were cut at a thickness of 5 μm . Sections were stained with Nissl and periodic acid-Schiff (PAS) stains. The degree of neuronal loss in the subiculum, CA1, CA2/3, CA4 (end plate) and dentate gyrus was evaluated on Nissl stained sections using a four-point rating scale according to the degree of nerve cell loss, using the Wyler classification²⁶. In this grading system, grade 0 corresponds to normal hippocampus; grade 1 corresponds to mild mesial temporal damage (MTD) with hippocampal neuronal loss of 10% in areas CA1, CA3, and CA4; grade 2 corresponds to moderate MTD with neuronal loss between 10 and 50%; grade 3 corresponds to marked MTD or classical HS with neuronal loss of 50%; and grade 4 corresponds to total MTD or HS with neuronal loss of 50% involving all sectors of the hippocampal pyramidal cell layer, dentate gyrus, subiculum, and para-hippocampal gyrus. For additional analysis, grades 0–2 were considered to be non-HS, and grades 3 and 4 were considered to be HS. In this study all cases corresponded to grade 3 or classical HS.

Archival material obtained from normal post-mortem hippocampus of 5 subjects, 3 women and 2 men, mean age 45.8 ± 14 years, matched by sex and age ($p > 0.05$) were simultaneously processed as controls. The post mortem specimens were free from neurological injury, and did not carry a secondary diagnosis of drug or alcohol dependency. The time elapsed between death and autopsy never exceeded 6 h.

All of the used samples were obtained from patients assisted at the Epilepsy Center of Ramos Mejía Hospital, Buenos Aires. All patients selected and admitted to this study were evaluated by the same group of professionals to confirm the epileptogenic zone and determine inclusion in the epilepsy surgical program²⁷.

The study was conducted with the approval of the Ethics Committee of Ramos Mejía Hospital in accordance with the Ethical Standards laid down in the 1964 Declaration of Helsinki, and all the subjects submitted informed consent.

2.2. Tissue processing

The temporal lobe, comprising superior, middle and inferior gyri was separated from the rest of the brain in post mortem controls, and the anterior temporal lobe extirpated during surgery, (anterior temporal lobectomy) were fixed in formalin for 5 days.

After that, tissue blocks (thickness: 5 mm) were made following coronal planes and were embedded in paraffin. All sections were cut at 7 μm with a microtome, stretched in water at 40 °C and mounted on slides, deparaffined in xylene, hydrated and stained with hematoxylin and eosin.

2.3. Immunohistochemistry

After deparaffinizing, the sections were briefly treated according to the following procedure: a 15-min wash in distilled water; then an incubation in a microwave oven twice for 5 min in a citric acid solution (0.1 mol/L citric acid monohydrate and 0.1 mol/L trisodium citrate dihydrate), pH 6.0; after that a 2-fold 5-min wash in phosphate-buffered saline (PBS), the sections were incubated for 30 min in 0.5% (v/v) hydrogen peroxide in ethanol to quench endogenous peroxidases.

Afterwards, they were incubated overnight in a humid chamber with rabbit anti-doublecortin (DCX, Sigma, St Louis,) diluted 1:200 in PBS Triton X-100 and 0.1% (w/v) sodium azide. The complex was detected using supersensitive multilink-HRP/DAB kit from BioGenex (QD000-5L) following the vendor's procedure. After dehydration the sections were mounted with Permount medium and coverslipped.

2.4. Image analysis

Quantification of neurons expressing DCX was determined by computerized image analysis. The images were acquired by a SONY Power Had 3CCD colour video camera system from a Zeiss AxioPhot microscope.

Images were digitalized with a resolution of 768×494 pixels and were analyzed using J Image analysis program.

Ten fields per section were evaluated. The total number of cells counted ranged between 2.000 and 3.000 per case. All images were captured under identical lighting and magnification conditions and processed by Image J freeware. After shading correction, an automatic discrimination procedure was performed and the mean grey value (MGV) of specific labeling and the background was measured. The specific MGV was then defined as the difference between the background MGV and the MGV of the discriminated profiles. The mean density and SD were calculated for each case.

Other parameters analyzed were, the total neuronal reactivity area and the number of reactive cells per field. All these parameters were determined in DG granular cells, and in CA1 pyramidal neurons. The average reactive size and the standard deviation were determined in each case.

2.5. Statistical analysis

A comparison between MGV, mean area, and the number of DCX-R cells reactive cells was determined in DG and CA1 areas of hippocampus of epileptic patients and controls.

Student T test was used to determine the statistical significance of the differences, $p < 0.05$ was considered significant. SPSS for Windows was used to perform the total statistical analysis.

3. Results

Clinical data of 9 patients with refractory temporal lobe epilepsy and hippocampal sclerosis included in this study is resumed in Table 1.

In both controls and epileptic hippocampus, DCX positive cells with neuronal morphology were found among granular cells in dentate gyrus (DG), pyramidal layers and hippocampus hilus.

Morphological changes (granular cell dispersion, and alterations in dendrite morphology) and quantitative differences (mean

Table 1

Clinical data of 9 patients with refractory TLE and HS.

Patient	Age of epilepsy onset	Time of epilepsy duration before surgery	MRI	Neuropsychological assessment	Psychiatric assessment
1	9	31	RHS	Normal	Normal
2	27	13	RHS	Normal	Depression
3	28	17	RHS	Deficit of verbal memory	History of depression and psychosis
4	17	31	RHS	Deficit of visual memory	Anxiety disorder
5	4	16	LHS	Deficit of non verbal memory	Normal
6	9	31	RHS	Normal	Normal
7	2	30	RHS	Deficit of verbal memory	History of psychosis
8	12	30	RHS	Normal	History of depression
9	23	12	RHS	Deficit of verbal memory	History of psychosis

MRI: magnetic resonance image. RHS: Righth Hippocampal Sclerosis. LHS: left hippocampal sclerosis.

grey value, reactive area, and total number of DCX-positive cells), were determined in DG and in CA1 of control and hippocampal sclerosis sections (HS).

3.1. Morphological changes

Morphological changes in DCX-positive cells were observed in granular cells of dentate gyrus and in pyramidal layers (CA1) in hippocampal sclerosis tissue compared with normal controls.

In DG of controls, DCX-positive granular cells had a typical granular morphology with a high DCX immunoreactivity in the somas. Rows of DCX-positive neurons without dispersion were observed among DG layers. On the contrary, in DG of HS, DCX-positive granular cells had an important dispersion, and many of these reactive neurons, were localized into the hilus and into the adjacent molecular layer (Fig. 1). Other important difference found in HS, was the presence of a DCX positive dendrites arborization directed into the molecular layer. This arborization phenomenon was not observed in controls (Fig. 1).

In pyramidal layer, DCX-positive cells with pyramidal morphology were detected in both control and epileptic sections;

DCX-positive neurons in CA1 of epileptic patients showed reactive somas and longer tortuous apical dendrites in addition many reactive fibers crossing along CA1 were observed. These observations were not present in normal hippocampus where DCX was only expressed in neuronal somas (Fig. 2).

Quantitative differences; MGV (mean grey value), DCX reactive area, and mean number of DCX-positive cells in DG and CA1:

Significantly lower staining intensity, measured as Mean Grey Value, (MGV) was observed in DCX-positive neurons localized in DG and in CA1 of epileptic hippocampus compared with normal controls. Furthermore, lower DCX reactive area was observed in pyramidal layers of CA1, and also a reduced in the mean number of DCX-positive cells were determined in DG compared to normal hippocampus ($p < 0.05$) (Fig. 3).

4. Discussion

This study was performed in hippocampal tissue obtained from patients with refractory TLE and HS who underwent neurosurgery. TLE is characterized by spontaneous recurrent seizures originated from the temporal lobe. The precise causes of TLE are unknown

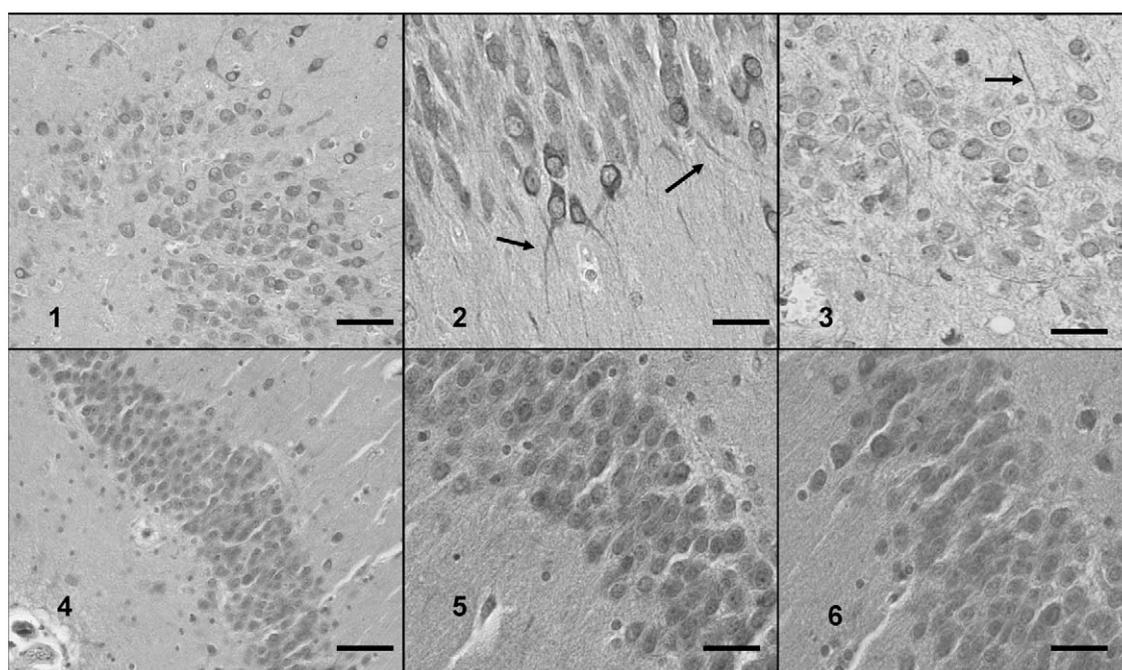


Fig. 1. (1, 2, 3) Dentate Gyrus of Epileptic patients with TLE and HS. DCX positive granular cells have an important dispersion and a dendrite arborization directed into the molecular layer. (4, 5, 6) Dentate Gyrus of Controls. DCX positive granular cells had a linear distribution with a high DCX reactivity in the somas and no dispersion among DG layers. (1, 4) Low magnification, scale bar: 60 μm . (2, 3, 5, 6) High magnification, scale bar: 30 μm .

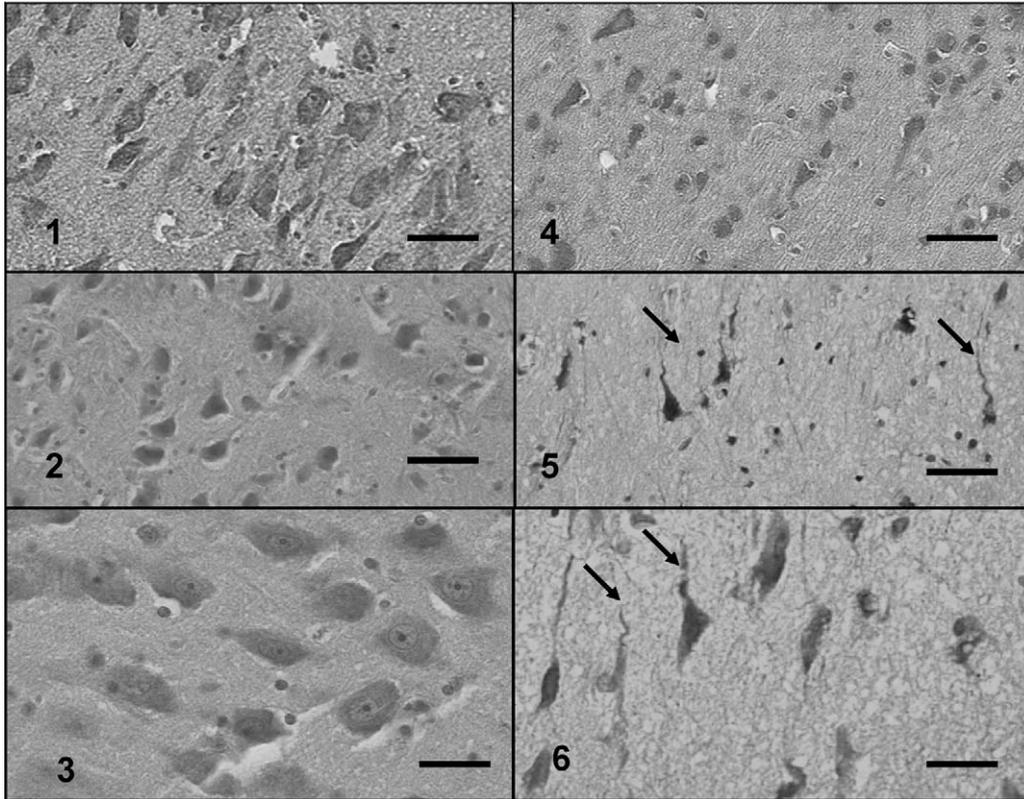
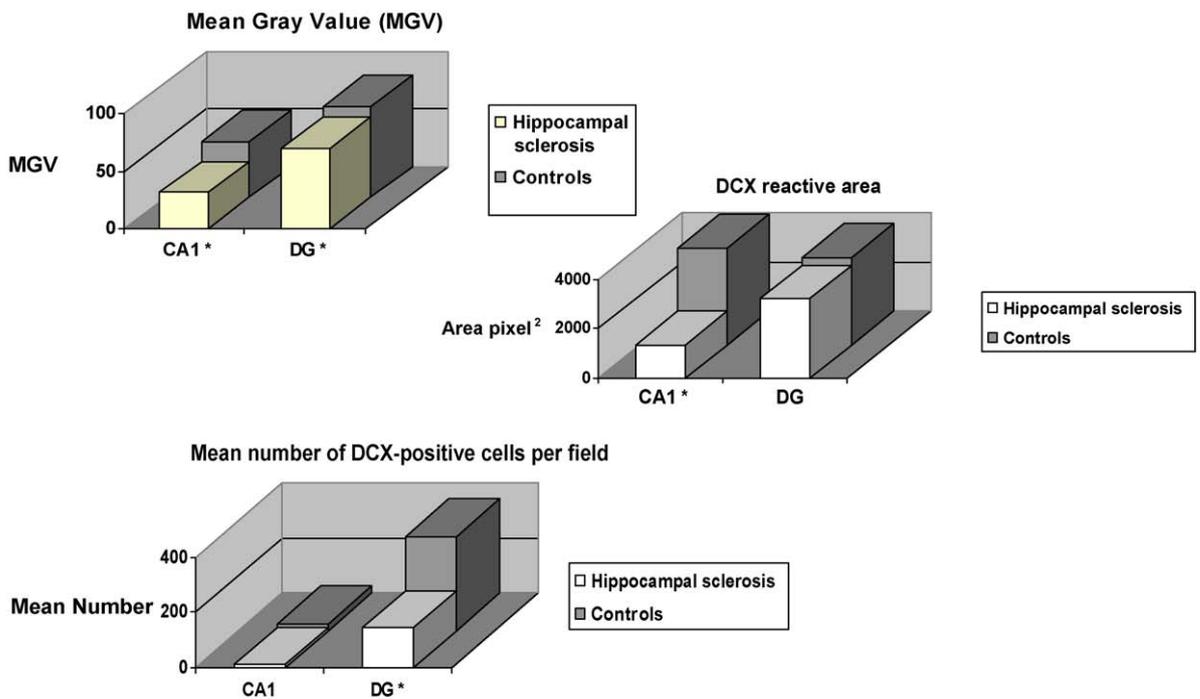


Fig. 2. (1, 2, 3) DCX positive cells with pyramidal morphology localized in CA1 of control hippocampus. (4, 5, 6) DCX positive cells with pyramidal morphology localized in CA1 of TLE patients with HS. DCX positive cells show smaller reactive somas with longer tortuous apical dendrites. (1, 2, 3, 4) Low magnification. Scale bar: 60 μm . (5, 6) High magnification. Scale bar: 30 μm .



DG: Dentate gyrus
 CA1: Pyramidal Layer
 * $p < 0,05$

Fig. 3. Quantitative differences of DCX immunoreactivity in DG and CA1 of hippocampus with hippocampal sclerosis and normal hippocampus (controls).

however TLE with hippocampal sclerosis (HS) occurs after an initial precipitating injury such as brain injury, tumours, status epilepticus and febrile seizures¹. Histopathological studies of HS demonstrated a reduction of gabaergic interneurons²⁸; a partial degeneration of CA1 and CA3 pyramidal neurons³¹; and aberrant sprouting and hippocampal reorganization^{29,30}.

Over the last decade altered dendritic remodeling and changes in dentate gyrus neurogenesis has emerged as another hallmark of TLE, however controversial results were found in acute versus chronic models of epilepsy; while in acute experimental models, NG and plasticity is clearly enhanced, it has not been established what happens in chronic experimental models of epilepsy and in human chronic epilepsy with HS¹.

In this preliminary study, the results of quantitative measurements of cells expressing DCX demonstrated a decrease in DCX expression in human hippocampus with HS compared to normal postmortem controls. A significant reduction in DCX expression in epileptic tissue was found in DG and in CA1 areas according to the MGv (mean grey value), and besides the mean number of DCX-positive neurons was significantly reduced in DG, while the DCX reactive area was significantly reduced in CA1 regions. These results may represent a reduction in the hippocampal neuroplasticity and neurogenesis in response to the exposure to chronic ictal activity. DCX has been considered a marker of newly generated neurons in the adult dentate gyrus, as well it has been considered useful for determining the changes in dentate NG in human hippocampal tissue, where it is not possible to make *in vivo* analysis with BrdU¹¹.

In accordance with our results, previous studies performed in experimental models of chronic epilepsy found a decreased number of DCX-positive neurons and a reduced level of DCX mRNA after 4 to 6 weeks of kainic acid (KA) administration¹⁷. Interestingly, animals exhibiting greater frequency and intensity of epileptic seizures, demonstrated more dramatic loss of DCX-positive cells^{16,17,32}.

In human tissue of epileptic patients a previous study found an absence of positive cells for PSA-NCAM (polysialic acid neural cell adhesion molecule) which identifies newly formed neurons, in TLE patients with severe hippocampal neuronal loss, but an increase of PSA-NCAM positive cells were found in TLE patients with mild neuronal loss¹⁴. Also a reduction of PSA-NCAM expression was reported in granular layers of dentate gyrus in children with frequent seizures who underwent epilepsy surgery¹⁵.

Furthermore, in agreement with the results of our study, a significant reduction in DCX mRNA synthesis was found in DG of patients with TLE and HS¹⁸. On the contrary, Liu et al., 2008 found an increase of DCX positive cells in temporal lobe cortex of patients with TLE, suggesting an up-regulation of DCX in temporal cortex with a functional significance in the pathophysiology of epilepsy¹⁹. However, the analysis of the hippocampal homogenates performed by these authors, showed no significant differences of DCX positive cells¹⁹. In this study, we did not analyze hippocampal homogenates, we determined DCX expression in hippocampal sections obtained after therapeutic lobectomy of patients with refractory TLE.

In this investigation, the analysis of the arrangement of DCX immunoreactive cells showed granular cell dispersion and alterations in dendrite morphology of DCX positive cells. In relation to these findings, newborn cells positive for Musashi-1 (expressed by post-mitotic cells) with ectopic position (in the hilus and in the CA3 area), were described in adult epileptic tissue obtained after hippocampectomy⁷. Also in patients with TLE high cypstatin C expression associated with greater numbers of PSA-NCAM positive newborn cells were found in the molecular layer of DG, although the overall number was decreased, indicating that the newborn cells migrate to abnormal locations³³.

It is important to mention that an age dependent decrease of DCX expression in the normal and epileptic hippocampus has been reported^{11,18}. In our study epileptic patients and normal controls were matched by age and sex so that the observed differences were due to epilepsy and not to normal aging process.

The present findings in hippocampal tissue obtained from epileptic patients who underwent neurosurgery, are in accordance with animal models of chronic TLE and support the hypothesis that there is a decrease of DG neurogenesis in chronic epilepsy^{14–18,33}.

The extent of decrease in DG neurogenesis is more pronounced in animals exhibiting greater frequency of epileptic seizures¹⁶. Furthermore, the extent of decline in DG neurogenesis is directly proportional to the severity of the lesion in chronic TLE¹⁴. Related to these findings, all the patients of our study had a long history of severe and refractory epileptic seizures.

DCX expression has been associated with neuronal migration, neurite and axon elongation, synaptogenesis, and has been considered a marker for newly generated cells^{8,9,11}. Indeed, decreased DCX expression could be related to hippocampal neuronal loss described in the pathophysiology of HS; it has been proposed that both patterns of cell death, apoptosis and necrosis, may contribute to the sclerosing process of mesial temporal structures. However, as HS is considered a pre-existing lesion occurring before the onset of seizures, evidence also exists that HS progressively ripens over time in pathological, biochemical and electrophysiological terms³⁴. Additionally, decreased DCX expression could be also suggestive of a reduction in dentate gyrus neurogenesis, and may also implicates an abnormal migration of mature granule cells along a radial glial scaffold^{18,33}. The mechanisms by which chronic seizures induce a reduction in DCX expression and in DG neurogenesis are not clear, but it has been proposed that changes in the number of neuronal stem cells and changes in the microenvironment, including alterations in the levels of different neurotrophins are involved^{1,35}.

Decreased hippocampal neuroplasticity and neurogenesis in chronic epilepsy has been proposed as having potential repercussions in cognitive functions as learning and memory and in emotional behaviour such as depression: These alterations have been reported in behavioural experimental models of hippocampal sclerosis induced by KA^{16,31,36}, and in chronic epileptic patients with TLE who frequently develop depression, psychoses and cognitive deficits^{1,20,22,23}. In this study we did not determine a correlation between DCX abnormalities and memory and emotional disorders, because of the sample was very small. However, most of the patients (7/9) included in this study had memory deficits and/or psychiatric conditions, before surgery.

Strategies to enhance DG neurogenesis and neuroplasticity including physical exercise, exposure to enriched environment and antidepressant therapy, have been proposed as having benefits and are under intensive investigation¹. In conclusion, our study confirms a decrease in DCX expression in hippocampus of patients with HS and chronic and refractory TLE. These results suggest neuroplasticity and neurogenesis abnormalities in the hippocampus with potential consequences in cognitive and emotional functions.

Conflicts of interest

None.

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