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Neuronal and astrocytic tetraploidy is increased in drug-resistant epilepsy

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Abstract

Aims: Epilepsy is one of the most prevalent neurological diseases. A third of patients with epilepsy remain drug-resistant. The exact aetiology of drug-resistant epilepsy (DRE) is still unknown. Neuronal tetraploidy has been associated with neuropathology. The aim of this study was to assess the presence of tetraploid neurons and astrocytes in DRE.

Methods: For that purpose, cortex, hippocampus and amygdala samples were obtained from patients subjected to surgical resection of the epileptogenic zone. Post-mortem brain tissue of subjects without previous records of neurological, neurodegenerative or psychiatric diseases was used as control.

Results: The percentage of tetraploid cells was measured by immunostaining of neurons (NeuN) or astrocytes (S100β) followed by flow cytometry analysis. The results were confirmed by image cytometry (ImageStream X Amnis System Cytometer) and with an alternative astrocyte biomarker (NDRG2). Statistical comparison was performed using univariate tests. A total of 22 patients and 10 controls were included. Tetraploid neurons and astrocytes were found both in healthy individuals and DRE patients in the three brain areas analysed: cortex, hippocampus and amygdala. DRE patients presented a higher number of tetraploid neurons ($p = 0.020$) and astrocytes ($p = 0.002$) in the hippocampus than controls. These results were validated by image cytometry.

Conclusions: We demonstrated the presence of both tetraploid neurons and astrocytes in healthy subjects as well as increased levels of both cell populations in DRE patients. Herein, we describe for the first time the presence of tetraploid astrocytes in healthy subjects. Furthermore, these results provide new insights into epilepsy, opening new avenues for future treatment.

KEYWORDS
astrocytes, flow cytometry, neural cell cycle, polyploidy, refractory epilepsy, temporal lobe epilepsy, tetraploidy

Cristina Virginia Torres-Díaz and María C Ovejero-Benito contributed equally to the manuscript.

INTRODUCTION

Epilepsy is one of the main neurological diseases affecting over 50 million people worldwide. Despite the continuing emergence of new anticonvulsant medications (ACMs), the percentage of drug-resistant patients is difficult to reduce and remains approximately 25–30% [1]. The International League Against Epilepsy (ILAE) defined drug-resistant epilepsy (DRE) as a failure of adequate trials of two tolerated, appropriately chosen and used AC schedules (in monotherapies or in combination) to achieve sustained seizure freedom [2]. Several hypotheses could explain the processes occurring in the epileptogenic zone of DRE patients, based on disease-related mechanisms, drug-related mechanisms and genetic mechanisms, which may be interlinked [3].

Temporal lobe epilepsy (TLE) is one of the main types of epilepsy associated with drug resistance [4]. TLE usually progresses with hippocampal sclerosis (HS), which involves severe neuronal loss, especially in the hilar, cornus ammonis 1 (CA1) and CA3 regions [5], although it is not completely clear whether this loss is a cause or a consequence of this process [6]. In addition, HS is associated with gliosis, granule cell dispersion and mossy fibre sprouting [1, 7, 8].

Tetraploid neurons (with a DNA content equivalent to 4C) can be found in different regions of the healthy central nervous system, such as the cortex and hippocampus [9, 10]. These neurons have larger dendritic trees and somas than diploid neurons and contribute to cell variability, playing specific roles as long-distance projection neurons [9–12]. The process causing tetraploidy, cell cycle re-activation, has been observed in different neurodegenerative diseases such as Alzheimer's disease [13]. In fact, polyploid neurons are more prone to die [14–16], probably by progressing through the G2/M cell cycle checkpoint [12, 13, 17]. This suggests that in neurodegenerative diseases, where neurons become tetraploid, G2/M transition blockade may act as a survival mechanism for these neurons [13].

TLE is characterised by recurrent focal seizures originating from a network located along the mesial aspect of the temporal lobe, which is usually accompanied by temporal or HS [4]. HS is the main diagnosis among patients undergoing surgical resection for focal DRE [8]. Furthermore, neuronal death in the epileptogenic zone associated with HS occurs in conjunction with electrophysiological changes [1]. Thus, we hypothesised that tetraploid neurons may be involved in epilepsy and, more precisely, in HS. These neurons may be more prone to die, similar to tetraploid neurons in Alzheimer's patients [14, 15, 18]. Moreover, we hypothesised that astrocytes could also become tetraploid thereby contributing to the gliosis observed in HS.

Thus, the first objective of this study was to determine whether tetraploid neurons are involved in TLE. Secondarily, we analysed whether tetraploid astrocytes are present in the brain of DRE patients and control subjects and if they are also involved in TLE.

Key points

- We demonstrated the existence of tetraploid astrocytes in the healthy human brain.
- Neuronal tetraploidy is increased in the hippocampi of patients with drug-resistant epilepsy.
- Astrocytic tetraploidy is in the hippocampi of patients with drug-resistant epilepsy.

MATERIALS AND METHODS

Study subjects and ethical approval

The protocol and the Informed Consent Form of this study were approved by the Independent Clinical Research Ethics Committee of the Hospital Universitario de La Princesa. The study followed the STROBE guidelines and the Revised Declaration of Helsinki. Samples of the epileptogenic zone and the surrounding cortical area from DRE patients were collected and frozen in dry ice immediately after neurosurgical extraction. Brain tissue resection was defined intraoperatively, according to the presence of interictal epileptogenic discharges, according to acute electrocorticography. If the resection involved the amygdala, this tissue was also analysed. An extensive analysis of the patients' clinical records was performed. Inclusion criteria were resective neurosurgery of the epileptogenic zone and signing the informed consent. The recruitment period lasted 2 years, starting on September 2018.

Post-mortem tissue from subjects without previous records of neurological, neurodegenerative or psychiatric diseases, who had signed informed consents, was provided by the biobank 'Biobanco en Red de la Región de Murcia', BIOBANC-MUR. Control samples were processed following standard operating procedures with appropriate approval of the Ethics and Scientific Committees. Post-mortem intervals are detailed in Table S1. The control-to-case ratio was 1:2.3. The samples used in each analysis were as follows depending on the anatomical zone of seizure onset: hippocampal origin $n = 16$, cortical epileptogenic zone $n = 3$. The amygdala was only resected in 3 patients as this procedure was limited to those patients in whom the resection was justified to improve their outcome.

The region selection for both brain tissue types was standardised to avoid a possible bias derived from the extraction, which was visually controlled, particularly for the hippocampus. All the possible subregions represented in the sample were included.

Cell nuclei isolation and immunostaining

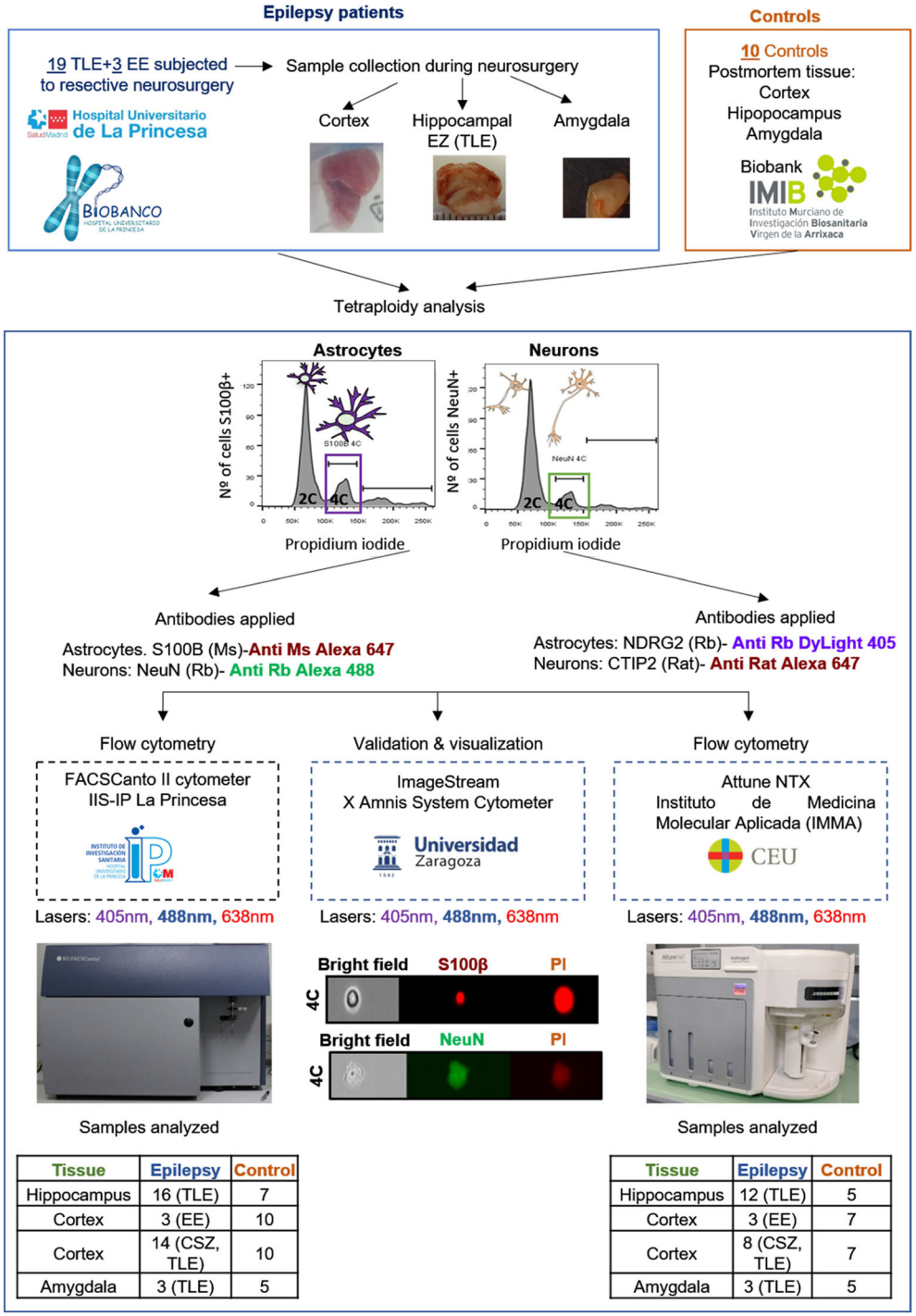
Samples consisting of 4–5 mm-edge cubes of human brain tissues were placed in Dounce homogenizers containing 3.0 ml ice-cold,

DNase-free PBS 0.1%Triton (PBT) with a protease inhibitor cocktail (Roche, Switzerland). Cell nuclei isolation was carried out following a modified form of a previously reported procedure [10, 19].

Immunostaining was performed by simultaneously adding primary and secondary antibodies to isolated unfixed nuclei in a medium containing 5% foetal calf serum (FCS) and 1.25 mg/ml bovine serum albumin (BSA). In control samples, the primary antibodies were excluded. Finally, the reaction was incubated overnight at 4°C in the dark. Immunostained nuclei were filtered through a 40 μm nylon filter, capable of retaining big aggregates but not nuclei. Then, the volume was adjusted to 350–800 μl with DNase-free PTx containing 40 μg/ml propidium iodide (PI, Sigma, USA) and 25 μg/ml DNAse-free RNAse I (Sigma, USA) and analysed in a Flow cytometer or an ImageStream X Amnis System Cytometer equipment.

Nuclear biomarkers of neurons and astrocytes were selected to stain only mature cells to differentiate tetraploid neurons or astrocytes from neural stem cells undergoing DNA synthesis or mitosis. Initially, NeuN and S100β were used to identify neurons and astrocytes, respectively. NeuN is a nuclear biomarker of adult neurons [10, 19].

Results were later validated with alternative biomarkers. CTIP2 was used as an alternative marker to detect neurons because it is expressed in a subpopulation of long-range projection tetraploid



Tissue	Epilepsy	Control
Hippocampus	16 (TLE)	7
Cortex	3 (EE)	10
Cortex	14 (CSZ, TLE)	10
Amygdala	3 (TLE)	5

Tissue	Epilepsy	Control
Hippocampus	12 (TLE)	5
Cortex	3 (EE)	7
Cortex	8 (CSZ, TLE)	7
Amygdala	3 (TLE)	5

FIGURE 1 Workflow of the experiments performed, the samples analysed and the equipment used for each determination. Abbreviations: 2C, diploid neurons; 4C, tetraploid neurons; CSZ, cortical surrounding zone to the epileptogenic zone; EE, extratemporal epilepsy; EZ, epileptogenic zone; Ms, mouse; PI, propidium iodide; Rb, rabbit; TLE, temporal lobe epilepsy

neurons [19]. Similarly, NDRG2 [20] and S100β [21] are also expressed in the nuclei of adult astrocytes.

The rabbit anti-NeuN polyclonal antibody (Merck, Germany) was diluted 1/800 and detected with a donkey anti-rabbit Alexa Fluor 488 (Invitrogen, UK). The mouse S100β monoclonal antibody [4C4.9] (Thermo Fisher Scientific, USA) was used at 1/70 dilution and detected with a goat anti-rabbit Alexa Fluor 647 (Invitrogen, USA).

The rat anti-CTIP2 monoclonal antibody [25B6] (Abcam, UK) was used at 6 μg/ml. The rabbit polyclonal anti-NDRG2 Antibody HPA002896 (Atlas antibodies) was used at 4 μg/ml. These antibodies were detected with a goat anti-rabbit DyLight 405 (Invitrogen, USA) and an anti-rat 647 (Atlas antibodies, Sweden) and used at 1/500.

Flow cytometry

NeuN and S100β analyses were carried out in the Flow Cytometry Unit of Hospital de La Princesa using a FACSCanto II Cytometer (BD Biosciences, USA). NDRG2 and CTIP2 analyses were carried out in an Attune™ NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific Inc., USA) in the Flow Cytometry Unit of 'Instituto de Medicina Molecular Aplicada' (IMMA, Universidad San Pablo CEU) (Figure 1).

Data were analysed with FACSDiva (BD Biosciences, USA), Thermo Scientific™ Attune™ NxT Software, (Thermo Fisher Scientific, USA) and FlowJo (Walter and Eliza Hall Institute of Medical Research, Australia) and displayed using logarithmic scaling. Tetraploidy was analysed as previously described focusing on the percentage of tetraploid neurons or astrocytes [10] (Figure S1). The total number of NeuN+ or S100β+NeuN− nuclei per epileptogenic zone analysed can be found in Table S3.

Tetraploid cells visualisation

An ImageStreamX Amnis System Cytometer (EMD Millipore, USA) that combines confocal microscopy and flow cytometry was used to visualise tetraploid neurons and astrocytes immunostained with NeuN and S100β as described above (Figure S2).

Statistical analysis

Quantitative variables are expressed as mean and standard deviation. Statistical comparison was performed by the Wilcoxon rank-sum test or analysis of variance (ANOVA) test as appropriate. The resulting comparison was represented by using boxplots combined with density plots, which allow the representation of the distribution according to the percentage of tetraploidy (wider densities imply a higher number of patients for that percentage). For clarity, the density plot is not shown for groups with low dispersion. The relation between clinical data and tetraploidy results was assessed by using the Pearson correlation coefficient. $p < 0.05$ was considered statistically significant. Statistical analyses were performed in R, version 4.0.3 (http://www.R-project.org).

RESULTS

Study population

Twenty-two DRE patients were subjected to neurosurgical resection of the epileptogenic zone and 10 controls were recruited (Table 1). Patients were previously treated with anticonvulsant drugs. Data on anticonvulsant treatment are summarised in Table S2. Nineteen patients were diagnosed with TLE, with an epileptogenic zone located in the hippocampus, and 3 patients had extratemporal epilepsy (EE). The anatomical findings observed in all samples from TLE patients were compatible with HS. For the EE cases, there was no evidence of cortical dysplasia in their samples. Subpial gliosis was observed in all EE cases, and in one case, there were ectopic neurons in the white matter, as the only significant alteration. Most of the patients (81%) achieved Engel I/II [22] 6 months after neurosurgery and thus were considered responders (Table 1).

Neuronal tetraploidy

Initially, the percentage of tetraploid neurons present in the epileptogenic zone was estimated as the proportion of positive cells for the neuronal marker NeuN using flow cytometry [10, 19]. The number of cytometry determinations varied among the different experimental conditions due to experimental issues (see Methods, Figure 1). Only mature NeuN+ S100β− neurons were considered. TLE patients ($n = 16$) showed a significant increase in the percentage of tetraploid neurons in the epileptogenic zone of the hippocampus compared with controls ($n = 7$, $p = 0.020$, Figure 2A). As

TABLE 1 Clinical and socio-demographic data of DRE patients and controls included in this study

		Patients	Controls
	Women (%)	9 (39%)	3 (30%)
	Age (years)	46 ± 9	60 ± 12
	Age at onset (years)	18 ± 14	NA
	Seizures/month (*n*)	7 ± 8	
	Anticonvulsant drugs (*n*)	5 ± 3	
Seizure aetiology	Structural	5 (23%)	
	Infectious	2 (9%)	
	Unknown	15 (68%)	
% Engel I/II	6 months	17 (81%)[a]	
	12 months	14 (88%)[a]	
	24 months	11 (85%)[a]	

Note: Data are presented as mean ± standard deviation or number (%). The outcome of neurosurgical resection was evaluated using Engel's classification at 6, 12 and 24 months after surgery. Patients were classified as responders to neurosurgery if they achieved Engel I and II. Abbreviations: DRE, drug-resistant epilepsy; NA, not applicable.
[a]Response was calculated for those patients whose clinical information was available.

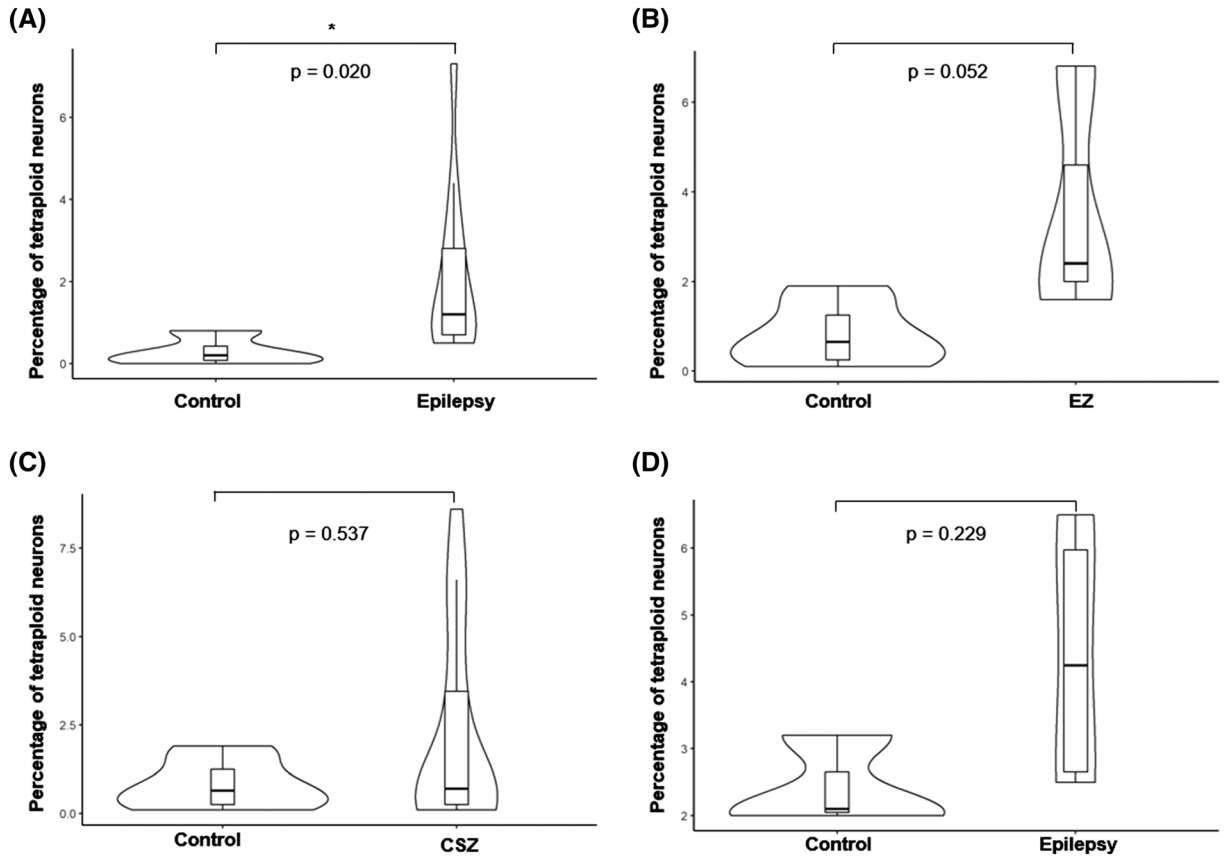


FIGURE 2 Percentage of tetraploid neurons in different brain regions of temporal lobe epilepsy (TLE) patients and controls. Cells from surgical samples were analysed for marker expression and tetraploidy by flow cytometry. The percentage of tetraploid neurons (NeuN+ S100β− cells) is shown for (A) the hippocampus of healthy controls ($n = 7$) and the epileptogenic zone of TLE patients ($n = 16$). (B) Cortex of healthy controls ($n = 10$) and epileptogenic zone of extratemporal epilepsy (EZ, $n = 3$). (C) Cortex of healthy controls ($n = 10$) and cortical surrounding zone (CSZ, $n = 14$) to the epileptogenic zone. (D) Amygdala of healthy controls ($n = 5$) and TLE patients ($n = 3$). Data are represented as violin box plots. Differences were analysed by the Wilcoxon rank sum exact test. *p < 0.05. Abbreviations: CSZ, cortical surrounding zone to the epileptogenic zone; EZ, epileptogenic zone

can be observed in the density plot (Figure 2A), in the control group, most subjects exhibited a percentage of tetraploid neurons lower than 1, which was increased to 1.5 in the epilepsy group. To determine whether the increase of neuronal tetraploidy was specific to the hippocampus, the percentage of tetraploid neurons was also analysed in EEs. An increase in the percentage of tetraploid neurons was observed in the cortical epileptogenic zone ($n = 3$) compared with the control cortex ($n = 10$). Although this difference was not significant, its p value was very close to the significance threshold ($p = 0.052$, Figure 2B). The percentage of tetraploidy in the amygdala could be assessed in patients who were subjected to amygdalohippocampectomy. No significant differences were observed between the amygdala of controls ($n = 3$) and patients ($n = 5$, $p = 0.229$, Figure 2D). Finally, to determine whether this increase in neuronal tetraploidy was specific to the epileptogenic zone, the surrounding region to the epileptogenic zone removed to access the hippocampus was also analysed. No significant differences were observed between the surrounding cortical regions of epileptic patients ($n = 14$) and control subjects ($n = 10$, Figure 2C). Similarly, the comparison between the epileptogenic cortical region ($n = 3$) of those patients with EE and the surrounding cortical zone of TLE patients ($n = 14$) showed no significant difference ($p = 0.577$, Figure S3a). Results of the tetraploidy analysis in double-positive (NeuN+ and S100β+) cells can be observed in Figure S4.

Moreover, to confirm the presence of tetraploid neurons in both patients and controls, tetraploid neurons were visualised using an ImageStream X Amnis System Cytometer equipment. This analysis reveals that tetraploid neurons from both controls and patients had a rounded morphology with only one nucleus with double DNA content (Figure 3A).

The presence of tetraploid neurons was also analysed in a subset of patients using a different biomarker of differentiated neurons (CTIP2), which has been shown to be enriched in the tetraploid population. Overall, no differences were found between controls and DRE patients in any of the regions analysed (hippocampus and cortex) (Figure S5).

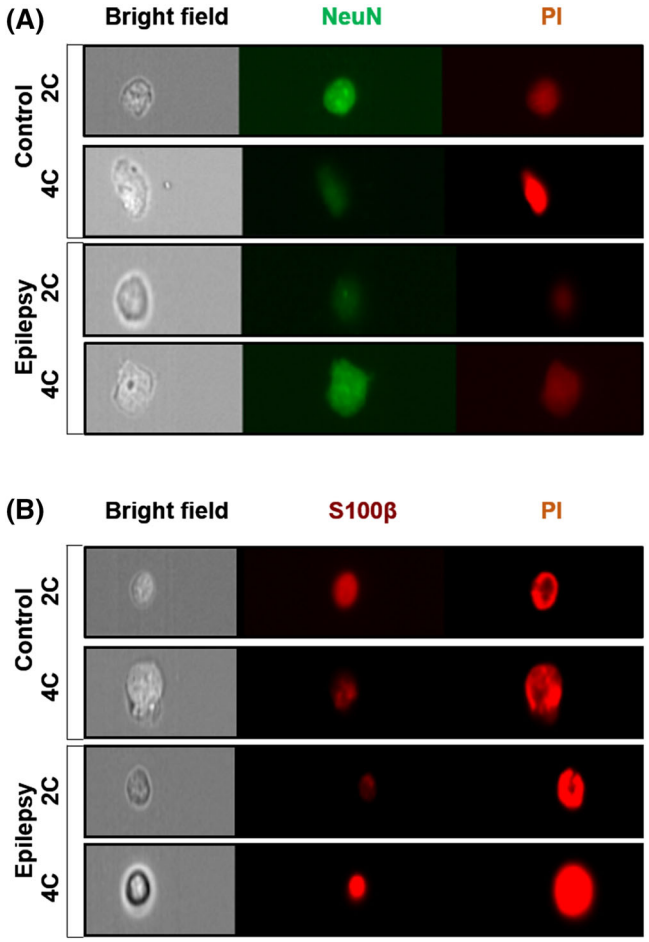


FIGURE 3 Visualisation of tetraploid neurons from temporal lobe epilepsy (TLE) patients and controls. (A) Cells from surgical samples were stained for NeuN and with PI, and images were acquired and analysed with an ImageStream X Amnis System Cytometer. Bright-field images (left panels) and NeuN (middle panels) and PI (right panels) staining are shown for NeuN+ cells from healthy control subjects (control) and TLE patients (epilepsy). (B) Cells from surgical samples were stained for S100β and with PI, and images were acquired and analysed with an ImageStream X Amnis System Cytometer. Bright-field images (left panels) and S100β (middle panels) and PI (right panels) staining are shown for S100β+ cells from healthy control subjects (control) and TLE patients (epilepsy). Abbreviations: 2C, diploid neurons; 4C, tetraploid neurons; PI, propidium iodide

Astrocytic tetraploidy

Although neuronal abnormalities are one of the main hallmarks of HS, glia also plays a key role in the epileptogenic zone. Thus, we wondered whether tetraploid astrocytes were present in DRE patients.

S100β-stained tetraploid astrocytes were found in the hippocampus, cortex and amygdala of healthy controls (Figures 3B and 4). Moreover, when the epileptogenic zone was the hippocampus ($n = 16$), levels of tetraploid S100β+ astrocytes were significantly increased compared with those of the control hippocampus ($p = 0.002$, $n = 7$, Figure 4A). No differences were found in the cortical epileptogenic zone ($p = 0.926$, Figure 4B). Although the mean of tetraploid astrocytes was increased in the cortex surrounding the epileptogenic zone and in the amygdala, this difference was not statistically significant ($p = 0.144$, Figure 4C; $p = 0.114$, Figure 4D). No significant differences were observed between the epileptogenic zone and the surrounding zone (Figure S3b).

S100β+ tetraploid astrocytes from the brain of epileptic patients and controls were visualised using an ImageStream X Amnis System Cytometer to confirm cytometry results. These astrocytes showed rounded morphology and only one nucleus (Figure 3B).

An alternative biomarker of differentiated astrocytes (NDRG2) was also used for tetraploidy analysis (Figure S6). The percentage of tetraploid astrocytes was higher in the epileptogenic zone of epilepsy patients (13.70 ± 5.48, $n = 12$) than in the control hippocampus (7.42 ± 5.10, $n = 5$), with a p value very close to the significance threshold ($p = 0.051$; Figure S6a). No significant differences were found in the percentage of NDRG2+ tetraploid astrocytes between the cortical epileptogenic zone and the control cortical tissue ($p = 0.905$, Figure S6b). Moreover, non-significant differences were observed between the control cortex and the surrounding zone, and in the amygdala between epilepsy patients and controls ($p = 0.152$; $p = 0.133$ respectively; Figure S6c,d).

Lastly, the relation between tetraploidy and clinical data is shown in Figure S7. The correlation between the percentage of tetraploid astrocytes and neurons was very strong for the amygdala ($r = 0.83$), cortex ($r = 0.98$) and hippocampus ($r = 0.99$). Surprisingly, there was a high correlation between the number of anti-convulsant drugs used by the patients and tetraploidy levels in the amygdala ($r = 0.83$ for astrocytes and $r = 1$ for neurons) but not in the other two areas. Moreover, there was a strong negative correlation between the number of seizures and the percentage of tetraploid neurons in the hippocampus ($r = -0.99$) and the percentage of tetraploid astrocytes in the hippocampus ($r = -0.97$) and the cortex ($r = -0.71$) (Figure S7b). As expected, there was a high correlation between age and tetraploidy in the cortex ($r = 0.88$ for astrocytes and $r = 0.95$ for neurons). An inverse correlation with age ($r = -0.99$ for astrocytes and $r = -0.86$ for neurons) was found in the amygdala, whereas no correlation was found in the hippocampus. Interestingly, there was a high inverse correlation between onset age and tetraploidy in the hippocampus ($r = -0.95$ for astrocytes and $r = -0.97$ for neurons) suggesting that early onsets implied higher percentages of tetraploidy. In the other two areas, no correlation with age of onset was found. Similarly, no association was observed between the percentage of tetraploidy and the Engel score 2 years after surgery (Table S4) or the history of febrile crises (Table S5).

DISCUSSION

In this study, we demonstrated the presence of both tetraploid neurons and astrocytes in TLE patients and control subjects. We also found a higher number of tetraploid neurons and astrocytes in the hippocampus of TLE patients.

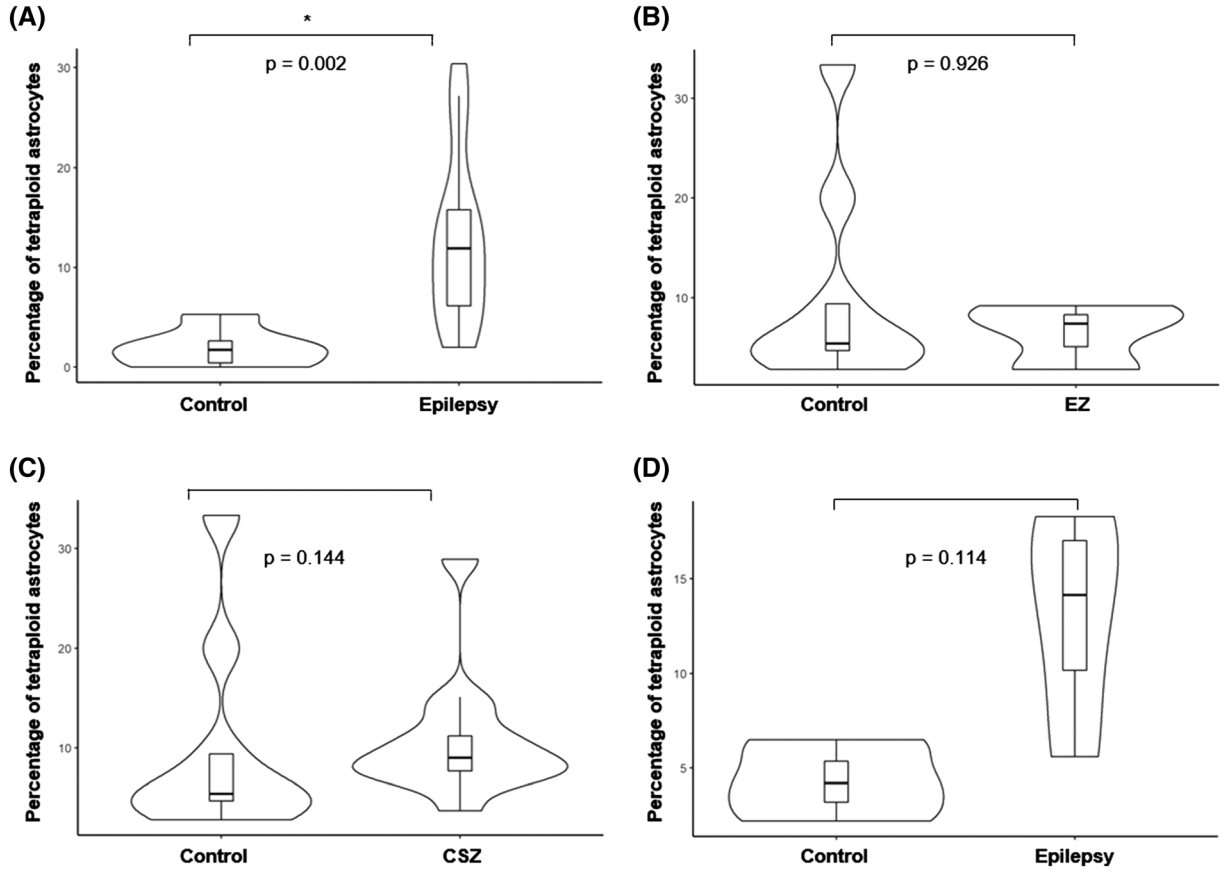


FIGURE 4 Percentage of tetraploid astrocytes in different brain regions of temporal lobe epilepsy (TLE) patients and controls. Cells from surgical samples were analysed for marker expression and tetraploidy by flow cytometry. The percentage of tetraploid astrocytes (S100β +NeuN− cells) is shown for (A) the hippocampus of healthy controls ($n = 7$) and the epileptogenic zone of TLE patients ($n = 16$). (B) Cortex of healthy controls ($n = 10$) and epileptogenic zone of extratemporal epilepsy (EZ, $n = 3$). (C) Cortex of healthy controls ($n = 10$) and cortical surrounding zone (CSZ, $n = 14$) to the epileptogenic zone. (D) Amygdala of healthy controls ($n = 5$) and TLE patients ($n = 3$). Data are represented as violin box plots. Differences were analysed by the Wilcoxon rank sum exact test. *p < 0.05. Abbreviations: CSZ, cortical surrounding zone to the epileptogenic zone; EZ, epileptogenic zone

As expected, tetraploid neurons were found in the cortex, hippocampus [10, 19] and amygdala of healthy controls. A significant increase in tetraploid neurons detected with NeuN was observed in TLE. We perceived a non-significant increment of tetraploid neurons in the surrounding region to the epileptogenic zone. The increase in extratemporal tetraploidy was not significant, although the p value was very close to the significance threshold ($p = 0.051$), probably due to the limited number of samples available ($n = 3$). To analyse tetraploidy, adult neurons were identified using the NeuN biomarker to differentiate them from adult neurogenesis, which is also impaired and abnormal in TLE [23, 24]. Unfortunately, we could not confirm the increase of neuronal tetraploidy in the hippocampus of TLE patients with an alternative biomarker (CTIP2). Perhaps, this is because although CTIP2 levels in tetraploid neurons were higher than those reported in previous publications, this marker is only expressed in a subset of tetraploid neurons [10].

Cell cycle reactivation in neurons of epilepsy patients has been proposed to modulate apoptosis [25–27]. Several factors are involved in this process. For example, E2F1, which is implicated in neuronal cell cycle reactivation and DNA synthesis [12], is found in an abnormal location in the neuronal cytoplasm of TLE neurons [28]. Cyclin B, involved in G2/M transition, has been observed in the hippocampus of TLE patients [27]. In addition, BDNF, a blocker of G2/M transition through Cdk1 inhibition in physiological tetraploid neurons [17], is overexpressed during seizures [29, 30]. These tetraploid neurons may be more prone to die, as previously described in Alzheimer's disease [14, 15, 18], contributing to the massive apoptotic cell death observed in the hippocampus of TLE patients [28, 31]. Although the association between neuron tetraploidy and cell death could not be thoroughly analysed, our data suggest a reduction in the number of neurons in the hippocampus and the surrounding cortical region to the epileptogenic zone.

Most DRE studies have focused on neurons [32]. However, astrocytes modulate synaptic plasticity and electrical properties in the brain in physiological conditions [33–36]. Recent studies have revealed that reactive astrocytes actively contribute to seizure activity in epileptogenesis [32]. During this process, astrocytes undergo morphological, molecular and functional modifications that allow the development of

astrogliosis, along with changes in the expression pattern of various proteins, such as transporters, receptors and enzymes, some of which are involved in astrocyte-neuron and astrocyte-astrocyte signalling [32]. In the 60s, there was controversy regarding the existence of tetraploid astrocytes and neurons [37–40]. Due to the lack of reproducible methods of DNA quantification, different researchers stated that it was impossible to reach a conclusion about this topic [41]. We have observed the presence of tetraploid astrocytes in different regions (cortex, hippocampus and amygdala) of the central nervous system of healthy controls and TLE patients using two different biomarkers (S100β and NDRG2) and two techniques (flow cytometry and image cytometry). We have confirmed that tetraploid astrocytes are increased in TLE. Tetraploid astrocytes may be bigger than diploid astrocytes, thereby contributing to the hypertrophy/hyperplasia of astrocytes found in the epileptogenic zone in TLE [42, 43]. Recently, polyploid astrocytes with abnormal mitosis were described in animal models of epilepsy [44] and Drosophila [45]. These studies found that multinucleated astrocytes could survive long periods and even re-enter mitosis [44]. These results suggest that possible complementary mechanisms caused by insults generated by seizures could alter the cell cycle.

Different processes suggesting cell cycle reactivation and proliferation in astrocytes have been observed in TLE patients [42, 43, 46]. In accordance with this observation, we found an increase in the number of astrocytes in all of the brain regions analysed. Surprisingly, unlike in neurons, NGF and p75 promote cell cycle arrest in astrocytes in epilepsy [47]. Moreover, in mouse models of TLE, BDNF and TrkB regulate severity and neuronal activity [48], which may contribute to G2/M blockade in tetraploid astrocytes in a similar way to the modulation in tetraploid neurons [17].

In accordance with previous results [10], we found a high correlation between age and cortical tetraploidy (in neurons and astrocytes) and between lower onset age and high levels of tetraploidy. Thus, if patients show an early onset of epilepsy, they may accumulate higher levels of tetraploidy. Moreover, an association between tetraploid neurons and tetraploid astrocytes was detected. This fact may be potentially explained because epilepsy may stimulate overexpression of factors that induce cell cycle re-entry (such as neurotrophins) [12, 19, 49] and tetraploidy in both cell populations. Similarly to recent treatments proposed for Alzheimer's [50], we hypothesise that the modulation of tetraploidy could be a potential treatment for DRE.

Limitations

This work has several limitations. The first and most relevant is the sample size. The number of patients, mainly DRE, is limited by the number of neurosurgical resections. This limitation is balanced by the exhaustive study of the patient clinical records and the fact that the tissue sample was fresh thus precluding potential biases caused by formalin fixation. Second, the control sample came from a brain tissue biobank; therefore, the possible effect of death on post-mortem tissue cannot be ruled out. The lack of availability of post-mortem brain tissue from drug-sensitive epilepsy patients or epilepsy patients not treated with anticonvulsant drugs hampered the analysis of the effect of epilepsy on the percentage of tetraploidy. Third, our results may have a bias regarding the tissue regions analysed. Despite all the tissues being visually controlled and the extraction being standardised, the selection of exactly the same anatomical structures in different samples was challenging, in particular for surgical brain samples, because clinical practice rules limited the amount of tissue extracted to the minimum necessary to improve the patient's condition. Fourth, although astrocytes are the brain cell type with the highest S100β expression [21], we observed that a subpopulation of neurons also expressed this marker [51]. Thus, we had to focus on S100β+/NeuN− astrocytes. Nevertheless, we validated our results with an alternative biomarker, NDRG2, which is mainly expressed in the nuclei of adult astrocytes [20]. Fifth, specific astrocyte states (e.g., reactive or non-reactive) could not be analysed in this study, due to the limitations of the current methodology for cell analysis. GFAP is one of the main biomarkers of reactive astrocytes [52] but is also expressed in neural stem cells [53]; accordingly, this biomarker does not allow for the differentiation of tetraploid astrocytes from neural stem cells. Sixth, the present study does not rule out the possible increase of tetraploid neurons and astrocytes in drug-sensitive patients; the obvious limitation of tissue samples from drug-sensitive patients hinders the comparison between drug-sensitive and DRE. Taking this all together, further studies are needed to confirm our results.

Conclusions

The presence of tetraploid astrocytes in healthy subjects is a groundbreaking discovery that could provide new insights into the study of the brain. The role of these cells in the structure and function of the brain remains unexplored and should be better studied in the future. Furthermore, both tetraploid neurons and astrocytes are increased in DRE. Accordingly, understanding their role in the pathogenesis of this disease could pave the way to new treatment lines for these patients.

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CONFLICT OF INTEREST

JMF is a shareholder (7.16% equity ownership) of Tetraneuron, a biotech company exploiting his patent on the phosphorylation of the Thr-248 and/or Thr-250 residues of the transcription factor E2F4 as a therapeutic target in pathological processes associated with somatic polyploidy. MC Ovejero-Benito has potential conflicts of interest (research support) with Leo Pharma. Nevertheless, these conflicts of interest are not related to the present study. The other authors have no relevant financial or non-financial interests to disclose.

ETHICS STATEMENT

The protocol and the Informed Consent Form of this study were approved by the Independent Clinical Research Ethics Committee of the 'Hospital Universitario de La Princesa'. The study followed the STROBE guidelines and the Revised Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

ASC performed the formal analysis, methodology and visualisation; PSJ and IGC performed the investigation and resources; PP, MN and MdT performed the resources; JMF and MDPM performed the investigation; CVT performed the resources, project administration and funding acquisition; MCOB performed the conceptualisation, formal analysis, methodology, investigation, visualisation, validation, project administration, funding acquisition, supervision and writing—original draft. All authors have read, reviewed and approved the final manuscript.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/nan.12873.

DATA AVAILABILITY STATEMENT

All data produced in the present study are available upon reasonable request to the authors.

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REFERENCES

1. Amin U, Benbadis SR. Avoiding complacency when treating uncontrolled seizures: why and how? *Expert Rev Neurother*. 2020;20(3):1-9. doi:10.1080/14737175.2020.1713100
2. Kwan P, Arzimanoglou A, Berg AT, et al. Definition of drug resistant epilepsy: consensus proposal by the ad hoc task force of the ILAE commission on therapeutic strategies. *Epilepsia*. 2010;51(6):1069-1077. doi:10.1111/j.1528-1167.2009.02397.x
3. Löscher W, Potschka H, Sisodiya SM, Vezzani A. Drug resistance in epilepsy: clinical impact, potential mechanisms, and new innovative treatment options. *Pharmacol Rev*. 2020;72(3):606-638. doi:10.1124/pr.120.019539
4. Baulac M. MTLE with hippocampal sclerosis in adult as a syndrome. *Rev Neurol (Paris)*. 2015;171(3):259-266. doi:10.1016/j.neurol.2015.02.004
5. Wieser H-G, ILAE Commission on Neurosurgery of Epilepsy. ILAE commission report. Mesial temporal lobe epilepsy with hippocampal sclerosis. *Epilepsia*. 2004;45(6):695-714. doi:10.1111/j.0013-9580.2004.09004.x
6. Thom M, Sisodiya SM, Beckett A, et al. Cytoarchitectural abnormalities in hippocampal sclerosis. *J Neuropathol Exp Neurol*. 2002;61(6):510-519. doi:10.1093/jnen/61.6.510
7. Fang M, Xi Z-Q, Wu Y, Wang X-F. A new hypothesis of drug refractory epilepsy: neural network hypothesis. *Med Hypotheses*. 2011;76(6):871-876. doi:10.1016/j.mehy.2011.02.039
8. Blumcke I, Spreafico R, Haaker G, et al. Histopathological findings in brain tissue obtained during epilepsy surgery. *N Engl J Med*. 2017;377(17):1648-1656. doi:10.1056/NEJMoa1703784
9. Lopez-Sanchez N, Frade JM. Genetic evidence for p75NTR-dependent tetraploidy in cortical projection neurons from adult mice. *J Neurosci*. 2013;33(17):7488-7500. doi:10.1523/JNEUROSCI.3849-12.2013
10. López-Sánchez N, Fontán-Lozano Á, Pallé A, et al. Neuronal tetraploidization in the cerebral cortex correlates with reduced cognition in mice and precedes and recapitulates Alzheimer's-associated neuropathology. *Neurobiol Aging*. 2017;56:50-66. doi:10.1016/j.neurobiolaging.2017.04.008
11. López-Sánchez N, Ovejero-Benito MC, Borreguero L, Frade JM. Control of neuronal ploidy during vertebrate development. In: Kubiak JZ, ed. *Cell Cycle in Development*. Springer Berlin Heidelberg; 2011:547-563. doi:10.1007/978-3-642-19065-0_22
12. Morillo SM, Escoll P, de la Hera A, Frade JM. Somatic tetraploidy in specific chick retinal ganglion cells induced by nerve growth factor. *Proc Natl Acad Sci U S A*. 2010;107(1):109-114. doi:10.1073/pnas.0906121107
13. Frade JM, Ovejero-Benito MC. Neuronal cell cycle: the neuron itself and its circumstances. *Cell Cycle*. 2015;14(5):712-720. doi:10.1080/15384101.2015.1004937
14. Mosch B, Morawski M, Mittag A, Lenz D, Tarnok A, Arendt T. Aneuploidy and DNA replication in the normal human brain and Alzheimer's disease. *J Neurosci off J Soc Neurosci*. 2007;27(26):6859-6867. doi:10.1523/JNEUROSCI.0379-07.2007
15. Arendt T, Brückner MK, Mosch B, Lösche A. Selective cell death of hyperploid neurons in Alzheimer's disease. *Am J Pathol*. 2010;177(1):15-20. doi:10.2353/ajpath.2010.090955
16. Barrio-Alonso E, Hernández-Vivanco A, Walton CC, Perea G, Frade JM. Cell cycle reentry triggers hyperploidization and synaptic dysfunction followed by delayed cell death in differentiated cortical neurons. *Sci Rep*. 2018;8(1):14316. doi:10.1038/s41598-018-32708-4
17. Ovejero-Benito MC, Frade JM. Brain-derived neurotrophic factor-dependent cdk1 inhibition prevents G2/M progression in differentiating tetraploid neurons. *PLoS ONE*. 2013;8(5):e64890. doi:10.1371/journal.pone.0064890
18. Arendt T. Cell cycle activation and aneuploid neurons in Alzheimer's disease. *Mol Neurobiol*. 2012;46(1):125-135. doi:10.1007/s12035-012-8262-0
19. López-Sánchez N, Frade JM. Genetic evidence for p75NTR-dependent tetraploidy in cortical projection neurons from adult mice. *J Neurosci off J Soc Neurosci*. 2013;33(17):7488-7500. doi:10.1523/JNEUROSCI.3849-12.2013
20. Flügge G, Araya-Callis C, Garea-Rodriguez E, Stadelmann-Nessler C, Fuchs E. NDRG2 as a marker protein for brain astrocytes. *Cell Tissue Res*. 2014;357(1):31-41. doi:10.1007/s00441-014-1837-5

21. Brozzi F, Arcuri C, Giambanco I, Donato R. S100B protein regulates astrocyte shape and migration via interaction with Src kinase *. *J Biol Chem*. 2009;284(13):8797-8811. doi:10.1074/jbc.M805897200
22. Engel JJ, Ness V, Rasmussen. Outcome with respect to epileptic seizures. In: *Surgical Treatment of the Epilepsies*; 1993:609-621.
23. Siebzehnrubl FA, Blumcke I. Neurogenesis in the human hippocampus and its relevance to temporal lobe epilepsies. *Epilepsia*. 2008;49-(Suppl 5):55-65. doi:10.1111/j.1528-1167.2008.01638.x
24. Thom M, Martinian L, Williams G, Stoeber K, Sisodiya SM. Cell proliferation and granule cell dispersion in human hippocampal sclerosis. *J Neuropathol Exp Neurol*. 2005;64(3):194-201. doi:10.1093/jnen/64.3.194
25. Timsit S, Rivera S, Ouaghi P, et al. Increased cyclin D1 in vulnerable neurons in the hippocampus after ischaemia and epilepsy: a modulator of in vivo programmed cell death? *Eur J Neurosci*. 1999;11(1):263-278. doi:10.1046/j.1460-9568.1999.00434.x
26. Koeller HB, Ross ME, Glickstein SB. Cyclin D1 in excitatory neurons of the adult brain enhances kainate-induced neurotoxicity. *Neurobiol Dis*. 2008;31(2):230-241. doi:10.1016/j.nbd.2008.04.010
27. Nagy Z, Esiri MM. Neuronal cyclin expression in the hippocampus in temporal lobe epilepsy. *Exp Neurol*. 1998;150(2):240-247. doi:10.1006/exnr.1997.6753
28. Fiala M, Avagyan H, Merino JJ, et al. Chemotactic and mitogenic stimuli of neuronal apoptosis in patients with medically intractable temporal lobe epilepsy. *Pathophysiol off J Int Soc Pathophysiol*. 2013;20(1):59-69. doi:10.1016/j.pathophys.2012.02.003
29. Lin TW, Harward SC, Huang YZ, McNamara JO. Targeting BDNF/TrkB pathways for preventing or suppressing epilepsy. *Neuropharmacology*. 2020;167:107734. doi:10.1016/j.neuropharm.2019.107734
30. Koyama R, Ikegaya Y. To BDNF or not to BDNF: that is the epileptic hippocampus. *Neurosci Rev J Bringing Neurobiol Neurol Psychiatry*. 2005;11(4):282-287. doi:10.1177/1073858405278266
31. Xu S, Pang Q, Liu Y, Shang W, Zhai G, Ge M. Neuronal apoptosis in the resected sclerotic hippocampus in patients with mesial temporal lobe epilepsy. *J Clin Neurosci off J Neurosurg Soc Australas*. 2007;14(9):835-840. doi:10.1016/j.jocn.2006.08.002
32. Riquelme J, Wellmann M, Sotomayor-Zárate R, Bonansco C. Gliotransmission: a novel target for the development of antiseizure drugs. *Neurosci Rev J Bringing Neurobiol Neurol Psychiatry*. 2020;26(4):1073858420901474-309. doi:10.1177/1073858420901474
33. Dulla CG. Losing touch with your astrocytes can cause epilepsy. *Epilepsy Curr*. 2015;15(6):349-350. doi:10.5698/1535-7511-15.6.349
34. Mederos S, Perea G. GABAergic-astrocyte signaling: a refinement of inhibitory brain networks. *Glia*. 2019;67(10):1842-1851. doi:10.1002/glia.23644
35. Perea G, Sur M, Araque A. Neuron-glia networks: integral gear of brain function. *Front Cell Neurosci*. 2014;8:378. doi:10.3389/fncel.2014.00378
36. Robel S. Astroglial scarring and seizures: a cell biological perspective on epilepsy. *Neurosci Rev J Bringing Neurobiol Neurol Psychiatry*. 2017;23(2):152-168. doi:10.1177/1073858416645498
37. Herman CJ, Lapham LW. DNA content of neurons in the cat hippocampus. *Science*. 1968;160(3827):537. doi:10.1126/science.160.3827.537
38. Lapham LW. Tetraploid DNA content of Purkinje neurons of human cerebellar cortex. *Science*. 1968;159(3812):310-312. doi:10.1126/science.159.3812.310
39. Museridze DP, Svanidze IK, Macharashvili DN. Content of DNA and dry weight of the nuclei of neurons of the external geniculate body and retina of the eye in Guinea pigs. *Sov J Dev Biol*. 1975;5(3):269-272.
40. Swift H. Quantitative Aspects of Nuclear Nucleoproteins**Aided by grants from the U. S. Public Health Service, and the Wallace C. and Clara A. Abbott Memorial Fund. In: Bourne GH, Danielli JF, eds. *International Review of Cytology*, Vol. 2. Academic Press; 1953:1-76. doi:10.1016/S0074-7696(08)61028-1
41. Swartz FJ, Bhatnagar KP. Are CNS neurons polyploid? A critical analysis based upon cytophotometric study of the DNA content of cerebellar and olfactory bulbar neurons of the bat. *Brain Res*. 1981;208(2):267-281. doi:10.1016/0006-8993(81)90557-6
42. Binder DK, Steinhäuser C. Astrocytes and epilepsy. *Neurochem Res*. 2021;46(10):2687-2695. doi:10.1007/s11064-021-03236-x
43. de Lanerolle NC, Lee T-S, Spencer DD. Astrocytes and epilepsy. *Neurother J am Soc Exp Neurother*. 2010;7(4):424-438. doi:10.1016/j.nurt.2010.08.002
44. Sosunov A, Wu X, McGovern R, Mikell C, McKhann GM, Goldman JE. Abnormal mitosis in reactive astrocytes. *Acta Neuropathol Commun*. 2020;8(1):47. doi:10.1186/s40478-020-00919-4
45. Nandakumar S, Grushko O, Buttitta LA. Polyploidy in the adult drosophila brain. *eLife*. 2020;9:e54385. doi:10.7554/eLife.54385
46. Liu JYW, Matarin M, Reeves C, et al. Doublecortin-expressing cell types in temporal lobe epilepsy. *Acta Neuropathol Commun*. 2018;6(1):60. doi:10.1186/s40478-018-0566-5
47. Cragnolini AB, Volosin M, Huang Y, Friedman WJ. Nerve growth factor induces cell cycle arrest of astrocytes. *Dev Neurobiol*. 2012;72(6):766-776. doi:10.1002/dneu.20981
48. Fernández-García S, Sancho-Balsells A, Longueville S, et al. Astrocytic BDNF and TrkB regulate severity and neuronal activity in mouse models of temporal lobe epilepsy. *Cell Death Dis*. 2020;11(6):411. doi:10.1038/s41419-020-2615-9
49. Alvim MKM, Morita-Sherman ME, Yasuda CL, et al. Inflammatory and neurotrophic factor plasma levels are related to epilepsy independently of etiology. *Epilepsia*. 2021;62(10):2385-2394. doi:10.1111/epi.17023
50. López-Sánchez N, Garrido-García A, Ramón-Landreau M, Cano-Daganzo V, Frade JM. E2F4-based gene therapy mitigates the phenotype of the Alzheimer's disease mouse model 5xFAD. *Neurother J am Soc Exp Neurother*. 2021;18(4):2484-2503. doi:10.1007/s13311-021-01151-1
51. Steiner J, Bernstein HG, Bielau H, et al. Evidence for a wide extra-astrocytic distribution of S100B in human brain. *BMC Neurosci*. 2007;8(1):2. doi:10.1186/1471-2202-8-2
52. Escartin C, Galea E, Lakatos A, et al. Reactive astrocyte nomenclature, definitions, and future directions. *Nat Neurosci*. 2021;24(3):312-325. doi:10.1038/s41593-020-00783-4
53. Yamaguchi M, Seki T, Imayoshi I, et al. Neural stem cells and neuro/gliogenesis in the central nervous system: understanding the structural and functional plasticity of the developing, mature, and diseased brain. *J Physiol Sci JPS*. 2016;66(3):197-206. doi:10.1007/s12576-015-0421-4

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