

# Calcium crystal formation in the epileptic focus of an *in vivo* epilepsy model

## Formación de cristales de calcio dentro del foco epiléptico de un modelo de epilepsia *in vivo*

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**ABSTRACT:** Epilepsy represents a significant health concern, affecting an estimated 2 million individuals in Mexico —patients enduring more than 70 seizures daily—. In the present study, refractory epilepsy was induced in Wistar rats by repeated stimulation with pentylenetetrazol (PTZ), ranging from one to one hundred stimulations. Hippocampal tissue was analyzed via scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS). The formation of solid calcium carbonate ( $\text{CaCO}_3$ ) crystals within the epileptic focus were observed. Notably, the concentration of  $\text{CaCO}_3$  exhibited a direct correlation with the number of stimulations, suggesting a progressive increase over the course of these events. This study puts forth a hypothesis elucidating the mechanism behind  $\text{CaCO}_3$  crystal formation in response to PTZ-induced seizures. Further research is needed to ascertain whether there exists a correlation between the dysregulation of calcium ions ( $\text{Ca}^{2+}$ ) in epilepsy and the formation of  $\text{CaCO}_3$ . This inquiry opens avenues for deeper understanding and potential advancements in the diagnosis of epilepsy and related neurological disorders.

**KEYWORDS:** calcification, calcium carbonate, epilepsy, Kindling model, SEM, EDS.

**RESUMEN:** La epilepsia representa un importante problema de salud, al estimarse su afectación a 2 millones de individuos en México —los pacientes sufren más de 70 convulsiones diarias—. En el presente estudio se indujo epilepsia refractaria en ratas Wistar mediante estimulación repetida con pentilentetrazol (PTZ), de una a cien estimulaciones. El tejido del hipocampo se analizó mediante microscopía electrónica de barrido (SEM) y espectroscopía de rayos X de energía dispersiva (EDS). Se observó la formación de cristales sólidos de carbonato cálcico

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(CaCO<sub>3</sub>) dentro del foco epiléptico. En particular, la concentración de CaCO<sub>3</sub> mostró una correlación directa con el número de estimulaciones, lo cual sugiere un aumento progresivo en el curso de estos eventos. Este estudio plantea una hipótesis, la cual elucida el mecanismo subyacente a la formación de cristales de CaCO<sub>3</sub> en respuesta a las convulsiones inducidas por PTZ. Es necesario seguir investigando para determinar si existe una correlación entre la desregulación de los iones de calcio (Ca<sup>2+</sup>) en la epilepsia y la formación de CaCO<sub>3</sub>. Esta investigación abre vías para una comprensión más profunda y posibles avances en el diagnóstico de la epilepsia y los trastornos neurológicos relacionados.

**PALABRAS CLAVE:** calcificación, carbonato de calcio, epilepsia, modelo Kindling, SEM, EDS.

## Introduction

Epilepsy is a chronic neurological disorder that manifests in recurrent, unprovoked seizures, profoundly affecting the lives of millions worldwide (Beghi, 2020; Begley *et al.*, 2022; Falco-Walter, 2020). According to the World Health Organization (WHO), epilepsy affects approximately 50 million people globally, making it one of the most common neurological conditions (WHO, 2024). In Mexico alone, an estimated 2 million individuals are affected by epilepsy, with some patients enduring as many as 70 seizures daily (Pesqueira *et al.*, 2023; Guzmán-Jiménez *et al.*, 2020; Rubio *et al.*, 2021). Such frequent seizures can lead to significant cognitive, emotional, and social impairments, exacerbating the overall burden of the disease on both patients and healthcare systems (Strzelczyk *et al.*, 2023; Vonck *et al.*, 2023; Rozensztrauch and Kołtuniuk, 2022). The management of epilepsy typically involves anticonvulsant medications, which help control seizures in the majority of cases (Riva *et al.*, 2021; E. Perucca 2021; Ghosh *et al.*, 2021). However, about 30% of patients do not respond to these medications, a condition known as refractory or drug-resistant epilepsy (Guery and Rheims, 2021; Lerche, 2020; Sultana *et al.*, 2021; Kwan *et al.*, 2010). Refractory epilepsy represents a particularly challenging aspect of the disorder, as it often results in frequent and severe seizures that are difficult to manage, leading to progressive neurological damage over time (Janson and Bainbridge, 2021; Anzellotti *et al.*, 2020; Singh, Mishra and Goel, 2021). The development of effective treatments for refractory epilepsy requires a deeper understanding of the underlying mechanisms that contribute to the persistence of seizures and the failure of pharmacological interventions.

One of the primary challenges in epilepsy research is elucidating the complex pathophysiological processes that underlie seizure activity. Several factors, including genetic predisposition, abnormal neuronal firing, and imbalances in neurotransmitter systems, have been implicated in the development of epilepsy (Rho and Boison, 2022; P. Perucca, Bahlo and Berkovic, 2020; Symonds and McTague, 2020). Seizures induce alterations in mesial-temporal structures, leading to neuronal loss and hippocampal size reduction, with 80% of complex partial seizures originating in the temporal lobe (Toro Pérez *et al.*, 2020). The hippocampus, a region of the brain critically in-

volved in memory formation and spatial navigation, is also one of the most affected areas during seizures (Wu *et al.*, 2020; Pires *et al.*, 2021; Girardi-Schappo *et al.*, 2021). Repeated seizure activity can lead to significant damage in this region, which has been linked to cognitive decline and memory impairments in both animal models and human patients with epilepsy (Ives-Deliperi and Butler, 2021; Novak, Vizjak and Rakusa, 2022; Engel *et al.*, 2013).

Seizures have a profound impact on the normal ion flow in the brain, particularly in the temporal lobe, as the region frequently involved in epilepsy. During a seizure, there is an excessive, synchronized firing of neurons, disrupting the delicate balance of intracellular and extracellular ions that is essential for normal neuronal function (Grisar, 1984; Spampinato *et al.*, 2004; Scharfman, 2007). This disruption primarily involves key ions such as sodium ( $\text{Na}^+$ ) (Catterall 2014), potassium ( $\text{K}^+$ ) (Brenner and Wilcox, 2012), chloride ( $\text{Cl}^-$ ) (Auer *et al.* 2020), and calcium ( $\text{Ca}^{2+}$ ) (Dreier and Heinemann, 1991; Steinlein, 2014), with modifications on the intracellular and extracellular concentration of the ions as depicted in table 1. Normally, the flow of these ions across neuronal membranes is tightly regulated by ion channels and transporters to maintain resting membrane potential and to propagate action potentials in a controlled manner. However, during seizure activity, this regulation breaks down. For instance, there is a massive influx of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  into neurons, which leads to depolarization and the uncontrolled firing of action potentials (Oyrer *et al.*, 2018; Sun *et al.*, 2022). The excessive influx of  $\text{Ca}^{2+}$  into neurons is particularly damaging, as calcium plays a critical role in various intracellular signaling pathways, including neurotransmitter release, gene expression, and activation of enzymes like proteases and phospholipases, which can lead to neuronal injury and cell death if dysregulated (Raimondo *et al.*, 2015; Benaim *et al.*, 2020). Concurrently, the efflux of  $\text{K}^+$  and accumulation of extracellular  $\text{K}^+$  contribute to further depolarization and prolong seizure activity by lowering the threshold for subsequent action (Ding *et al.*, 2024; Heinemann *et al.*, 1986). This imbalance between intracellular and extracellular ion concentrations creates a vicious cycle of excitotoxicity, wherein excessive excitatory neurotransmitter release, particularly glutamate, further exacerbates ionic imbalances and neuronal hyperexcitability (Chen *et al.*, 2023; Sarlo and Holton, 2021; Ben-Ari, 2002). The temporal lobe, due to its dense network of excitatory circuits, is especially vulnerable to these changes, and prolonged or recurrent seizures can lead to long-term alterations in ion channel function, synaptic plasticity, and even structural damage, contributing to the development of chronic epilepsy (Helmstaedter *et al.*, 2003; Elger, Helmstaedter and Kurthen, 2004; Lévesque, Ragsdale and Avoli, 2019). Understanding these ion imbalances is crucial for developing therapeutic interventions aimed at restoring ionic homeostasis in epilepsy patients.

**Table 1.**  $[ion]_{intra}$  and  $[ion]_{extra}$  (intracellular and extracellular, free ion concentrations). And  $E_{pds}$  energy potential gradient.

Typical rest				The typical peak during a seizure			Reference
Ion	$[ion]_i$	$[ion]_e$	$E_{ion}$	$[ion]_i$	$[ion]_e$	$E_{ion}$	
K <sup>+</sup>	96 mM	4 mM	−85 mV	94 mM	12 mM	−55 mV	Jiang and Haddad (1991)
Na <sup>+</sup>	10 mM	145 mM	+71 mV	55 mM	139 mM	+25 mV	Dreier and Heinemann (1991)
Ca <sup>2+</sup>	70 nM	2 mM	+137 mV	700 nM	100 μM	+66 mV	Jiang and Haddad (1991)
Cl <sup>−</sup>	7 mM	145 mM	−80 mV	26 mM	152 mM	−47 mV	Raimondo <i>et al.</i> (2015)

Source: Author’s elaboration.

In the current study, the effects of repeated seizures on calcium homeostasis and its potential link to the formation of calcium compounds crystals within the brain, specifically in the hippocampus, were evaluated in a well-established animal model of epilepsy, in which seizures were induced in Wistar rats through repeated administrations of pentylenetetrazol (PTZ), a chemical convulsant (Samokhina and Samokhin, 2018; Dhir, 2012). PTZ has been widely used in epilepsy research to induce seizures in rodents, as it reliably produces seizure activity that mimics certain aspects of human epilepsy, including both acute and chronic phases (Löscher, 2017). The hippocampal tissue from the rats was analyzed using scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS) to investigate potential structural changes in response to PTZ-induced seizures. SEM and EDS are powerful tools for the observation and quantification of mineral formations in brain tissue, providing high-resolution imaging and precise elemental analysis (Lewis *et al.*, 2019; Cheville and Stasko, 2014). SEM enables detailed visualization of the ultrastructure of tissues, including the identification of abnormal mineral deposits, such as calcium crystals, by producing images with a resolution far beyond that of light microscopy. The high magnification and depth of field in SEM make it ideal for studying the three-dimensional morphology of mineral formations at the micro- and nanoscale (Goldstein *et al.*, 2018). Complementing SEM, EDS provides the ability to perform elemental analysis by detecting the characteristic X-rays emitted from a sample when it is bombarded by an electron beam (Wyroba *et al.*, 2015). This allows for the precise identification and quantification of specific elements, such as calcium, in biological tissues (Newbury, 2005). EDS operates based on the principle that each element emits X-rays at unique energy levels when excited by an electron beam, thus enabling the detection of the elemental composition of the sample (Schneider *et al.*, 2010). The combination of SEM and EDS is particularly beneficial for studying pathological mineralization in brain tissue, as it not only reveals the spatial distribution and structure of these deposits but also allows for the quantitative analysis of their elemental makeup (Tsolaki and Bertazzo, 2019). This dual capability is crucial for understanding

the role of mineral formations in neurological conditions, such as epilepsy, where calcium dysregulation may contribute to disease progression.

The findings of the study revealed the formation of solid calcium carbonate ( $\text{CaCO}_3$ ) crystals within the epileptic foci of the hippocampus, a novel observation in epilepsy research. Interestingly, the concentration of  $\text{CaCO}_3$  crystals was found to correlate directly with the number of PTZ stimulations, suggesting that the repeated seizure activity led to a progressive accumulation of these crystals over time. This raises several important questions regarding the potential mechanisms underlying  $\text{CaCO}_3$  crystal formation and its implications for the pathophysiology of epilepsy.

## Materials and methods

### *In vivo* refractory epilepsy model

The kindling animal model was used as an *in vivo* refractory epilepsy model (Singh, Mishra and Goel, 2021). A total of 30 Wistar rats (180–260 g) were injected intraperitoneally with subconvulsive doses (35 mg/kg) of pentylenetetrazol (PTZ) dissolved in saline water daily. Following every injection, seizure activity was monitored for a duration of 20 minutes. The resultant seizures were categorized as follows: stage 0 denoting no observable response; stage 1 involving twitching of the face and ears; stage 2 consisting of jerking in the forelimbs and face; stage 3 encompassing myoclonic jerks and lifting; stage 4 characterized by clonic convulsions leading to the animal falling onto its side; stage 5 involving intense, repetitive tonic-clonic seizures. Animals were deemed to have undergone kindling once they displayed a sequence of three consecutive episodes at stage 4 or 5 (Spiller and Racine, 1994). Five groups ( $n = 6$ ) were designated according to the number of kindling stimulations ( $K$ ) that were induced: (i)  $K = 5$ ; (ii)  $K = 8$ ; (iii)  $K = 22$ ; (iv)  $K = 34$ ; (v)  $K = 60$ . The groups were compared to a control group ( $n = 6$ ) that did not receive any treatment.

### Histological procedures

At the 6-month mark after the analysis, the rats were euthanized with sodium pentobarbital (60 mg/kg) and intracardially perfused with 100 mL of 0.1 M phosphate buffer, followed by 100 mL of paraformaldehyde (4% w/v in 0.1 M PBS, PFA). Following fixation in 4% PFA for 12 hours, the brains were subjected to cryoprotection by undergoing consecutive 24-hour incubations in solutions containing 10%, 20% and 30% sucrose (dissolved in phosphate buffer). Subsequently, the brains were coronally sliced at a thickness of 10  $\mu\text{m}$  using a cryostat and then preserved in an antifreeze solution. The sections were dyed using the Hematoxylin-eosin technique.

### Measurement of crystals in brain tissue

Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS) techniques were used to measure crystal formation in extracted tissues.



For this, sections extracted from the frontal lobe were carefully chosen and treated with osmium tetroxide (1% in PB) for an hour at 4 °C. They were subsequently dehydrated using increasing concentrations of 70% ethanol supplemented with 1% uranyl acetate. After complete dehydration, the sections were immersed in propylene oxide and then exposed to a mixture of propylene oxide and Durcupan resin. Following a 48-hour polymerization period at 56 °C, the sections were observed under a light microscope to identify areas of interest. These areas were chosen for further processing: re-embedding and cutting into ultrathin sections for electron microscopy. To determine the appropriate region of the frontal lobe for ultrathin sectioning, semithin sections (500 nm thick) were stained using toluidine blue. The ultrathin sections (60 nm) were sliced using a Leica Ultracut UCT microtome, placed onto Formvar-coated single slot copper grids, and then stained with uranyl acetate and lead citrate.

The morphological characteristics and chemical composition of the crystals in the tissues were examined using a JEOL 5600 LV microscope, which was equipped with energy-dispersive X-ray spectroscopy (EDS) for elemental analysis. The SEM's ability to detect electron beam interactions with both organic and inorganic materials enabled detailed exploration of contrast variations and morphologies (Knott *et al.*, 2008; Knott, Rosset and Cantoni, 2011; Kempen *et al.*, 2015). For semi-quantification, the sample was measured at 20 kV, and secondary electrons (SE) were used to capture micrographs. Using a magnification of 2000x, initial micrographs were taken on which EDS analysis was performed to quantify signal intensity as counts per second based on the study area (2000  $\mu\text{m}^2$ ). For each Kindling stimulus value, three tissue samples were measured, and three micrographs were taken from different areas to ensure the measurements were representative of the entire tissue. The standard deviation of the intensities was used as the relative error. The signal intensity for each measured element was taken as its concentration over the standardized area, allowing for a correlation between intensity and the quantity of crystals present in the study area.

The determination of the compound type comprising the crystal was performed using point-based EDS, with the largest crystals used as reference points. The compound type was identified by consulting relevant literature and correlating the elements with their relative concentrations.

### Statistical analysis

The statistical value was represented as the mean  $\pm$  the standard deviation associated with the counts per second for each element in the 2000  $\mu\text{m}^2$  measurement area. Once the data were obtained, logarithmic regressions were performed in the form  $I_{\text{Ca}} = k \ln(K) + I_0$  where  $I_{\text{Ca}}$  represents the signal intensity of calcium in the measurement area,  $K$  is the number of Kindling stimulations,  $k$  is a constant that relates  $K$ , the PTZ concentration and the intensity, and  $I_0$  is the initial calcium concentration at 0 Kindling stimulations. The logarithmic relationship that yielded a coefficient of determination greater



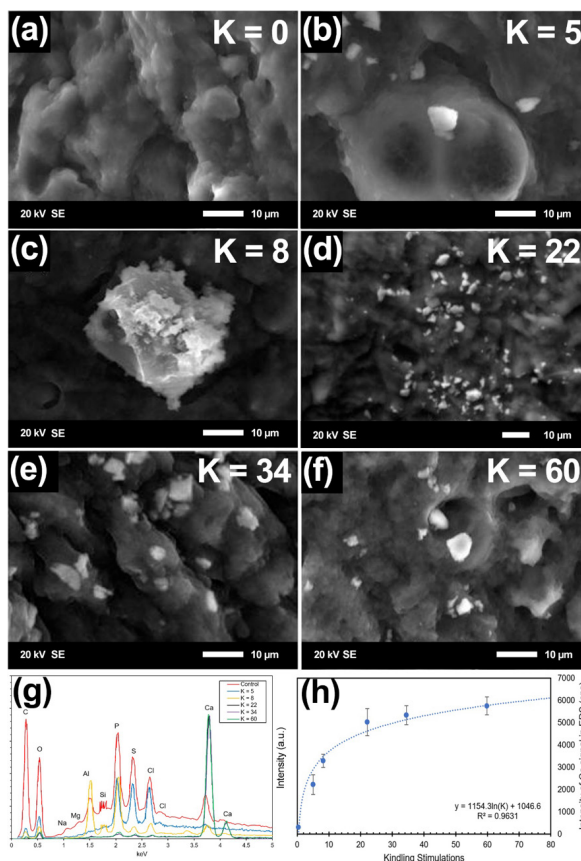
than 0.96 was identified. For determination of statistical differences in intensity, a one-way ANOVA was carried with a significance of 0.95.

## Results

### Calcium crystals analysis

Figure 1 shows representative microscopies obtained for the different groups according to the number of stimulations: K = 0 (1a); K = 5 (1b); K = 8 (1c); K = 22 (1d); K = 34 (1e) and K = 60 (1f). Control rats did not exhibit microscopic calcium crystal formation (figure 1a). In contrast, crystals in a wide range of sizes, from 5 to 20  $\mu\text{m}$  in width, were observed in the rest of the tissues.

**Figure 1.** Representative SEM micrographs showing calcium carbonate crystals in brain tissue of epileptic rats at different Kindling stimulations.



(a) Healthy Wistar rat amygdala (K = 0); (b) K = 5; (c) K = 8; (d) K = 22; (e) K = 34; (f) K = 60; (g) EDS spectra of samples, and, (h) calcium intensity per 2000  $\mu\text{m}^2$  of tissue as a function of Kindling stimulations. SE = secondary electrons.

Source: Author's elaboration.

An EDS analysis was performed to identify and quantify the elements present in the identified crystals. Figure 1g shows the EDS spectra of the tissues analyzed as a function of the number of stimulations performed. Similarly, figure 1h presents a comparison between calcium intensity in counts per second and the number of stimulations (average values  $\pm$  standard deviation): the dotted line reflects the fitted curve after a logarithmic regression. The graph suggests that calcium concentration (as determined by signal intensity) increases as a matter of the number of Kindling stimulations, suggesting a relationship between  $\text{Ca}^{2+}$  dysregulation and the severity of the epileptic seizures in the animal. The fitted curve reflects that calcium intensity follows a logarithmic behavior in relation to the number of Kindling stimulations, with a constant value of 1154.3 that correlates the concentration of PTZ and the number of Kindling stimulations.

Point EDS on the largest crystals observed for each tissue made it possible to identify the proportion of the remaining elements with respect to the amount of calcium observed. Table 2 shows the comparison of elemental ratios for rat tissues with  $K = 0$ , 22 and 34.

From the stoichiometric quantification of the atomic ratios for each element (table 2) it was determined that the crystals could be calcium carbonates or carboxylates, since only significant differences in calcium intensity were identified with respect to the control group without Kindling stimulations. Other potential calcium compounds include hydroxyapatite, a common compound found in bone structures with formula  $\text{Ca}_5(\text{PO}_4)_3(\text{OH})$ ; however, the significant decrease in the phosphorous signal associated with the increase in Kindling stimulations could indicate that crystals are not composed of such element. Since these were organic compounds, key elements such as carbon or oxygen were ruled out given the incapacity of the equipment to discern between organic and inorganic elements between the crystal formation.

**Table 2.** Punctual EDS quantification of elements present in calcium crystals.

Element	K = 0 (cps)*	K = 22 (cps)*	K = 34 (cps)*
Na	6.4 $\pm$ 1.5	10.9 $\pm$ 1.1	12.1 $\pm$ 1.3
Mg	2.8 $\pm$ 0.6	1.7 $\pm$ 0.2	1.5 $\pm$ 0.2
Al	21.3 $\pm$ 5.8	12.8 $\pm$ 1.3	30.4 $\pm$ 3.2
S	74.1 $\pm$ 12.1	36.1 $\pm$ 13.0	46.9 $\pm$ 7.0
Cl	77.4 $\pm$ 16.2	47.9 $\pm$ 23.8	49.1 $\pm$ 15.4
P	80.1 $\pm$ 32.1	1.8 $\pm$ 0.1 <sup>†</sup>	2.1 $\pm$ 1.4 <sup>†</sup>
Ca	72.6 $\pm$ 12.0	4000.1 $\pm$ 2600 <sup>†</sup>	5006.6 $\pm$ 1466 <sup>†</sup>
K	5.3 $\pm$ 0.8	8.5 $\pm$ 0.9	9.1 $\pm$ 0.8

\* Values are expressed as the average cps  $\pm$  standard deviation.

<sup>†</sup> Statistically significant values with  $p < 0.05$ .

Source: Author's elaboration.



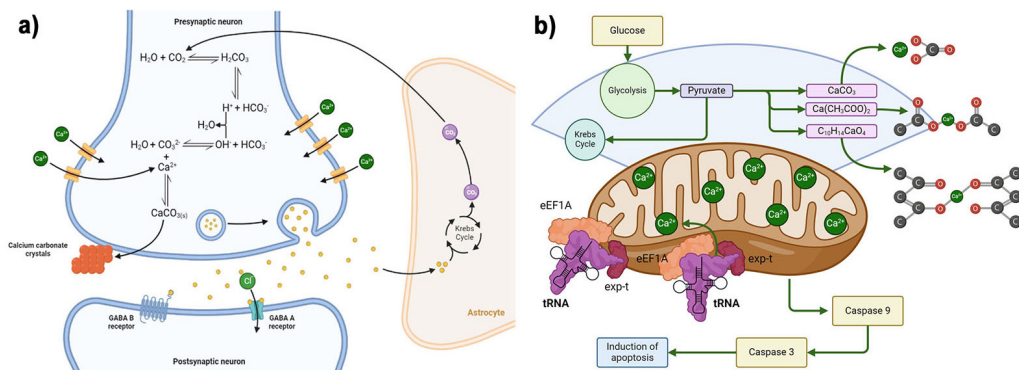
## Discussion

Calcium carbonate is a common inorganic compound that is typically associated with skeletal structures in the human body, such as bones and teeth (Ohgushi *et al.*, 1992; Bushinsky and Lechleider, 1987; Harvey, Zobitz and Pak, 1988). The presence of  $\text{CaCO}_3$  crystals in brain tissue, however, is highly unusual and has not been extensively documented in the context of epilepsy. We observed large solid crystals in epileptic seizure-induced rats. Detailed spectroscopic analysis showed that these structures were mainly composed of  $\text{CaCO}_3$ . One possible explanation for the formation of these crystals is related to the dysregulation of calcium ions ( $\text{Ca}^{2+}$ ) during seizure activity (Wojda, Salinska and Kuznicki, 2008; Akita and Fukuda, 2020; Kovac *et al.*, 2017). Seizures are known to cause excessive neuronal firing, leading to an influx of  $\text{Ca}^{2+}$  into neurons and astrocytes (Steinlein, 2014b; Siesjö, 1986). This influx can trigger a cascade of events, including the activation of calcium-dependent enzymes and signaling pathways that may contribute to neuronal damage (Yu, Chang and Tan, 2009; Zündorf and Reiser, 2011; Gleichmann and Mattson, 2011). The prolonged dysregulation of calcium homeostasis during recurrent seizures could potentially create conditions conducive to the precipitation of  $\text{CaCO}_3$  crystals within the brain tissue.

A literature analysis suggests a hypothesis on the formation of  $\text{CaCO}_3$  crystals. Synaptic processes are highly regulated under normal conditions. However, metabolism in the epileptic patient has as its main cellular manifestation what is known as the paroxysmal depolarizing shift (PDS) (Kubista, Boehm and Hotka, 2019; Hotka and Kubista, 2019; Tryba *et al.*, 2019; Tian *et al.*, 2005). Electrophysiological studies have shown evidence associated with a calcium ion ( $\text{Ca}^{2+}$ )-mediated depolarization, which results in voltage gated sodium channels opening and action potentials (Hotka and Kubista, 2019b). This depolarization is followed by a process of hyperpolarization mediated by  $\text{Ca}^{2+}$ -dependent potassium channels, resulting in a massive entrance of  $\text{Ca}^{2+}$  in neurons (Tryba *et al.*, 2019b).

Carbon dioxide ( $\text{CO}_2$ ) molecules released throughout the citric acid cycle may interact with intracellular water to produce carbonic acid ( $\text{H}_2\text{CO}_3$ ); this same acid dissociates into protons ( $\text{H}^+$ ) and acid carbonate ( $\text{HCO}_3^-$ ), which reacts with hydroxyl ions ( $\text{OH}^-$ ) generated by the same dissociation of the existing water to produce water ( $\text{H}_2\text{O}$ ) and bicarbonate ions ( $\text{CO}_3^{2-}$ ) (Becker, 1936). The high internalization of  $\text{Ca}^{2+}$  during PDS favors the interaction of  $\text{CO}_3^{2-}$  ions with  $\text{Ca}^{2+}$  ions, a reaction that culminates in the formation of  $\text{CaCO}_3$  species, which are susceptible to their own polymerization and, thus, the formation of defined crystalline superstructures that fixes in the extra-neuronal tissue (Wiechers, Sturrock and Marais, 1975). In this sense, although  $\text{H}_2\text{CO}_3$  acts as a defense mechanism in an attempt to compensate the polarization imbalance by calcium ions, the attempt is insufficient, and the charge decompensation leads to the appearance of epileptic seizures. The above is summarized in figure 2.

**Figure 2.** Hypothesis on the formation of calcium carbonate crystals.



(a) Formation of calcium carbonate crystals from  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$ . (b) Mitochondria function during a series of epileptic seizures and the impact of  $\text{Ca}^{2+}$  dysregulation on cell death through apoptosis. Source: Author's elaboration.

Other groups have reported the formation of calcium superstructures derived from CNS conditions (Froberg, Dorion and McMartin, 2006; Campbell *et al.*, 1999). However, until the present study, calcium crystal formation of any kind had not been reported in brains with epilepsy. As indicated above, dysregulation of neurotransmission mechanisms attributable to the calcium ion can lead to the formation of these crystals, and this phenomenon is not exclusive to epilepsy and can occur in other CNS pathologies.

The formation of  $\text{CaCO}_3$  crystals could represent a protective response by the brain to sequester excess  $\text{Ca}^{2+}$  ions, thereby preventing further damage. Calcium sequestration mechanisms have been observed in other tissues, such as muscle and bone, where excess calcium is stored in the form of calcium salts (Kraus-Friedmann, 1990; Lenton *et al.*, 2015; Moore *et al.*, 1975; Movsesian, 1998). It is plausible that a similar mechanism may be at play in the brain during prolonged seizure activity, although further research is needed to confirm this hypothesis. Furthermore, such crystal formation in the hippocampus also raises the possibility that these crystals could serve as a biomarker for epilepsy progression or severity. The direct correlation between the number of Kindling stimulations and the concentration of  $\text{CaCO}_3$  suggests that the accumulation of these crystals may reflect the cumulative effects of repeated seizures on the brain. If confirmed in human studies,  $\text{CaCO}_3$  crystals could potentially be used as a diagnostic tool to monitor disease progression in patients with epilepsy. Additionally, the presence of  $\text{CaCO}_3$  could offer insights into the long-term effects of refractory epilepsy and may inform the development of new therapeutic strategies aimed at restoring calcium homeostasis in the brain.

Notably, the identification of  $\text{CaCO}_3$  crystals in the hippocampus further opens new avenues for investigating the broader implications of calcium dysregulation in neurological disorders. Calcium is a key signaling molecule in-

volved in various physiological processes, including synaptic transmission, gene expression, and cell death (Clapham, 1995; Dodd, Kudla and Sanders, 2010). Dysregulation of calcium signaling has been implicated in a wide range of neurological conditions, including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS) (Bojarski, Herms and Kuznicki, 2008; Wang, Shi and Wei, 2017; Calvo-Rodriguez, Kharitonova and Bacskai, 2020; Cali, Ottolini and Brini, 2014; Ludtmann and Abramov, 2018; Schapira, 2013; Leal and Gomes, 2015; Grosskreutz, Van Den Bosch and Keller, 2010; Kawamata and Manfredi, 2010). The findings of the current study suggest that calcium dysregulation may also play a central role in the pathophysiology of epilepsy, potentially through the formation of  $\text{CaCO}_3$  crystals. Further research into the molecular mechanisms governing calcium homeostasis in the brain could provide important insights into the development of novel treatments for epilepsy and other neurological disorders.

Finally, further analyses are needed by means of SEM and spectroscopy evaluation (such as EDS) in tissues of neurodegenerative diseases, neoplasms, among others, that due to their nature associated to calcium metabolism could lead to the formation of these complexes. Similarly, it is necessary to continue research on the formation of  $\text{CaCO}_3$  crystals observed in the present work to corroborate the mechanism of formation and determine whether it is a neuroprotective process or a symptom of the deregulatory process.

## Conclusions

The present work reports the formation of solid  $\text{CaCO}_3$  was observed for the first time in an animal model of epilepsy, determining a direct relationship in the increase of calcium concentration as a function of the number of stimulations. It is hypothesized that  $\text{CaCO}_3$  is synthesized from calcium and carbonate ions present in synapses during the dysregulation observed in the pathological molecular processes of epilepsy. It remains unknown whether this phenomenon is a process of neuroprotection against the massive depolarization that occurs during epileptic seizures, so it is necessary to further investigate this hypothesis. However, the discovery of calcium carbonate ( $\text{CaCO}_3$ ) crystals in the hippocampal tissue of PTZ-treated rats represents a significant advancement in our understanding of the pathophysiological changes that occur in response to repeated seizure activity. The correlation between the number of PTZ stimulations and the concentration of  $\text{CaCO}_3$  crystals suggests that calcium dysregulation may play a key role in the progression of epilepsy. While the precise mechanisms underlying  $\text{CaCO}_3$  crystal formation remain unclear, this study opens new avenues for research into the role of calcium homeostasis in epilepsy and other neurological disorders. Further investigation is needed to determine whether  $\text{CaCO}_3$  crystals could serve as a biomarker for epilepsy progression and to explore the potential therapeutic implications of restoring calcium balance in the brain.

## Contributions by author

*Tessy López-Goerne*: Conception and design of the article, methodological development, data mining, analysis and interpretation, writing of the original draft. Review and final editing of the text.

*Rafael Valiente*: Writing the original draft, review and final editing of the text.

*Emilio Chávez*: Writing the original draft, review and final editing of the text.

*Antonella González-Bondani*: Writing the original draft, review and final editing of the text.

*Joaquín Manjarrez*: Methodological development, data mining, analysis and interpretation.

*Francisco J. Padilla-Godínez*: Data mining, analysis and interpretation, writing of the original draft. Review and final editing of the text.

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