UNIVERSIDADE FEDERAL DE ALAGOAS

INSTITUTO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

MAISA DE ARAÚJO COSTA

EFEITO NEUROPROTETOR DA LECTINA EXÓGENA GALECTINA-1 NO *STATUS EPILEPTICUS*

MACEIÓ-AL

2018

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Artigo para defesa de Mestrado apresentado ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal de Alagoas como requisito parcial para a obtenção do grau de mestre em Ciências da saúde.

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MACEIÓ-AL

2018

Catalogação na fonte
Universidade Federal de Alagoas
Biblioteca Central
Bibliotecária Responsável: Janis Christine Angelina Cavalcante

C837e Costa, Maisa de Araújo.
Efeito neuroprotetor da lectina exógena galectina-1 no *Status Eplepticus* / Maisa de Araújo Costa. – 2018.
34 f. : il., grafs., tabs.

Orientador: Olagide Wagner de Castro.
Coorientadora: Adriana Ximenes da Silva.
Dissertação (Mestrado em Ciências da Saúde) – Universidade Federal de Alagoas. Instituto de Ciências Biológicas e da Saúde. Programa de Pós-Graduação em Ciências da Saúde, Maceió, 2018.

Bibliografia: f. 28-31.

1. Eplepcia. 2. Galectin-1. 3. *Status Eplepticus*. I. Título.

CDU: 616.853

Universidade Federal de Alagoas
Instituto de Ciências Biológicas e da Saúde
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Efeitos neuroprotetor da lectina exógena galectina-1 no status epilepticus

Dissertação submetida ao corpo docente do Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal de Alagoas e aprovada em 06 de março de 2018.

Banca Examinadora

Prof. Dr. Olagide Wagner de Castro (Orientador)

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RESUMO

A microinjeção de pilocarpina intra-hipocampal (H-PILO) induz Status Epilepticus (SE) que pode levar a convulsões recorrentes espontâneas (SRS) e neurodegeneração em roedores. Estudos utilizando modelos animais têm demonstrado conhecimento de como as lectinas medeiam uma variedade de atividades biológicas com benefícios neuronais, especialmente a galectina-1 (Gal-1) que foi identificada como um composto neuroprotetor eficaz. Aqui, administramos galectina-1 (Gal-1) em ratos Wistar e avaliamos a gravidade do SE, incidência de CRE e padrões neurodegenerativos na região do hipocampo. Observamos redução na inflamação e neurodegeneração 24 horas e 15 dias após SE. No entanto, houve um aumento na neurodegeneração após 30 dias de SE. No geral, nossos dados sugerem que o Gal-1 tem efeito neuroprotetor no estágio inicial da epileptogênese e fornece novos conhecimentos sobre o papel das lectinas exógenas na epilepsia do lobo temporal.

ABSTRACT

Intrahippocampal pilocarpine microinjection (H-PILO) induces Status Epilepticus (SE) that can lead to spontaneous recurrent seizures (SRS) and neurodegeneration in rodents. Studies using animal models have advanced knowledge of how lectins mediate a variety of biological activities with neuronal benefits, especially galectin-1 (Gal-1) that has been tentatively identified as an effective neuroprotective compound. Here, we administrated galectin-1 (Gal-1) to Wistar rats and evaluated the severity of the SE, incidence of SRS and neurodegenerative patterns in the hippocampal region. We observed a reduction in inflammation and neurodegeneration 24 hours and 15 days after SE. However, there was an increase in neurodegeneration after 30 days of SE. Overall, our data suggest that the Gal-1 has neuroprotective effects in the early stage of epileptogenesis and provides new insights into the roles of exogenous lectins in temporal lobe epilepsy.

NEUROPROTECTIVE EFFECT OF EXOGENOUS LECTIN GALECTIN-1 IN *STATUS EPILEPTICUS*

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SUMÁRIO

INTRODUCTION

Temporal Lobe Epilepsy (TLE) is one of the most common types of epilepsy. It is recognized by focal seizures that may progress to secondary generalization, occurring in temporal lobe structures such as the hippocampus, amygdaloid complex and surrounding areas [1–4]. Most patients with TLE are characterized by prolonged seizures or Status Epilepticus (SE), especially in the first years of life. SE is defined as continuous and self-sustaining seizures lasting 30 minutes or more [1,5]. Furthermore, SE is an initial precipitating insult to the development of spontaneous recurrent seizures (SRS) [6].

Intrahippocampal microinjection of pilocarpine (H-PILO) in the animal model promotes a number of behavioral, electrographic and histological changes that are characteristic of TLE [7–9]. Wet dog shakes (WDS) is a typical behavior that occurs in animals after microinjection of H-PILO, indicating the progression of limbic seizures towards the generalized strategy of seizure control [10,11]. Other studies have showed that several inflammatory processes are simultaneously occurring in the brain of human patients and animal models during SE and epileptogenesis [12–14]. Moreover, a rapid increase of glial fibrillary acidic protein (GFAP)-immunoreactivity is observed, as well as an increase in inflammatory mediators [12]. A classic pattern of neurodegeneration is also observed in some areas of the hippocampus after SE, such as hilus of the dentate gyrus (DG), CA3 and CA1 [8,15,16].

Antiepileptic drug (AED) therapy can interrupt SE in most cases. However, about 30-40% of TLE patients are refractory to medical treatment [17]. In this context, advances in knowledge about new drugs are urgently needed to improve novel targeted therapies of TLE.

Recent studies have indicated that galectin-1 (Gal-1) may play a neuroprotective role after cerebral ischemia, reducing the astrocyte proliferation and apoptosis [18]. Gal-1 is a lectin

protein and is involved in the regulation of cell adhesion, proliferation, programmed cell death and immune responses [19]. Cytosolic reduced Gal-1 is released by Schwann cells and becomes oxidized in the extracellular space. The oxidized form of Gal-1 (not reduced) is responsible for promoting initial axonal growth in peripheral nerves after axotomy [20]. Macrophages are also stimulated by oxidized Gal-1, which secrete a factor that promotes axonal growth and Schwann cell migration, as well as enhancing peripheral nerve regeneration [21,22]. Administration of Gal-1 in the subventricular zone of the lateral ventricle increased neurogenesis and improved sensorimotor function after focal ischemia [23]. In addition, Gal-1 induces differentiation and inhibits proliferation of astrocytes: the production of BDNF is subsequently enhanced by differentiated astrocytes. Such elevated production can play an important role in the survival, differentiation and synaptic plasticity of neurons and may protect against neuronal loss after injury [24]. Additionally, glial protein fibrilar acid positive (GFAP+) astrocytes upregulate expression of Gal-1 in the glial scar [25].

Gal-1 has been observed to stimulate basal and kainate-induced proliferation of neural progenitors in the dentate gyrus of adult mouse hippocampus [26]. These data suggest that Gal-1 could potentially be used to treat some types of neurodegenerative pathologies, including TLE [22].

The purpose of this study was to evaluate the performance of pre-treatment with Gal-1 on in the H-PILO model. Specifically, we evaluated: i) the behavior of individuals with acute and chronic phase of epilepsy, and; ii) the amount of WDS before and during SE. We also analyzed the pattern of GFAP+ inflammation 24 hours after SE in different hippocampal subfields. Finally, we evaluated FJ+ neuronal damage at 24 h, 15 and 30 days after SE, in order to evaluate the possible neuroprotective role of exogenous Gal-1.

RESULTS

Development of SE rate

In the VEH + PILO group, 28 of 35 (80%) animals that received microinjections of PILO developed SE, and three died because of the severity of seizures; in Gal-1 + PILO group, 27 of 38 (71%) animals developed SE, and none died. Animals that did not develop SE were not included in the behavioral analysis; including those that received VEH microinjections instead of PILO (VEH + VEH) and therefore did not develop SE.

Analyze of latency time and WDS

Animals did not immediately have limbic seizures after microinjection of PILO. This latency to seizure onset (Fig. 1A) was unchanged in GAL-1 + PILO (20.8 ± 4.5) when compared with VEH + PILO (31.8 ± 5.9). As soon as animals received the PILO administration they began to develop intense head and body shakes, known as 'wet dog shakes' (WDS). The number of WDS (Fig. 1B) remained similar between GAL-1 + PILO (63.6 ± 12.3) in relation to VEH + PILO (82.6 ± 17.4).

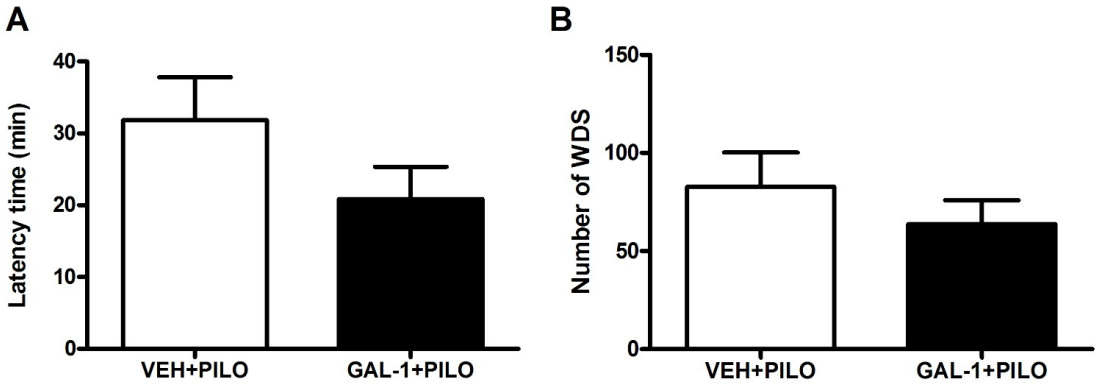

Figure. 1 A) was unchanged in GAL-1 + PILO (20.8 ± 4.5) when compared with VEH + PILO (31.8 ± 5.9) and Figure 1B) remained similar between GAL-1 + PILO (63.6 ± 12.3) in relation to VEH + PILO (82.6 ± 17.4) Test t, unpaired, p> 0.05. Fonte: autor.

Classification and temporal pattern of limbizures

The behavioral data showed that both groups had seizures of the same severity index, according to the Racine scale (1972). Seizures increase in severity during SE [9], though few studies have been able to provide a comprehensive explanation for this behavioral pattern. Hence, we will now detail the seizure patterns of the VEH+ PILO and GAL-1+PILO groups (Fig. 2A and 2B).

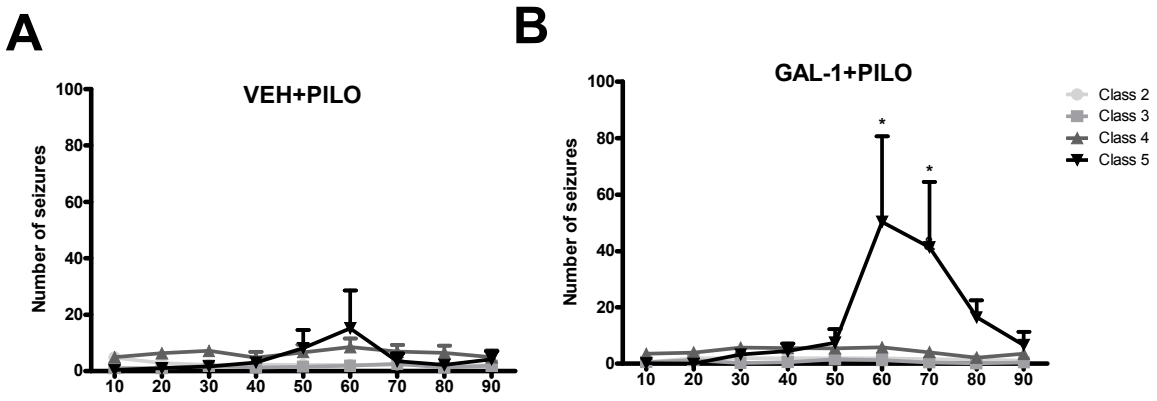

Figure. 2 Analysis of seizures evolution during 90 minutes on (A) VEH+PILO and (B) GAL-1+PILO groups. Fonte: autor.

To quantify the severity of seizures we considered the number and time classes of seizures. GAL-1+PILO had a smaller number of class 2 seizures (14.6 ± 2.0) in relation to VEH+PILO (30.3 ± 4.4) (Fig. 3A). Similarly, the total time in class 2 (Fig. 3C) was lower in the GAL-1+PILO group (2121 ± 507.3) when compared with VEH+PILO group (4902 ±

146.9). GAL-1 treatment lead to less time ($p < 0.05$) with class 2 seizures in the two first ten minutes of SE (Fig. 3E).

GAL-1+PILO subjects also had less class 4 seizures (14.5 ± 2.9) compared to VEH+PILO rats (54.3 ± 14.5) (Fig. 3B). However, total time in class 4 (Fig. 3D) was the same in GAL-1+PILO (2015 ± 486.2) compared with VEH+PILO (1140 ± 291.1) (Fig. 3D). GAL-1 subjects had fewer class 4 seizures ($p < 0.05$) in the three first ten minutes of SE (Fig. 3F).

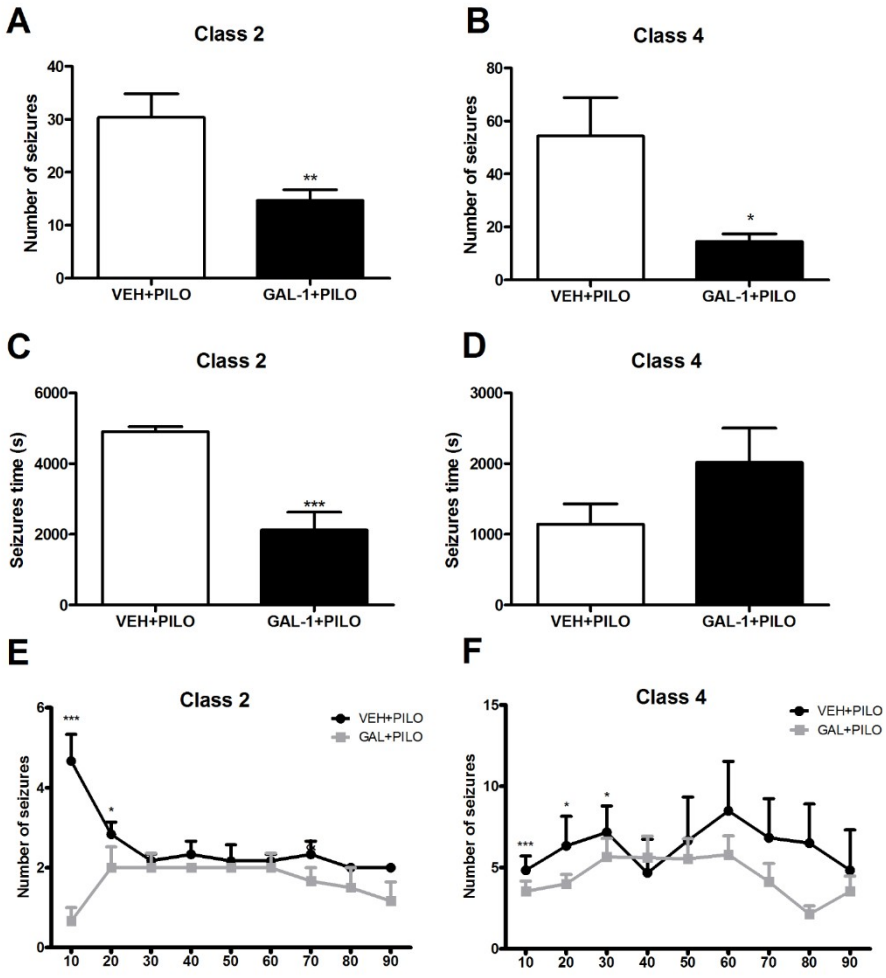

Figura. 3 A) Number of seizure the animals presented class 2 in the SAL + PILO groups, 30.33 ± 4.470; GAL + PILO, 14.67 ± 2.044; B) Number of seizure the animals presented class 4 in the SAL + PILO groups, 54.33 ± 14.50; GAL + PILO, 14.50 ± 2.952; C) Total time of class 2 in the SAL + PILO group, 4902 ± 146.9; GAL + PILO, 2121 ± 507.3; D) Total class 4 time in the SAL + PILO groups, 1140 ± 291.1; GAL + PILO, 2015 ± 486.2) E) Class 2 number observed per window in groups SAL + PILO (in black) and GAL + PILO (in gray). T-test, unpaired, $p < 0.05$. * and *** compare the groups SAL + PILO and GAL + PILO in times 10 and 20; (F) Class number 4 observed per window in

groups SAL + PILO (in light green) and GAL + PILO (in dark green). T-test, unpaired, p <0.05. * compares the SAL + PILO and GAL + PILO groups at times 10, 20 and 30. ANOVA. Fonte: autor.

Assessment of spontaneous recurrent seizures (SRSs)

Immediately after SE, SRSs were recorded. Animals that did not develop SE were again excluded from this analysis. The frequency of SRS in the GAL-1+PILO group remained unchanged (4.8 ± 1.0) in relation to the VEH+PILO group (4.4 ± 0.7) (Fig. 4A). Similarly, time of SRS was the same in the GAL-1+PILO group (69.4 ± 25.6) compared to the VEH+PILO group (128.2 ± 31.8) (Fig. 4B).

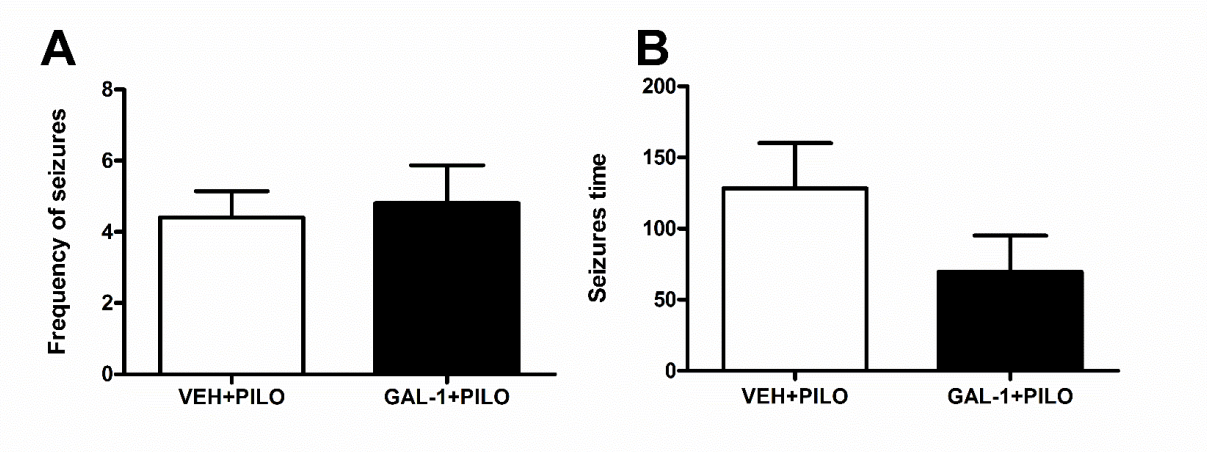

Figure. 4 A) Analysis of the frequency of CREs SAL + PILO (4.8 ± 1.0) and GAL + PILO (4.4 ± 0.7); B) Duration of CREs groups SAL + PILO (128.2 ± 31.89) and GAL + PILO (69.40 ± 25.61). T-test, unpaired, p <0.05. Fonte: autor.

Neurodegeneration: FJ-C+ neurons in the DG hilus and CA3 and CA1 regions of the hippocampus after 24 h, 15 and 30 days of SE

In order to analyze the action of galectin-1 in relation to neurodegeneration after SE, we performed the FJ-C staining procedure. As expected, the VEH+VEH group had no FJ-C+ cells in the DG hilus, CA3 and CA1 regions of hippocampus.

After 24h of SE, the number of FJ-C+ neurons decreased in the DG hilus of GAL-1+PILO (8.2 ± 2.3) individuals when compared to the VEH+PILO group (141.5 ± 45.1) (Fig. 5 C), indicating a possible neuroprotective role in this specific region and time. However, the overall amount of FJ-C+ cells remained unchanged in CA3 (70.8 ± 34.9) and CA1 (126.8 ± 77.6) of GAL-1+PILO rats, in relation to the same areas of VEH+PILO rats (CA3, 170.2 ± 61.1; CA1, 221.0 ± 52.7) (Fig. 5 F e I).

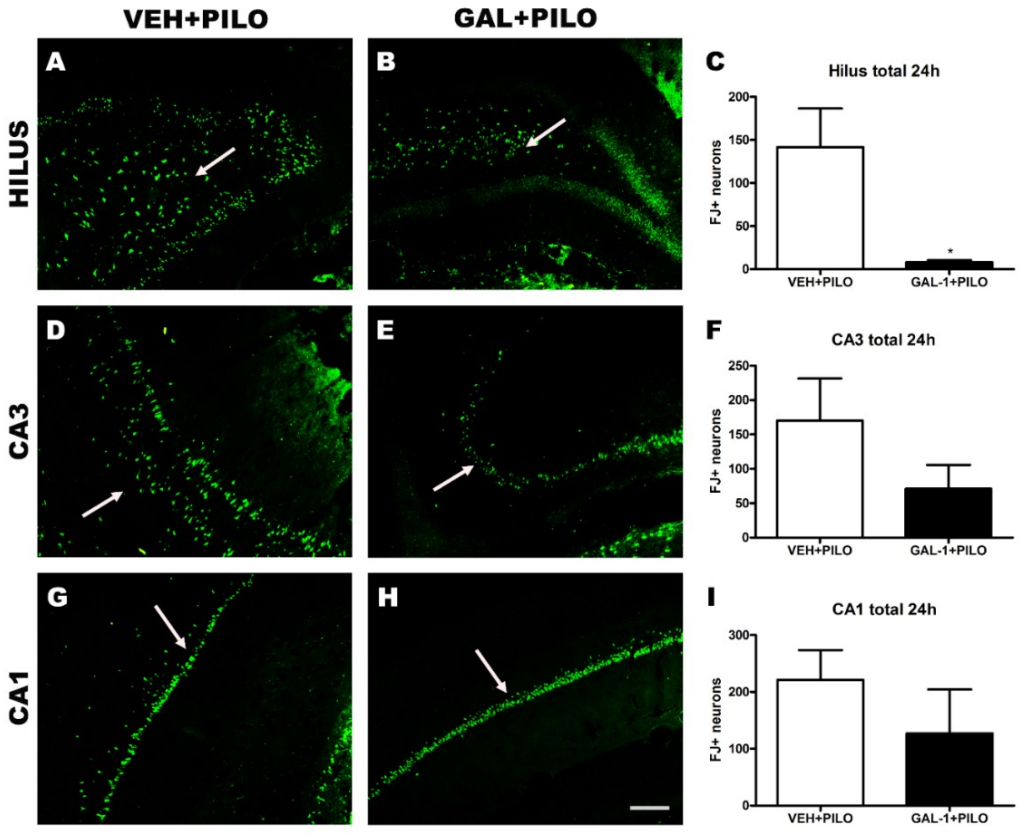

Figure. 5 Hippocampal regions of FJ + H-PILO and GAL-1 model of 24h days after SE: (A) Hilus 10x, SAL + PILO (141,5 ± 45,12) B)GAL-1+ PILO (8,200 ± 2,311); (C) Total number of FJ +

no hilus; (D) CA3 10x, SAL + PILO (170.2 ± 61.10) (E) GAL + PILO (70.83 ± 34.95); (F) Total number of FJ + in CA3; (G) CA1 10x, SAL + PILO (221.0 ± 52.73) (H) GAL + PILO (126.8 ± 77.60); (I) Total number of FJ + in CA1. T-test, unpaired, p <0.05. * compared to SAL + PILO. Arrows represent the counted locations, hilus, CA3 and CA1, respectively. Calibration bar 10 μm (A-H). Fonte: autor.

Similarly, 15 days after SE, GAL-1 rats had a decreased number of FJ-C+ cells in CA1 (91.2 ± 71.6) compared with the same region of the VEH+PILO (344.8 ± 77.0) group (Fig. 6I). However, the number of FJ-C+ neurons kept unshaken in the DG hilus (21.6 ± 21.6) and CA3 (105.2 ± 66.2) of GAL-1+PILO regarding to similar regions of VEH+PILO (DG hilus, 61.8 ± 29.9; CA3, 263.2 ± 81.1) (Fig. 6C e F).

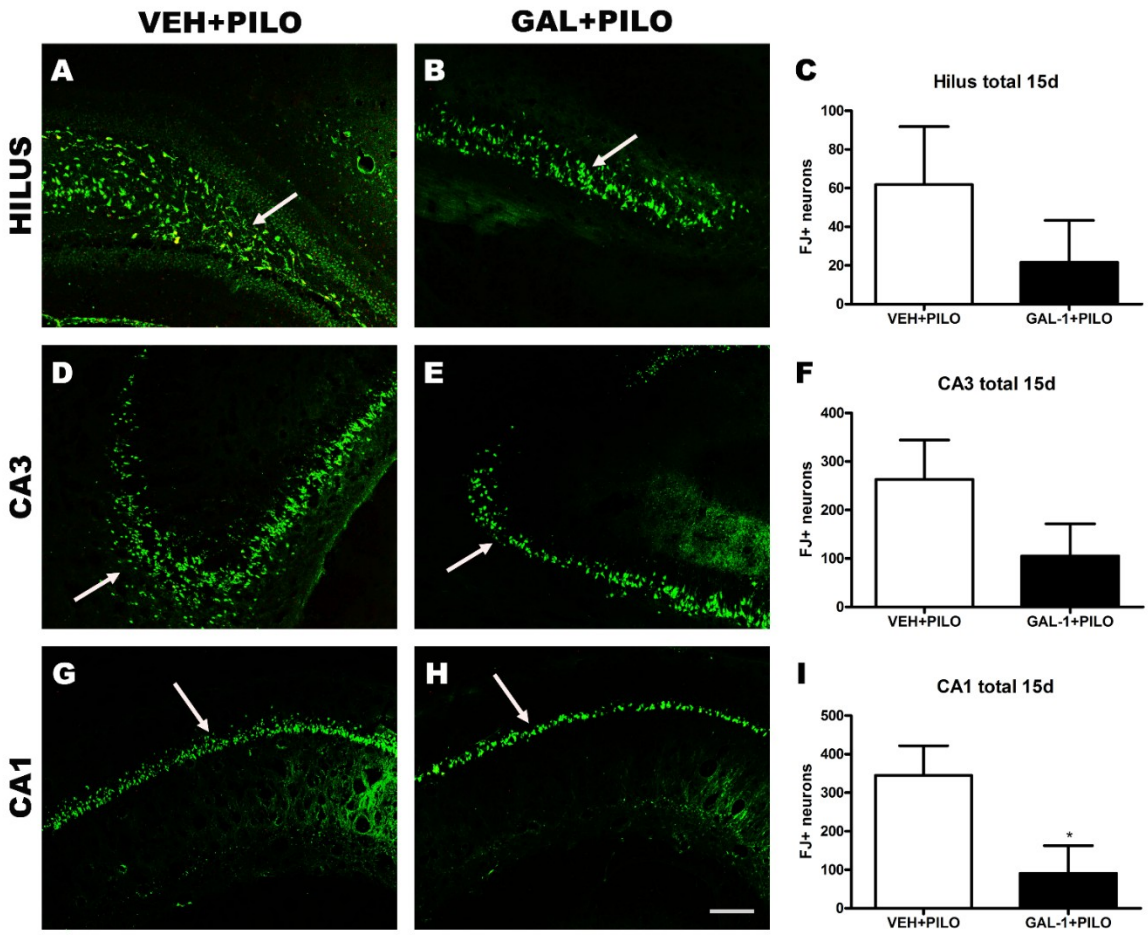

Figura. 6 Hippocampal regions of FJ + H-PILO and GAL-1 model 15 days after SE (A) Hilus 10x, SAL + PILO (61.83 ± 29.94) (B) GAL + PILO (21.67 ± 21.67) (C) Total number of FJ + no hilus; (D) CA3 10x, SAL + PILO (263.2 ± 81.16) (E) GAL + PILO (105.2 ± 66.23) (F) Total number of FJ + in CA3; (G) CA1 10x, SAL + PILO (344.8 ± 77.05) (H) GAL + PILO (91.20 ± 71.67) (I) Total

number of FJ + in CA1; T-test, unpaired, p <0.05. * compared to SAL + PILO. Arrows represent the counted locations, hilus, CA3 and CA1, respectively. Calibration bar 10 μm (A-H). Fonte: autor.

Neurodegeneration increased after 30 days of SE. In GAL-1+PILO group there was a higher quantity of FJ-C+ cells in CA1 (219.9 ± 81.8) region in comparison to this area in the VEH+PILO group (22.1 ± 22.1) (Fig. 7I). Nevertheless, amount of degenerating neurons was unchanged in the DG hilus (9.7 ± 9.7) and CA3 (89.5 ± 38.0) of GAL-1+PILO individuals when compared with the VEH+PILO group (DG hilus, 0.1 ± 0.1; CA3, 27.4 ± 27.4) (Fig. 7C e F).

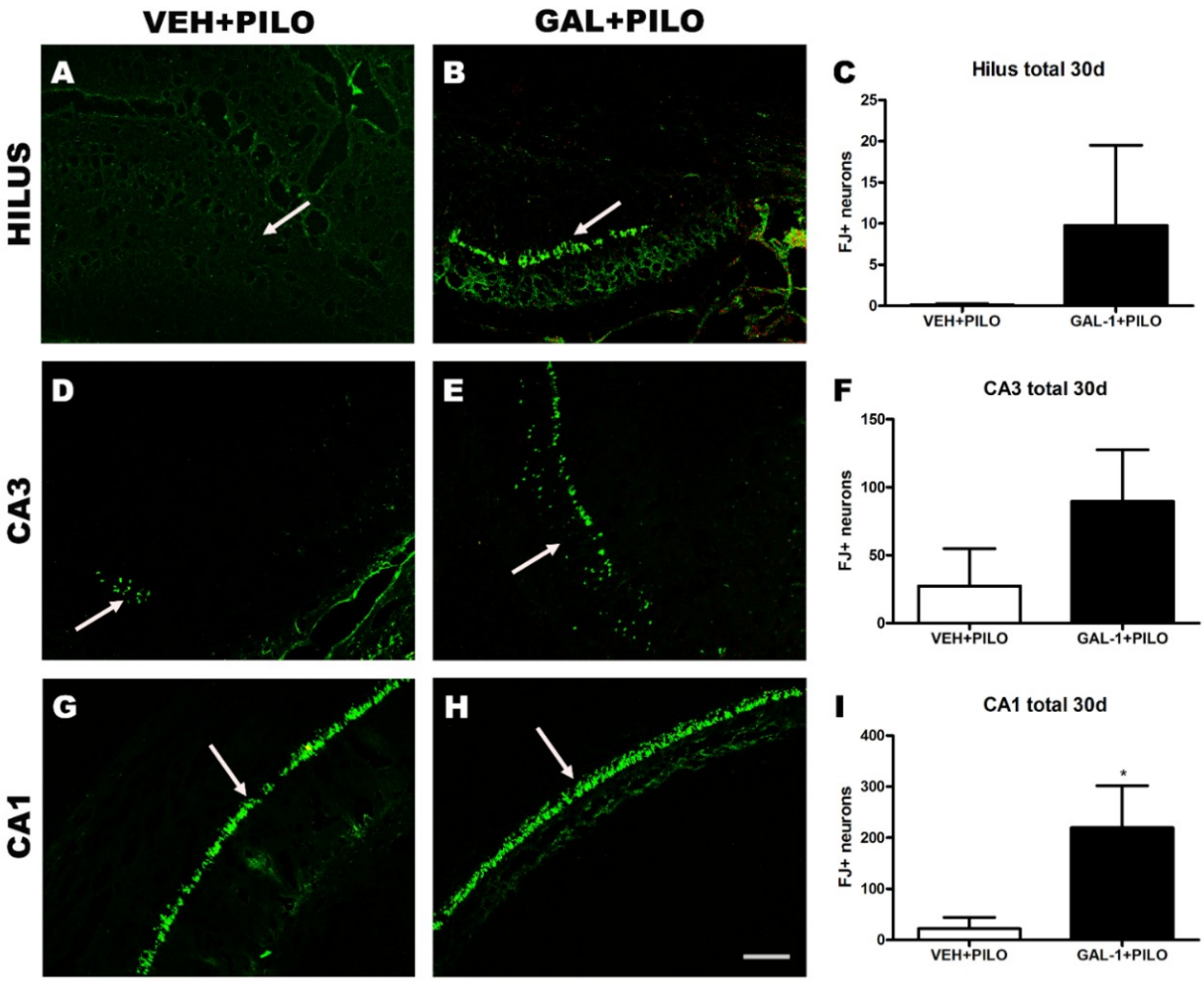

Figure. 7 Hippocampal regions of FJ + H-PILO and GAL-1 model 30 days after SE (A) Hilus 10x, SAL + PILO (0.1429 ± 0.1429) (B) GAL + PILO (9,750 ± 9,750); (C) Total number of FJ + no hilus; (D) CA3 10x, SAL + PILO (27.43 ± 27.43) (E) GAL + PILO (89.57 ± 38.02); (F) Total number of FJ + in CA3; (G) CA1 10x, SAL + PILO (22.14 ± 22.14) (H) GAL + PILO (219.9 ± 81.89); (I) Total number of FJ + in CA1; T-test, unpaired, p <0.05. * compared to SAL + PILO. Arrows represent the counted locations, hilus, CA3 and CA1, respectively. Calibration bar 10 µm (A-H). Fonte: autor.

Immunofluorescence for GFAP

After 24 hours of SE, the amount of GFAP+ cells was lower in the regions of SO (190±27.7), SP (65±6.2) and SR (179.3±26.6) of the GAL-1+PILO group than the same areas in the VEH+PILO group (SO, 382.3±48.8; SP, 170.3±22.3; SR, 442.7±62.4). The number of GFAP+ cells remained unchanged in hilus of DG of GAL-1+PILO individuals compared to those in the VEH+PILO group (Fig. 8).

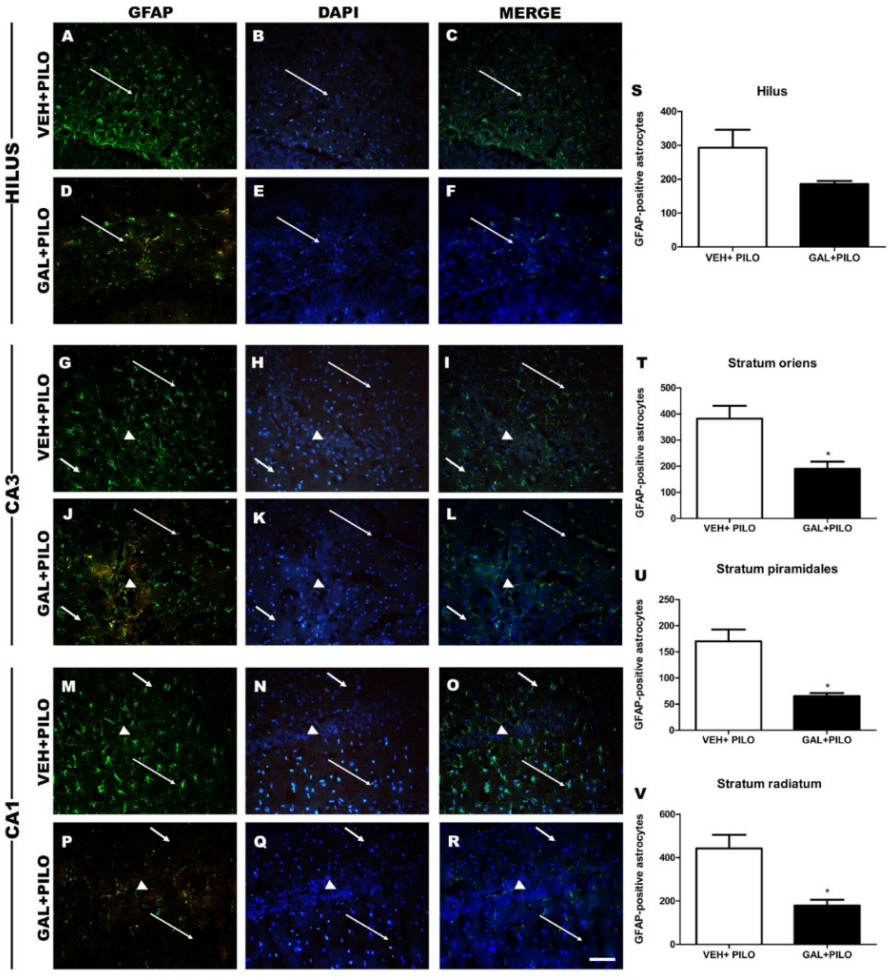

Figure. 8 Expression of GFAP in hippocampal astrocytes after induction of SAL + PILO and GAL + PILO. A, D (green) distribution of GFAP positive cells in the dentate gyrus hilus after 24h of SE. DAPI B, E (blue), and their fused images are shown (C, F), G shows the amount of GFAP + neurons in the hilus .; H, K (green) distribution of GFAP positive cells in CA3 (SO, SP, SR) after 24 h of SE. DAPI (I, L, blue), and their fused images are shown (J, M), N G shows the amount of GFAP + neurons in SO; O, R (green) distribution of GFAP-positive cells in CA1 (SO, SP, SR) after 24 h of SE. DAPI P, S (blue), and their fused images are shown (Q, T), U shows the amount of GFAP + neurons in SP and V G shows the amount of GFAP + neurons in SR; T-test, unpaired, $p < 0.05$. * compared to SAL + PILO. Arrows represent the hilus counted sites, CA3 and CA1, respectively. Scale bars = 50 mum. Fonte: autor.

DISCUSSION

TLE is the most common type of epilepsy[6]. Many Patients with TLE are refractory to medical treatment, so it is important to search for new drug therapies to effectively treat seizures and protect the brain [7]. Furthermore, it is known that SE is an initial precipitating injury that can initiate TLE and trigger a cascade of histological and biochemical alterations [27]. SE remains a therapeutic challenge and is associated with high mortality and morbidity; these seizures soon become self-sustaining and pharmacoresistant [28,29]. Hence, developing new therapeutics is essential to combat the damage associated with epileptic seizures. Our data indicate that intrahippocampal pretreatment of GAL-1 decreases FJ-C+ neurons in the DG hilus after 24h of SE and in CA1 region of hippocampus 15 days after SE.

On average, latency after PILO injection lasts 15 to 30 minutes until the appearance of behavioral seizures[30]. We observed that GAL-1+PILO and VEH+PILO groups spent had a

latency period of 10 to 35 minutes until the onset of epileptic seizures, corroborating the literature (Fig. 1A).

Our animals showed WDS during the latency period, which is strongly correlated with the septo-hippocampal system. Previous researchers have observed that activation of septo-hippocampal system decreases in response to repeated stimulation in the hippocampus.[10]Although the number of WDS remained unchanged, the frequency of WDS decreased after the beginning of SE in both groups, as previously described (Rodrigues et al., 2005) (Fig. 1B).

Behavioral patterns of VEH+PILO and GAL-1+PILO groups were analyzed in detail to ascertain if pre-treatment with Gal-1 before SE induction SE decreased motor seizures. Gal-1 did not prevent the seizures or influence the severity of seizures. Because of this, we adopted a more comprehensive analysis of behavior, focusing on the evolution of seizures, as well as individual and comparative observations of some classes of Racine's scale (1972).

During the 90 minutes of SE, animals in the VEH+PILO and GAL-1+PILO groups showed similar behaviors (Fig. 2). Seizures are initially focal and centralized in areas of brain responsible for behavior and expressions as facial automatism, head and neck myoclonus, being characterized as the first classes according to Racine's scale (1972). However, as SE develops, other areas of brain are recruited, developing generalized motor seizures. Limbic centers are then recruited (intermediate classes), following involvement of extra-limbic regions, resulting in final classes of Racine's scale [31–33].

In animals pretreated with Gal-1, we observed a delay in the development of generalized seizures in comparison to VEH+PILO animals, which showed a progression in classes 2 and 4 seizures (Fig. 3E e 3F). In the GAL-1+PILO group we noted milder behavior and significantly less frequent seizures after the onset of SE at times 10-20 min and 10-30 min

of class 2 and 4 seizures, indicating a possible decrease in severity of the seizures at these specific times when compared with the VEH+PILO group.

Acute insult of SE can cause functional and morphological changes up to the appearance of SRSs. In the present study, some animals developed self-sustained seizures and spontaneous seizures - noticeable from the fourth day after SE[34]. It should be noted that this data includes only the seizures that occurred in the six hours filmed and is therefore likely to underestimate the number of seizures (Fig. 4A and 4B).

This type of sub-chronic and chronic epilepsy are useful to compare the occurrence of seizures over longer periods of time and offers advantages over other models, since partial seizures after neuronal damage are difficult to control. Indeed, damage in the DG can initiate cascades of events that eventually lead to mossy fiber sprouting and excitation of new granule cells, causing SRSs[53.] Although Gal-1 did not prevent the generation of SRSs it was effective in decreasing neuronal death 15 days after SE, supporting previous reports that behavioral changes are not related to neuronal death[8] .

Cell loss in hippocampus has been related to axonal reorganization in the dentate gyrus, where mossy cells of DG innervate the pyramidal cells of CA3 and "mossy cells" of hilus designed for inner molecular layer of DG on hippocampal sclerosis, thereby establishing a recurring circuit with granule cells [35]. Some studies suggest that sprouting of mossy fibers is a substrate for the genesis of epileptic seizures in humans and experimental models, and have a role in the reorganization of epileptogenic tissue [35,36].

Previous studies indicate that inflammatory cytokines are involved in pathogenesis of epilepsy, being a possible reason for cell loss observed in intrahippocampal chemical models compared to other animal models involving SE [37]. Recent studies using animal models have shown that these cytokines have a deleterious effects on neurons when altering neuronal

excitability, producing toxic mediators and increasing the impermeability of blood-brain barrier [38,39]. IL-1β can induce NMDA receptor activation, thereby enhancing calcium influx into neurons, and leading to neuronal hyperexcitability and neurodegeneration [40].

Inflammatory cell adhesion also plays a role in seizures pathogenesis, revealing that expression of vascular cell adhesion molecules is high and adhesion of leukocytes to endothelial cells is increased in cerebral blood vessels after seizures induced by PILO. This is mediated by P-selectin - glycoproteins similar to mucin and integrins of leukocyte[41] and results in a cascade of events, including cerebral inflammation and epileptogenesis.

Neurotrophic factors such as BDNF play an important role in cell changes. BDNF determines cell differentiation of neuronal subpopulations during development, and its receptor (TrkB) increases in the hippocampus after seizures. It is therefore likely that BDNF is involved in cellular changes observed in hippocampal sclerosis [42].

We observed neuronal death in the pyramidal cells of CA1 and CA3, as well as in interneurons of the DG hilus in both groups after 24 hours and 15 days of SE. It is worth noting that the epileptic condition affects the number of cells and their survival in the DG hilus after 24 hours of SE [43]. Thus, Gal-1 seems to be important for neuronal protection of the hilus at this specific time (Fig. 5C). Furthermore, it is known that number of CA1 pyramidal cells of the hippocampus decreases for at least 2 months after SE, depending on the epileptic condition [43]. As noted in the results, degeneration of CA1 decreased in animals pre-treated with Gal-1 after 15 days of SE (Fig. 6I.).

The long-term role of exogenous Gal-1 in the brain is not well known. Our results showed that after 30 days of SE neuronal death appeared to rise in areas that Gal-1 had previously seemed to protect (Fig. 7 I). Two different hypotheses may account for this. First, it could be a compensatory mechanism. Under homeostatic conditions, Gal-1 contributes to

normal physiology. However, during chronic inflammation there is an increase of intestinal epithelial cells binders for this lectin and Gal-1 intensifies the pro-apoptotic effect on these cells[44]. Alternatively, Gal-1 can induce axonal degeneration, and plays a key role in the accumulation of immune cells after injury[45]. Despite all these reports, further studies should be conducted with chronic treatment with GAL-1. Additionally, another factor can be taken into account that is the fact that SRSs progression occurs during 30 days. Oxidized Gal-1 is capable of causing local axonal degeneration, leading to release of cytokines and other factors that promote recruitment and activation of immune cells. The cascade of proinflammatory cytokines initiated by Gal-1-induced degeneration could lead to flow of neutrophils and macrophages, causing apoptosis of damaged cells during epileptogenisis.

All layers (CA, DG and subiculum) of the hippocampus contain astrocytes and neuron-astrocyte interactions play an important role in the development and functional activity of this organ [46]. In patients with TLE, there is marked glial proliferation in the hippocampus, usually associated with hippocampal sclerosis. Such reactive gliosis causes a substantial increase in GFAP, characterized by hypertrophy and proliferation of glial cell bodies - possibly caused by seizures [47]. The increase in GFAP is a biochemical indicator of the transformation of normal glial cells into reactive glial cells [48]. Moreover, in addition to an increase in GFAP expression, SE promotes the hypertrophy of reactive astrocytes [49,50]. Thus, enhanced expression of GFAP plays an important role in the progression of epilepsy.

It has also been observed that Gal-1 expression is higher in activated astrocytes (located in CA3 subregion and DG of hippocampus) after kainate-induced seizure, with an associated proliferation of neural progenitor cells in adult rat hippocampi [26]. Based on our findings, Gal-1 decreases the amount of GFAP-positive astrocytes after 24 hours of SE, indicating a possible immunomodulatory role in the acute phase of epileptogenesis (Fig. 8).

Based on our results we tentatively propose that endogenous Gal-1 may decrease the severity of seizures, though they are not sufficient to prevent the expression of SRSs. Furthermore, Gal-1 may decrease the number of FJ+ neurons in the hilus and CA1 of hippocampus after acute and subchronic periods of epilepsy. Thus, taken together our findings suggest that Gal-1 has a neuroprotective effect that is both region- and time-dependent. to the potential benefits include a possible softening of the initial seizures, as well as class 4 seizures. These data support the hypothesis that Gal-1 plays a role during SE, possibly enhancing the survival of neural cells during epileptogenesis. If confirmed, our results provide the basis for a new therapeutic strategy for decreasing neuronal death in epilepsy through pre-treatment with Gal-1.

METHODS

Animals Model

Male Wistar rats (n=64, 240–340 g) from the Central Bioterium at Federal University of Alagoas (UFAL) were submitted to a light-dark cycle of 12/12 hours (lights on at 07:00 AM and lights off at 07:00 PM) with food and water *ad libitum*. All experiments were designed to minimize suffering and to minimize the number of animals used. All experiments were approved by the Ethical Committee for Animal Experimentation of the UFAL (Protocol 19/2014).

Surgical procedure

Animals were anesthetized with thiopental sodium (40 mg/kg, i.p [Cristália®]) and, prior to surgery, they received 0.1 mL/100g of veterinary pentabiotic (Fort Dodge®, subcutaneous). All individuals received local anesthetic (lidocaine with epinephrine, subcutaneous [Astra®]) after fixing on stereotaxic. Cannula was implanted stereotaxically in the DG hilus according to the following coordinates: - 6.30 mm anterior-posterior (AP, reference: bregma); 4.50 mm medial-lateral (ML, reference: sagittal sinus); - 4,50 mm dorso-ventral (DV, reference: dura mater) [8,32,51].

Synthesis of Galectin-1

Galectin-1 was obtained from a bacterial culture (*E. coli*, strain M-15) transformed with a PQU-50 plasmid containing the complete gene (dimeric form) or point mutations in position 2 (serine-cysteine monomeric form). Expression induction of this protein by bacteria was performed by adding B-D-thioglycolate-piranozídeo (IPTG). The supernatant of a culture of bacteria was subjected to affinity chromatography on agarose-gel lactose. This process was monitored by reading OD (280nm) and electrophoresis. The bacteria were provided from the Glycobiology Laboratory of the Medical University of Atlanta-USA. Synthesis was carried out at Sao Paulo University in Ribeirao Preto.

Gal-1 and pilocarpine microinjetions

Study animals received the drug (Gal-1, PILO) or its vehicle (VEH), intra-hipoccampally. The rats were divided into 3 experimental groups: VEH+VEH (n=12), VEH+PILO (n=25) and GAL-1+PILO (n=27).

After being gently immobilized, animals received 1µL of Gal-1 (5mg/µL) or VEH (saline 0.9%) in the left DG hilus 30 minutes before the administration of 1 µL PILO (1.2mg/µL) to induce limbic seizures (GAL-1+PILO or PZN+PILO) or 1 µL VEH (VEH+VEH or GAL-1+VEH). We administer the drugs using a 5µL syringe (Hamilton Company, Reno, NV, USA) connected to a microinjection pump (Harvard Apparatus PHD 2000, Holliston, MA, USA) at a speed of 0.5 µl/min. All animals were rescued with diazepam (5 mg/kg; i.p.) 90 minutes from SE onset.

Behavioral analysis

The main purpose of the behavioral analysis was to evaluate the neurophysiological changes occurring in animals subjected to SE for 90 minutes - sufficient time to induce neurodegeneration [8]. Animals were placed in an acrylic transparent box and behavioral activity was recorded by video camera (Full HD Digital Camcorder Sony DCR-PJ6). Behavior was analyzed according to Racine's scale (1972) and the following stages were recorded: (0) immobility, (1) facial movements, (2) head nodding, (3) forelimb clonus, (4) rearing, (5) rearing and falling. The severity of seizures and number of WDS was quantified before and during SE according to Melo et al. (2016). We then evaluated spontaneous recurrent seizures (SRS). Animals were observed over 30 days during which they were video monitored for 6 hours daily in acrylic boxes with food and water *ad libitum*.

Tissue sampling

After 24 hours, 15 or 30 days of SE induction, animals were injected with an overdose of sodium thiopental, and were transcardially perfused with 0.1 M phosphate-buffered saline

pH 7.4 (PBS) and 4% paraformaldehyde solution in PBS. Brains were then processed [32] and sections were cut (30 µm thickness) using a cryostat (Leica CM 1850; temperature ranging from -18 to -22°C). These sections were used for FJ-C staining [8,32,52].

FJ-C staining procedure

To analyze neuronal death, brain sections were immersed in 100% ethanol for 3 min, followed by 1 min in 70% ethanol and 1 min in distilled water. After this step, during 15 min on a rotating platform, slides were transferred to a solution of 0.06% potassium permanganate. Slides were rinsed three times for 1 minute in distilled water and were transferred to the FJ staining solution (0.0001%) for 30 min. After staining, sections were again rinsed three times in distilled water (1 min) and the slides were cover slipped using *fluoromount* (EMS). Finally, sections were analyzed and images captured using a fluorescence microscope (Nikon DS RI1).

Immunofluorescence

We used an antibody that binds to glial fibrillary acidic protein (GFAP). After PILO-induced SE there is an increase in the number of astrocytes (astrogliosis) due to neuronal death caused by lesions in the hippocampal formation. Immunofluorescence was used to visualize these glial cells, making possible its estimate in the regions of stratum oriens (SO), piramidales (SP) and radiatum (SR), as well as hilus of dentate gyrus.

Brain tissue slices were submerged for 10 minutes in methanol and washed with PBS 1x (2 times in 10 minutes). An antigenic rescue was then performed with citrate buffer (pH 6) for 10 minutes (output 6) and, after a cooling period (30 minutes, room temperature), immersed in the same solution. After cooling, sections were incubated in an autofluorescence blocking solution with PBS/glycine 3% (1h, room temperature), followed by a second blocking of nonspecific sites with fish skin gelatin in 0.05% PBS 1X and equine serum and 1.5% (1h, room temperature). Shortly thereafter, they were incubated with the primary

antibody rabbit anti-GFAP Sigma (1: 250) diluted in fish skin gelatin in 0.05% in PBS 1X (overnight, 4° C). In the second stage, the slices were washed with PBS 1x (2 times in 10 minutes), followed by incubation with Alexa 594 secondary antibody (anti-rabbit, 1: 2000) diluted in fish skin gelatin 0.05% in PBS 1X (1h , room temperature). Sections were washed with PBS 1x (2 times 5 minutes) and incubated with DAPI (Fluoroshield With DAPI Mounting Medium, ab104139, Abcam®, USA) (1:1000, diluted in PBS 1x; 15 minutes, room temperature) in order to mark the cell nuclei. Finally, the sections were washed (PBS 1x, 5 times 2 minutes) and used as mounting medium PBS/glycerol. To control for binding specificity, sections were subjected to the same protocol with omission of anti-GFAP antibody. Sections were examined and images captured using a fluorescence microscope (Nikon DS RI1).

Cell counting

Neuron and astrocytes were counted using ImageJ software (Wayne Rasband; Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA). Fluoro-Jade C positive (FJ-C +) cells were counted in three different coordinates of the hippocampus: CA1, CA3 and the DG hilus, (AP −2.56 mm; AP −3.30 mm and AP −6.30 mm; Paxinos and Watson, 2007), as showed by Castro et al., (2011). These regions were selected because of their high sensitivity to neurodegeneration. GFAP+ cells were counted in of SO, SP and SR, as well as the hilus of the dentate gyrus. All cells were counted on the contralateral hippocampus, because animals that receive microinjection of PILO develop a scar around the microinjection site due to mechanical lesions caused by cannulae and the local action of PILO.

Statistical analysis

All experimental values are presented as mean ± SEM and a significance level of 5% (described as $P < 0.05$) was adopted for all statistical tests. To analyze WDS patterns and the FJ + cells we performed an unpaired t test. To determine the evolution of seizures, we compared the classes of Racine's scale by one-way analysis of variance (ANOVA), followed by Student Newman Keuls post-hoc test (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA).

Acknowledgements

This research was supported by a grant from CAPES/CNPq (#458143/2014) and FAPEMIG; MA Costa and Melo IS was recipient of a FAPEAL fellowship. We would like to thank our collaborators at the Dental Research Center in Biomechanics, Biomaterials and Cell Biology (CPbio).

Author Contributions

MIS. collected the data, conceived the research hypothesis and wrote the manuscript. C-MN; FJNS; C-APM and OTL assisted with research assays, data collection and manuscript writing. SCEA and HJM performed and data analysis of ionic quantification of saliva. C-SL; AEMG,A-SAC, A-BR and RLP performed the Eletrochemical analyses, Raman and FTIR Spectroscopy and analytical analysis and wrote the manuscript. COW, GLR, SAX and BE were involved in conceiving the study, data analysis and interpretation, as well as reviewing and editing all parts of the final document for publication. S-SR was involved in conceiving and designing the study, conceived the research hypothesis and wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

The authors have no conflict of interest to disclose. The authors declare no competing financial interests.

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FIGURE LEGENDS

Figure 1. Latency (minutes) to SE (A) and number of WDS before and during the SE (B). Latency to SE and WDS remained unchanged when compared both groups. Unpaired t test, $p > 0.05$.

Figure 2. Evolution of SE in VEH + PILO (A) and GAL-1 + PILO (B). Number at all classes of Racine's scale for window (10 min) in VEH + PILO and GAL-1 + PILO. Two-way ANOVA, Bonferroni post-test, $p < 0.001$. (B) * compared with classes 2, 3 and 4.

Figure 3. Total seizures number and time of classes 2 and 4 during SE. GAL-1+PILO group had a smaller total number of seizures at classes 2 and 4 compared VEH+PILO (A and B), however, in relation, pretreatment with Gal-1 decreased seizures time only in class 2 (C and D). We analyzed the number at classes 2 and 4 of Racine's scale for window (10 min) in VEH + PILO and GAL-1 + PILO in order to observe the severity of seizures with more details. In the beginning of SE, GAL-1+PILO group had a lower number of seizures at classes 2 (E) and 4 (F). Unpaired t test, $p < 0.05$. *, ** and *** compared with VEH + PILO.

Figure 4. Analysis of frequency and duration of CRE. After pretreatment with Gal-1, frequency and duration of CRE maintained not significantly when compared to VEH+PILO groups during 30 days then SE. Unpaired t test, $p > 0.05$.

Figure 5. Qualitative and quantitative analysis of FJ-C+ neurons in the DG hilus, CA3 and CA1 region of hippocampus after 24 hours of SE. (C) Gal-1 was able to decrease number

of FJ-C+ neurons in the DG hilus 24h after SE. (F and I) There was no change in other areas. All areas in VEH + PILO (A, D, G) and GAL-1 + PILO (B, E, H). Total number of FJ-C+ in the DG hilus (C), CA3 (F) and CA1 (I). Arrows represent the DG hilus (A-B), CA3 (D-E) and CA1 (G-H) region. (A-D) Magnification, 100×; scale bar, 100 μm. Unpaired t test, $p < 0.05$. * compared with VEH + PILO.

Figure 6. Qualitative and quantitative analysis of FJ-C+ neurons in the DG hilus, CA3 and CA1 region of hippocampus after 15 days of SE. (I) Gal-1 decreased the number of FJ-C+ neurons in CA1 area 15 days after SE. (C and F) There was no change in other areas. All areas in VEH + PILO (A, D, G) and GAL-1 + PILO (B, E, H). Total number of FJ-C+ in the DG hilus (C), CA3 (F) and CA1 (I). Arrows represent the DG hilus (A-B), CA3 (D-E) and CA1 (G-H) region. (A-D) Magnification, 100×; scale bar, 100 μm. Unpaired t test, $p < 0.05$. * compared with VEH + PILO.

Figure 7. Qualitative and quantitative analysis of FJ-C+ neurons in the DG hilus, CA3 and CA1 region of hippocampus after 30 days of SE. (I) Gal-1 increased the number of FJ-C+ neurons in CA1 area 15 days after SE. (C and F) There was no change in other areas. All areas in VEH + PILO (A, D, G) and GAL-1 + PILO (B, E, H). Total number of FJ-C+ in the DG hilus (C), CA3 (F) and CA1 (I). Arrows represent the DG hilus (A-B), CA3 (D-E) and CA1 (G-H) region. (A-D) Magnification, 100×; scale bar, 100 μm. Unpaired t test, $p < 0.05$. * compared with VEH + PILO.

Figure 8. Qualitative and quantitative analysis of GFAP+ astrocytes in hilus, stratum oriens, stratum piramidales and stratum radiatum of hippocampus 24 hours after SE. Gal-1 was able to reduce the number of GFAP+ astrocytes in SO (T), SP (U) and SR (V) regions of CA3 (G-L) and CA1 (M-R). However, number of GFAP+ astrocytes remained unchanged in hilus of DG (A-F, S). Arrows represent the DG hilus (A-F). Small arrows represent SO region; arrowheads, SP region; larger arrows, SR region. (A-R) Magnification, 100×; scale bar, 100 μm. Unpaired t test, $p < 0.05$. * compared with VEH + PILO.

UNIVERSIDADE FEDERAL DE ALAGOAS

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

PARECER CONSUBSTANCIADO

PROJETO Nº 19/2014

TÍTULO: Análise da neuroplasticidade hipocampal em animais pré-tratados com galectina-1 e óleo essencial de *citrus limon* após status epilepticus induzido por microinjeção hipocampal de pilocarpina.

RESPONSÁVEL: Prof. Dr. Olagide Wagner de Castro.

OBJETIVO: Avaliação da gravidade do SE de acordo com a escala de Racine (1972) modificada por Pineal e Rovner (1978) e os padrões de fenômenos inflamatórios, neurogênese, neurodegeneração em regiões hipocampais nos animais pré-tratados com Gal-1, perfundidos 30 dias após SE. Além disso, será avaliado o efeito neuroprotetor e anti-inflamatório do OECL paralelo com o resveratrol, aplicados por meio de micro-injeções hipocampais.

SITUAÇÃO: APROVADO

PERÍODO DE VIGÊNCIA: 20/11/2014 a 20/11/2015

DADOS DO ANIMAL:

ESPÉCIE	LINHAGEM	QUANTIDADE
Rato Heterogênico	Wistar	76

Maceió, 19 de novembro de 2014.

Silvana Ayres Martins

Coordenadora da COMISSÃO

Profa. Dra. Silvana Ayres Martins
Coordenadora da Comissão de
Ética no uso de Animais
SIAPE 1120858