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Reduced hippocampal manganese-enhanced MRI (MEMRI) signal during pilocarpine-induced status epilepticus: Edema or apoptosis?

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Summary Manganese-enhanced MRI (MEMRI) has been considered a surrogate marker of Ca²⁺ influx into activated cells and tracer of neuronal active circuits. However, the induction of status epilepticus (SE) by kainic acid does not result in hippocampal MEMRI hypersignal, in spite of its high cell activity. Similarly, short durations of status (5 or 15 min) induced by pilocarpine did not alter the hippocampal MEMRI, while 30 min of SE even reduced MEMRI signal. Thus, this study was designed to investigate possible explanations for the absence or decrease of MEMRI signal after short periods of SE. We analyzed hippocampal caspase-3 activation (to evaluate apoptosis), T₂ relaxometry (tissue water content) and aquaporin 4 expression (water-channel protein) of rats subjected to short periods of pilocarpine-induced SE. For the time periods studied here, apoptotic cell death did not contribute to the decrease of the hippocampal MEMRI signal.

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signal. However, T₂ relaxation was higher in the group of animals subjected to 30 min of SE than in the other SE or control groups. This result is consistent with higher AQP-4 expression during the same time period. Based on apoptosis and tissue water content analysis, the low hippocampal MEMRI signal 30 min after SE can potentially be attributed to local edema rather than to cell death.

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Introduction

Status epilepticus (SE) is defined as a seizure that persists for a sufficient length of time or is repeated frequently enough that recovery between attacks does not occur (CCT-ILAE, 1981). This definition was based on the persistence or repetition of the epileptic seizures rather than on the duration, although experts agreed that it should last at least 30 min to evoke a chronic epileptic condition (Lemos and Cavalheiro, 1995).

In the pilocarpine model of temporal lobe epilepsy (TLE), SE onset is defined as a seizure that persists for at least 5 min, and usually it may persist from 4 to 24 h (Leite et al., 1990). Electroencephalographical (EEG) recordings immediately after intraperitoneal pilocarpine injection have shown that low-voltage, fast activity appears in the neocortex and amygdala, while theta rhythm is evident in the hippocampus. As the behavioral manifestation of seizures becomes more severe, the theta hippocampal activity is replaced by high-voltage spiking and fast activity. EEG recordings immediately after injection have shown that pilocarpine can induce ictal epileptic events and that these EEG patterns are correlated with behavioral changes that culminate into SE (for review, see (Curia et al., 2008)).

MRI is a noninvasive and high-resolution imaging modality that is considered the most sensitive and specific structural neuroimaging for epilepsy, allowing several neuropathological studies. There are many MRI techniques: T₁- and T₂-weighted imaging, functional MRI, manganese enhanced MRI (MEMRI), arterial spin labeling (ASL), diffusion tensor imaging (DTI) that can detect not only damage caused by status epilepticus but also plastic changes in the brain that occur in response to damage (for review, see (Gröhn et al., 2011)).

Manganese-enhanced magnetic resonance imaging (MEMRI) is based on the fact that Mn²⁺ is a paramagnetic substance that changes both transverse and longitudinal relaxation (Koresky and Silva, 2004; Silva et al., 2004) and thus can act as a contrast agent in magnetic resonance imaging (MRI). The ability of Mn²⁺ to compete with Ca²⁺ allows it to be a marker of increased cellular activity and to trace neuronal connections (Koresky and Silva, 2004; Pautler et al., 1998).

Many animal studies have found a strong correlation between local brain activity and manganese enhancement using specific stimuli and MnCl₂ systemically injected with and without transient breakdown of the blood–brain barrier (BBB) (Kuo et al., 2006; Lin and Koresky, 1997; Pautler and Koresky, 2002; Weng et al., 2007; Yu et al., 2005). This ability, added to the fact that Mn²⁺ clearly enhances the various subfields of the hippocampus (dentate gyrus (DG), CA1 (Cornu Ammonis) and CA3 (Aoki et al., 2004; Watanabe et al., 2004), suggests that MEMRI could act as an imaging marker of epileptic focus.

Considering the chronic phase of epilepsy in the kainate or pilocarpine models, recent reports provide evidence that the MEMRI hyperintensity in the DG is mostly correlated with mossy fiber sprouting (Immonen et al., 2008; Malheiros et al., 2012b; Nairismägi et al., 2005), or, alternatively, inversely correlated with the frequency of spontaneous recurrent seizures (Dedeuvaerdere et al., 2013). Although conflicting, these findings relate the MEMRI signal in the hippocampus to local increases of cell activity. However, it has been shown that manganese enhancement of the MRI signal is significantly decreased in the hippocampus in the acute and latent phases of the kainate model (Alvestad et al., 2007). These last results could be either attributed to cell damage or loss following the long duration of SE (Alvestad et al., 2007; Immonen et al., 2008). In an attempt to circumvent the possible causes of reduction in the MEMRI signal during SE (cell damage/loss), the status was fully blocked at progressive time periods after its onset (5, 15 or 30 min) and the cell activity and the MEMRI signal were evaluated. It resulted that short durations of status did not produce differences in the MEMRI signal (30 min of SE reduced MEMRI signal) despite increased c-fos expression (Malheiros et al., 2012a).

Based on these findings, the present study will further investigate possible causes (apoptosis cell death or tissue water content) for reduction of the MEMRI signal during SE induced by pilocarpine.

Methods

Animals and study design

All protocols were approved by the Animal Care Committee of the Universidade Federal de São Paulo (CEP 0750/07). Adult male Wistar rats (250–300 g) were housed 4 rats/cage and kept under controlled laboratory conditions (12 h light/12 h dark cycle with lights on at 07:00 a.m., temperature 22 ± 1 °C, air humidity 50–60%, ad libitum access to food and water). Pilocarpine hydrochloride (300 mg/kg, i.p. Vegeflora, Paraiba, Brazil) was systemically injected, and 30 min prior, animals were given scopolamine methyl bromide (1 mg/kg, i.p., Sigma, Saint Louis, MI, US) to reduce systemic cholinergic side effects. Pilocarpine animals developed SE on average 30 min after the injection. One hundred two animals were used in this study. The SE-related mortality was 22.3% and 24.7% of animals that received pilocarpine injection did not develop SE.
The study design is outlined in Fig. 1. Rats were divided into 6 experimental groups. Awake animals for Groups 1–4 received a solution of MnCl₂·4H₂O (1 M) diluted in bicine solution (100 mM in deionized water) with pH adjusted to 7.4 using NaOH. Final concentration of MnCl₂ was 100 mM. Freshly prepared MnCl₂ (60 mg/kg) was injected intraperitoneally 12 h prior to pilocarpine injection when Mn³⁺ has already reached the hippocampus and the Mn²⁺ accumulation is still ongoing (Lee et al., 2005).

Behavioral seizures development was observed based on the Racine scale (Racine, 1972). The SE onset was defined after 5 min of continuous seizure activity. At the end of different SE time periods (5, 15 or 30 min), all the experimental groups, including the controls, received a mixture of thionembutal + diazepam (30 + 10 mg/kg, i.p.). Behavioral analysis in this paper supports previous encephalographic results (Mello et al., 2006) showing that this mixture terminates the SE in up to 10 min.

Group 1 (n = 6 for each time period) and Group 3 (n = 4 for each time period) was designed to compare MEMRI with c-fos expression and caspase-3 assay, respectively, after SE. Group 2 (n = 8) and Group 4 (n = 4) served as controls for Groups 1 and 3, respectively. These groups underwent the same protocol but did not receive pilocarpine injections. Group 5 (n = 5 for each time period) received pilocarpine and Group 6 (n = 5) did not experience SE. These groups were used without MnCl₂ injection, and T₂-weighted MRI was acquired to compare their aquaporin-4 expression. Abbreviations: M-esc—scopolamine methyl bromide; SE—Status epilepticus; AQ4—aquaporin 4; Pilo—pilocarpine and thio + dz—thionembutal + diazepam.

Fig. 1 Temporal diagram depicting the groups, treatments, and procedures used in the present study. The animals were divided into six groups: Groups 1–4 received a solution of MnCl₂·4H₂O (60 mg/kg) 12 h prior to pilocarpine injection. Group 1 (n = 6 for each time period) and Group 3 (n = 4 for each time period) were designed to compare MEMRI contrast with c-fos expression and caspase-3 assay, respectively, after SE. Groups 2 (n = 8) and 4 (n = 4) served as controls for Groups 1 and 3, respectively. These groups underwent the same protocol but did not receive pilocarpine injections. Group 5 (n = 5 for each time period) received pilocarpine and Group 6 (n = 5) did not experience SE. These groups were used without MnCl₂ injection, and T₂-weighted MRI was acquired to compare their aquaporin-4 expression. Abbreviations: M-esc—scopolamine methyl bromide; SE—Status epilepticus; AQ4—aquaporin 4; Pilo—pilocarpine and thio + dz—thionembutal + diazepam.

MRI

All MRI (T₁ and T₂-weighted) were acquired 14 h after MnCl₂. To decrease the post-SE mortality rate, the animals
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Fig. 2  Hippocampal MEMRI for pilocarpine injected animals in the acute phase 5, 15 and 30 min after SE. T1-weighted MEMRI images (A and C) and MEMRI data (B). The regions of interest (ROIS) were drawn in the hippocampal sub regions DG (dentate gyrus), CA1 and CA3 (Cornu Ammonis), as represented in A. The DG was enlarged and converted from gray into a colored scale in C to show differences between the CTR and SE30 groups (**P < 0.01). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(even controls treated with the mixture of thionembutal + diazepam, as above described) were placed in a supine position on the surgical table, tracheotomised, intubated (tube with approximately 0.5 mm diameter), and connected to a respirator for small animals (model 7025, Ugo Basile) and ventilated with ambient air (21% FIO2), at a respiratory rate of 70 cycles/min and a volume of 3.5 mL/cycle. These ventilatory parameters were monitored during the image acquisitions according to physiological parameters of each animal.

Images were obtained in a 2 T/30 cm superconducting magnet 85310HR (Oxford Instruments, Abingdon, UK) interfaced with a Bruker Avance AVII console (Bruker-Biospin, Inc., Billerica, MA, USA) using Paravision 5.0 software. A crossed saddle radiofrequency coil (Papotti, 2006) was used as a head probe. T1-weighted FLASH (Fast Low-Angle Shot) sequence was used in animals (Groups 1–4) that were MnCl2 injected (TR = 200 ms, TE = 5.8 ms, flip angle = 90°, 4 means, 40 min/animal). A volume of 40 × 40 × 11.2 mm3 was covered with a 192 × 192 × 16 points, generating a spatial resolution of 208 × 208 × 700 μm3. T2 MSME (Multi Slice Multi Echo) sequence was acquired in Groups 5 and 6 with 208 × 208 μm2 spatial resolution to determine the hippocampal T2 relaxation time (4 averages; TR = 2000 ms, 15 equally spaced echoes, TE = 15–225 ms; FOV = 40 × 40 mm, 19 min/animal).

MRI data was analyzed using the Paravision 5.0 software. One author (JMM), blinded to the group’s identity has manually outlined the regions of interest (ROI). Alterations in the relative signal intensity of the dentate gyrus (DG), CA1 (Cornu Ammonis) and CA3 were quantified from a single coronal section in T1-weighted 3D images at the antero-posterior level of −3.6 mm from the bregma (Fig. 2A). The signals were calculated as the ratio between the intensity of the mean signal in the ROI and the intensity of the mean signal of the adjacent corpus callosum (baseline value). The increased intensity of the relative signal, when compared to control animals, was determined as MEMRI hyperintensity. A ROI utilized in this study, denominated DG, in reality includes the DG and the proximal portion of the CA3, because it was not possible to separate them for analysis (Immonen et al., 2008). T2 relaxation time was determined by drawing bilaterally the contour of the hippocampus to define the region of interest (ROI) on the MSME images. The hippocampal level was the same as used in the analysis of T1-weighted images (3.6 mm caudal to bregma). The software tool ISA (Image Sequence Analysis) was used and the T2 calculated from a monoexponential curve.

Brain tissue

C-fos immunohistochemistry

Animals for Groups 1 and 2 were perfused transcardially, just after MRI acquisitions, with saline followed by 4% formaldehyde in 0.1 M phosphate buffer (PB, Sigma-Aldrich, pH 7.4). After perfusion, the brains were then removed from the
skull and stored at 4 °C in 30% sucrose for 3–4 days. Coro- nal sections with 30 μm were cut on a cryostat and one of three consecutive coronal sections was pre-treated with hydrogen peroxidase, followed by normal goat serum (1:200) and 0.3% Triton X-100 for 30 min. Sections were then (1) incubated with primary antibody (rabbit anti c-Fos 1:3000; Vector Laboratories, CA) at room temperature for 24 h; (2) incubated with a secondary antibody (goat anti-rabbit IgG 1:200; Vector Laboratories, CA) for 2 h at room temperature; (3) treated with 1:100 avidin–biotin complex for 90 min and a nickel-intensified diaminobenzidine reaction. The sections were rinsed in phosphate buffer, dried and mounted on gelatin-coated slides and coverslipped.

Histological images were captured on a high-resolution digital camera (Nikon DXM1200), installed in a Nikon microscope (Eclipse E600FN) with a magnification of 10×. Immunohistochemical labeling of c-Fos in the DG, CA1 and CA3 was evaluated by quantitatively measuring grayscale values using the National Institutes of Health (NIH) Image J software (http://rsbweb.nih.gov/ij/index.html). The grayscale values for the hippocampal sub regions were compared to those for the adjacent corpus callosum (baseline value). Sections were assessed across three different levels (rostral, medium, and caudal) of the hippocampus bilaterally, corresponding to levels 2.8, 3.8, and 4.8 mm caudal to the bregma (Paxinos and Watson, 1998) to exclude any possible rostro-caudal variability.

Caspase-3 fluorometric assay
Caspase-3 activity was studied in n = 4 animals per group using the method described by Thornberry et al. (1997) recently modified by Belizário et al. (2001). Rats were decapitated just after MRI acquisitions with animals still under the effects of anaesthetic. The hippocampi were dissected at 4 °C and immediately added to 20 mM HEPES buffer (pH 7.4) that contained 2 mM EDTA, 0.1% CHAPS, 10% sucrose, 0.1% PMSF, 0.1% benzamidine, 0.1% antipain, 0.1% TLCK, 0.1% chemostatin and 0.1% pepstatin (5 mM homogenization buffer/mg tissue). Homogenates were obtained by mechanically disrupting the tissue three times on dry-ice, with thawing in an ice bath, interpolated by 1 min of moderate vortex shaking. Samples were centrifuged at 12,000 × g for 40 min at 4 °C to remove cellular debris. Total proteins were determined in the supernatants using the Bio–Rad Protein Assay (Bio-Rad Labs, Germany). Homogenates (100 mg/protein) were incubated at 37 °C with the tetrapeptide substrate: AspGlu-Val-Asp (Ac-DEVD-AMC, 4 mM) for caspase-3, in a final volume of 150 mL. For a negative control, homogenates were pre-incubated for 10 min at 37 °C with commercial inhibitor to caspase-3 (Ac-DEVD-CHO, 1 mM), followed by the addition of the respective substrate. Activity was measured continuously over 2 h on a FlexStation 3 (Molecular Probes) Spectrofluorimeter, using λex = 360 nm and λem = 465 nm. Results are expressed in activity (nmol AMC).

Aquaporin 4 expression assay
Brain tissues were homogenized in ice-cold isolation solution (200 mM mannitol, 80 mM HEPES, 41 mM KOH, pH 7.5) containing protease inhibitor cocktail. The homogenates were centrifuged at low speed (4000 × g) for 15 min at 4 °C to remove nuclei and cell debris. Protein concentrations were determined by the Bradford assay method (Bio-Rad Protein Assay kit; Bio-Rad Laboratories, Hercules, CA).

Brain samples were run on 12% polyacrylamide minigels. After transfer by electroelution to a hydrophobic polyvinylidene difluoride membrane (Amersham Hybond-P PVDF Membrane GE Healthcare, Buckinghamshire, UK), blots were blocked with 5% milk and 0.1% Tween 20 in TBS or 1 h. Blots were then incubated overnight with an anti-AQP4 (Santa Cruz, TX, US) antibody (1:10,000). The labeling was visualized with a horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG diluted 1:2000; Sigma) using Western blotting detection reagents, the enhanced chemiluminescence detection system ECL (GE Healthcare, Buckinghamshire, UK). The images were obtained using the chemiluminescence imaging system Alliance 4.2 (Uvitec, Cambridge, UK) and quantitative analysis of antibodies were performed using densitometry, normalizing the bands to actin (Santa Cruz, Texas, US; 1:2000 with anti-goat 1:10,000) expression.

Statistical analysis
All data are presented as the mean ± SEM (standard error of mean). A one-way analysis of variance (ANOVA) was followed by the Bonferroni post hoc test with the statistical significance set to  P < 0.05 for each analysis.

Results

MEMRI
In the dentate gyrus (DG) the MEMRI signal was similar among the SE5 (1.008 ± 0.008), SE15 (1.011 ± 0.010) and control (CTR) groups (1.031 ± 0.012) (Fig. 2B). In the same region, the SE30 MEMRI signal (0.979 ± 0.007, P < 0.01) was less than that of the CTR (Fig. 2B and C).

In the CA1 (Cornu Ammonis) field, the groups SE5 (0.938 ± 0.007), SE15 (0.947 ± 0.004) and SE30 (0.943 ± 0.008) displayed a MEMRI signal similar to that of the control group (0.961 ± 0.011).

In the CA3 field, as for the DG region, the MEMRI signal was similar among SE5 (0.981 ± 0.007), SE15 (0.993 ± 0.013) and control (1.010 ± 0.014) groups. However, the MEMRI signal of the SE30 group (0.953 ± 0.015) was significantly lower than the CTR (P < 0.05).

C-fos
Fig. 3 shows c-Fos immunohistochemical labeling in the DG (A-D) region, CA1 (E-H) and CA3 (I-L) fields. The densitometry analysis of c-fos labelled cells indicated that in the DG, more c-fos expressing cells occur in the SE5 (41.1 ± 4.5), SE15 (40.8 ± 5.8) and SE30 (40.3 ± 5.5) groups than in the control (10.3 ± 0.8, P < 0.05). Similar results were observed in the CA1 and CA3 fields. In the CA1 field, the mean number of c-fos labeled cells in the SE5 (26.9 ± 2.3), SE15 (31.8 ± 2.5) and SE30 (25.9 ± 3.6) groups is higher than in the control one (10.9 ± 0.7, P < 0.05); in the CA3 field the mean number of c-fos immuno-labelled cells was similarly
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Fig. 3 Hippocampal histological analysis of c-fos expression (gray scale values) for DG (A–D), CA1 (E–H) and CA3 (I–L) for control (CTR), SE5, SE15 and SE30 groups, respectively. Pilocarpine-treated animals showed intense staining in all hippocampal sub regions unlike the controls.

Fig. 4A summarizes fluorimetric caspase-3 activity (nmol AMC). There were no differences between all studied groups: SE5, 129.2 ± 9.4; SE15, 148.9 ± 19.7; SE30, 153.0 ± 8.4 and CTR, 140.6 ± 19.6.

Caspase-3

T2 relaxometry

Hippocampal MRI relaxometry (ms) findings are summarized in Fig. 4B. There were no differences between SE5 (62.3 ± 1.9) and SE15 (60.7 ± 1.9) groups when compared to control group (58.4 ± 2.6). In addition, T2 in the SE30 group (70.4 ± 2.2) is higher than in the control (P < 0.01) and SE15 (P < 0.05) groups.

Aquaporin-4 (AQP-4)

Aquaporin-4 expression, as measured by means of densitometry, was higher in the SE30 (120.6 ± 4.7) group than in the control (95.2 ± 5.6, P < 0.05) or SE15 (89.2 ± 8.4, P < 0.05), but similar to the SE5 (105.5 ± 0.95) group. There were no other differences between groups. Results are shown in Fig. 4C and D.

Discussion and conclusion

The MEMRI and c-fos expression data presented here are consistent with our own and others’ previous data, which showed decrease in relative signal intensity in T1-weighted MEMRI images in the hippocampus during pilocarpine and kainate SE (Alvestad et al., 2007; Immonen et al., 2008; Malheiros et al., 2012a). There are no identified MEMRI differences between pilocarpine-treated animals (SE5 and SE15) and controls for the dentate gyrus (DG), CA1 (Cornu Ammonis) and CA3 fields.

The experiments conducted here aimed to add some light to the proposed role of MEMRI as preclinical biomarker for the severity of epileptogenesis in animal models. Hippocampal MEMRI signal in the epileptic chronic phase was proposed to be related to local increases of cell activity after seizures (Dedeurwaerdere et al., 2013). Although the manganese enhancement was related to spontaneous seizures outcome in the chronic epilepsy phase, it does not seem to happen after acute induced seizures. It is noteworthy that the SE30 group displayed a decrease MEMRI signal even in the DG and CA3 areas, which contrasts with the high cell activity in all hippocampal sub regions (DG, CA1 and CA3) confirmed by c-fos expression. Immediate early gene c-fos
is a marker of neurons that have been recently active, e.g., during a seizure (Sheng and Greenberg, 1990). Therefore, the absence of hippocampal MEMRI for SE30 and MEMRI signal similar to controls for SE5 and SE 15 cannot be attributed to decreased neuronal activity.

One possible explanation for the absence of MEMRI signal in the hippocampus for SE30 could be related to cell loss, once in vivo MEMRI enhancement is proportional to the Mn$^{2+}$ taken into cells through voltage gated Ca$^{2+}$ channels (Silva et al., 2004). However, our current results show that there is no alteration in the levels of caspase-3 activity after pilocarpine-induced status, during the time period investigated here. Caspase-3 activation is a mechanism of cell death (apoptosis) induced by seizures and has been detected between 24 h and 7 days after pilocarpine-induced status epilepticus (SE) in neurons, primarily in the hippocampus (Weise et al., 2005), although other studies have shown that active caspase-3 occurs mainly in the astrocytes and to a lesser extent in neurons (Ferrer et al., 2000; Narkilahti et al., 2003). Regardless of which hippocampal cell population is more affected by caspase-3 activation, it was not detectable within a few hours after SE onset, thus suggesting that after different durations of status (5, 15 or 30 min), apoptotic cell death is not related to reduction of MEMRI signal in the hippocampus.

In addition to cell death, local edema is often found in epileptic hippocampus, so it could by itself alter the MEMRI signal output. The current results indicate that T$_2$ signal hyperintensity is indeed present in SE30 group, but not in the earlier time periods. This is maybe due to increases of water content in the hippocampus reducing the manganese signal. The hippocampus of patients with mesial temporal lobe epilepsy is often hardened and shrunken, a condition known as sclerosis. Magnetic resonance imaging (MRI) reveals an increase in the T$_2$-weighted signal, whereas the diffusion-weighted imaging shows a higher apparent diffusion coefficient in sclerotic hippocampi, indicative of an increased water flow into the hippocampus, and accumulation of water in the brain parenchyma. In another human study, hippocampal edema and neuronal cell loss appear together (Kumar et al., 2013). Therefore, a significant increase in aquaporin-4 (AQP-4) was observed in sclerotic, but not in non-sclerotic, hippocampi obtained from patients with medically intractable temporal lobe epilepsy (Lee et al., 2004). For animals, SE induced by kainic
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acid indicate significantly greater tissue edema (AQP-4) and T2 MRI changes (Lee et al., 2012).

AQP-4 is a water-channel protein expressed strongly in the brain, predominantly in astrocyte foot processes at the borders between the brain parenchyma and major fluid compartments, including cerebrospinal fluid (CSF) and blood. This distribution suggests that AQP-4 controls water fluxes into and out of the brain parenchyma (Papadopoulos and Verkman, 2007). In interstitial edema, AQP-4 might facilitate the removal of excess extracellular brain water (Bloch et al., 2006; Bloch et al., 2005; Papadopoulos et al., 2004). Here, the AQP-4 protein expression was higher in the SE30 group than in the other studied groups. The result obtained by AQP-4 in the SE30 group was consistent with T2 relaxation changes found in the same time period, indicating the relationship of both measures with brain edema.

In conclusion, the DG and CA3 MEMRI hypo signal seen in the SE30 group could be explained by increases of water content rather than cell death. We suggest that the T2 relaxation changes are attributable to brain edema. Decreases in brain edema and/or cellular swelling could be mediated by AQP-4, resulting in better clearance of water excess from brain tissue. The hippocampal edema presented after 30 min of status changes the MEMRI relaxation times so that, the MEMRI signal was lower in control group, thereby presenting a hypo signal on T1-weighted images.

Disclosures and ethics

None of the authors has any conflict of interest to disclose. We affirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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