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Sustained elevation of cerebrospinal fluid glucose and lactate after a single seizure does not parallel with mitochondria energy production

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ABSTRACT

Generalized seizures trigger excessive neuronal firing that imposes large demands on the brain glucose/lactate availability and utilization, which synchronization requires an integral mitochondrial oxidative capability. We investigated whether a single convulsive crisis affects brain glucose/lactate availability and mitochondrial energy production. Adult male Wistar rats received a single injection of pentylentetrazol (PTZ, 60 mg/kg, i.p.) or saline. The cerebrospinal fluid (CSF) levels of glucose and lactate, mitochondrial respirometry, [^{14}C]-2-deoxy-D-glucose uptake, glycogen content and cell viability in hippocampus were measured. CSF levels of glucose and lactate (mean ± SD) in control animals were 68.08 ± 11.62 mg/dL and 1.17 ± 0.32 mmol/L, respectively. Tonic-clonic seizures increased glucose levels at 10 min (96.25 ± 13.19) peaking at 60 min (113.03 ± 16.34) returning to control levels at 24 h (50.12 ± 12.81), while lactate increased at 10 min (3.23 ± 1.57) but returned to control levels at 360 min after seizures (1.58 ± 0.21). The hippocampal [^{14}C]-2-deoxy-D-glucose uptake, glycogen content, and cell viability decreased up to 60 min after the seizures onset. Also, an uncoupling between mitochondrial oxygen consumption and ATP synthesis via FoF1-ATP synthase was observed at 10 min, 60 min and 24 h after seizures. In summary, after a convulsive seizure glucose and lactate levels immediately rise within the brain, however, considering the acute impact of this metabolic crisis, mitochondria are not able to increase energy production thereby affecting cell viability.

1. Introduction

Seizures induce neurochemical alterations in the brain, which may involve increased glutamate concentrations in the synaptic cleft and hyperactivation of glutamate receptors (Meldrum, 1993). This neurotoxic mechanism has important implications for the structural and functional abnormalities found in epilepsies and seizures.

Pentylentetrazol (PTZ) has been used as a model to induce seizures by mechanisms that involve the inhibition of the GABAergic system, followed by a massive synaptic membrane depolarization and excess release of glutamate (Bruno et al., 2002). Such events require a rapid increase in energy support to the brain cells to normalize membrane potential, ionic gradients, and glutamate levels, otherwise neural cells may be exposed to a harmful environment, and thus, likely will be designated to die (Bromfield et al., 2006). In this context, an adequate supply of metabolic substrates must match the high-energy demands necessary to restore glutamatergic homeostasis (Mendelowitsch et al., 2001). Actually, synaptic glutamate excess is predominantly removed through astrocytic uptake, a process that stimulates glycolysis in astrocytes, and the extrusion of lactate to extracellular space to be used as fuel for neuronal activity (Danbolt et al., 2016; Pellerin and Magistretti, 1994).

Moreover, glucose from brain glycogen stores may also be diverted from oxidative degradation inside the mitochondria, leading to the

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generation of considerable amounts of lactate, which could then be released into the brain parenchyma (Schousboe et al., 2007; Waitt et al., 2017). Thus, it seems that microenvironmental alterations at level of cell compartments during and after the course of seizures limit the production of high amounts of energy through oxidative metabolism, even though it has been reported that improvement in lactate utilization by neurons exert neuroprotective roles in various excitotoxic events (Madji Hounoum et al., 2017; Patet et al., 2016; Schurr et al., 1997a). Beyond an energy source, a recent work proposed that lactate exerts a neuromodulatory role on excitatory synapses of the brain via GPR81 receptors (Lauritzen et al., 2014). This is particularly important, because generalized seizures trigger a transient hypoxia and increases non-oxidative glycolysis (Bromfield et al., 2006; Schurr et al., 1997a, 1997b). Therefore, along with the availability of cerebral glycogen, glucose and lactate, the engagement of these substrates on complementary metabolic pathways should be performed in synchronized manner to avoid unnecessary waste of energy and substrates mobilization (Bouzier-Sore et al., 2006; Darbin et al., 2005; Fornai et al., 2000; Walling et al., 2007). Based on this ground, it is imperative to consider the impact of a convulsive seizure on mitochondrial energy production, which is crucial for the maintenance of cells survival. Overall, the study of brain extracellular flux of metabolic substrates over time may be a feasible strategy to estimate the rate of mobilization after a convulsive event (Cornford et al., 2002; Darbin et al., 2005; Fornai et al., 2000), meanwhile the oxygen consumption by brain tissue preparations enriched in mitochondria provide insights regarding specific substrates utilization and potential deficits in particular oxidative complexes of the electron transport system.

In that context, cerebrospinal fluid (CSF) analysis has become a basic tool in the study of a number of parameters including brain metabolism and neurochemical abnormalities associated to pathological events (Busnello et al., 2006; Chow et al., 2005; Cruz Portela et al., 2002; Stefani et al., 2017). Accordingly, some studies highlight that CSF glucose and lactate levels and glucose/lactate ratio provide a better understanding of the balance between brain oxidative/non-oxidative metabolism and, in addition, presents an important role in the prediction of clinical neurological outcomes and in the evaluation of tissue stress in several neurological disorders (Darbin et al., 2005; Makoroff et al., 2005; Stefani et al., 2017).

Here, we examined in rats whether a single convulsive crisis affects brain glucose/lactate availability and mitochondrial energy production.

2. material and methods

2.1. Chemicals

Lactate and glucose kits were obtained from Katal Biotecnologica® (MG, Brazil). [^{14}C]-2-deoxy-D-glucose (2DG) was obtained from Dupont NEN (Boston, MA, USA). PTZ was obtained from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Animals

Male Wistar rats, 60–90 days old (200–300 g) were used. Animals (n = 88) were kept on a 12 h light: 12 h dark cycle (lights on at 07:00 a.m.) at a constant temperature of 22 ± 1°C. They were housed in plastic cages (five animals per cage) with water and food *ad libitum*. Procedures for the care and use of animals were adopted according to the regulations of the Guide for the Care and Use of Laboratory Animals (National Research Council, USA). The local ethics committee of our institution approved this study (CEUA#30124).

2.3. PTZ administration, cerebrospinal fluid (CSF) and tissue sampling

Rats received a single injection of PTZ (60 mg/kg, i.p., dissolved in saline), producing tonic-clonic seizures, only considered when animals displayed scores above 5 in the revised Racine seizure scale (Luttjohann et al., 2009). The animals that did not show tonic-clonic seizures were used as no-seizure group. The onset of convulsive crisis lasted around 2 min. Afterwards, animals were anesthetized with sodium thiopental (40 mg/kg i.p.) at 10, 30, 60, 120 or 240 min after the onset of seizures, followed by an immediate collection of CSF sample (40–60 µL per rat) by direct puncture of the cisterna magna with an insulin syringe (27 gauge × 1/2 in. length). CSF samples for lactate and glucose were centrifuged at 5000 × g for 5 min and the supernatant was stored at -70 °C until analysis. After CSF sampling, animals were killed by decapitation 10, 30, 60 min and 24 h after the onset of seizures, and hippocampi samples were used for quantification of [^{14}C]-2-deoxy-D-glucose uptake, glycogen levels, mitochondrial respiration, and cell viability. At least eight (n = 7–8) animals were used at each time point.

2.4. Measurements of CSF glucose and lactate levels

Glucose and lactate were assayed in CSF using a glucose oxidase methodology manufactured by Katal Biotecnologica, MG, Brazil. All samples and standard were carried out in duplicate in the same experiment. Calibration factors were determined using a standard of glucose (5.56 mmol/L) and lithium lactate (4.44 mmol/L). At least eight (n = 7–8) animals were used at each time point.

2.5. [^{14}C]-2-deoxy-D-glucose (2DG) uptake

Rats were euthanised by decapitation and their cerebral hippocampus was dissected over ice-cold Krebs-Ringer bicarbonate buffer. Hippocampus was cut in 0.3 mm slices using a McIlwain tissue chopper. The hippocampus slices (around 50 mg) were added to flasks (11cm^3) containing 0.5 mL Krebs-Ringer bicarbonate buffer, pH 7.4, 0.2 µCi of [^{14}C]-2-deoxy-D-glucose (2DG) and incubated at 35 °C in a metabolic shaker for 30 min. To stop the reaction the flasks were inserted on ice and the tissue was washed with ice-cold incubation buffer. Hippocamppi were dissolved in 200 µL of lysis buffer (NaOH 2 N), and the incorporated radioactivity was measured by liquid scintillation spectrometry. At least eight (n = 7–8) animals were used at each time point.

2.6. Measurement of glycogen levels

The samples of hippocampus (around 200 mg) were added to 2.0 mL of KOH 30% and heated at 100 °C until full dissolution of tissue, and then cooled in water. Ethanol (2.0 mL) was added and the mixture heated for 10 min at 70 °C. Next, the tubes were cooled on ice for 3 min and centrifuged at 3000 rpm for 5 min. Supernatant was discarded and 0.2 mL of HCl 5 N and 3.8 mL of distilled water were added to the pellet. The glycogen content was determined by the Krisman method (Krisman, 1962). The sample (50 µL) was mixed with iodine [I_2 0.01% and KI 0.1% in a saturated solution of $(NH_4)_2SO_4$] and the resultant absorbance was determined at 460 nm and compared with a glycogen standard. At least eight (n = 7–8) animals were used at each time point.

2.7. High resolution respirometry analysis

Animals subjected to PTZ or saline injection were euthanized and had left cerebral hippocampus collected 10 min, 60 min and 24 h after the convulsive event. Hippocampal tissue was then homogenized using 15 strokes of a Potter glass tissue homogenizer, as previously described (Burtscher et al., 2015), in a volume of 1 mL of ice cold buffer (Sucrose 320 mM, Tris 10 mM, EDTA 1 mM). Next, 50 µL of homogenate was resuspended to 2 mL in respiration buffer (100 mM KCl, 75 mM mannitol, 25 mM sucrose, 10 mM Tris-HC1, 5 mM phosphate, and 0.05 mM EDTA, pH 7.4) at a O2K High Resolution Respirometer (Oroboros Instruments, Innsbruck, Austria) chamber. Samples were submitted to a protocol consisting of sequential titrations of substrates, inhibitors and

uncouplers, to elicit specific mitochondrial respiratory states, where O_2 consumption (JO_2) was analyzed and assessed as a proxy of mitochondrial function. Mitochondrial respiratory state I is considered basal JO_2 sustained by stimuli and substrates endogenously present on samples. State IV was elicited due to addition of mitochondrial complex I and II substrates, respectively, Pyruvate 10 mM, Malate 10 mM, Glutamate 20 mM and Succinate 10 mM. Further, State III was stimulated by the addition of ATP synthase substrate ADP 2.5 mM. Mitochondrial O2 consumption that is not coupled to ATP production was assessed in State IV_O through the addition of Oligomycin 2 mM, an inhibitor of FoF1-ATP synthase (mitochondrial Complex V). Finally, KCN 5 mM was added to inhibit mitochondrial cytochrome C oxidase to indicate all residual non-mitochondrial oxygen-consuming reactions (ROX), and was subtracted from all previous states for correction. OxPhos coupling efficiency, that represents the capacity of ADP to control oxygen consumption from State IV to State III, was calculated as (JO_2 State III- JO_2 State IV) / JO_2 State III.

2.8. Statistical analysis

The data are presented as mean ± S.D. and the statistical analysis was performed with One-Way ANOVA followed by a post hoc Dunnett test. All results with a $P \leq 0.05$ were considered significant.

3. Results

CSF glucose and lactate levels in control animals were 68.08 ± 11.62 mg/dL and

1.17 ± 0.32 mmol/L, respectively (Fig. 1A and B). Animals injected with PTZ and did not present tonic-clonic seizures displayed CSF levels of glucose and lactate not significantly different from controls (data not shown). Fig. 1A and B show a rapid and sustained elevation in the CSF glucose and lactate levels in animals presenting tonic-clonic seizures. However, glucose and lactate levels dynamics displayed a distinct temporal profile. To date, glucose levels increased 10 min after PTZ injection (96.25 ± 13.19 mg/dL) peaking at 60 min (113.03 ± 16.34 mg/dL) then returning to control levels at 24 h (50.12 ± 12.81 mg/dL) (Fig. 1A), while lactate levels also increased at 10 min post injection (3.23 ± 1.57 mmol/L) but returned to control level only at 360 min (1.58 ± 0.21 mmol/L) (Fig. 1B). Also, PTZ increased lactate/glucose ratio 10 min after seizure (ratio > 2:1), followed by a 1:1 ratio at 60 min. From 60 min to 24 h the CSF lactate/glucose ratio was persistently elevated (ratio > 2:1) (Fig. 2A).

Considering that tonic-clonic seizures may increase lactate release from skeletal muscle, we also investigated whether peripheral glucose and lactate levels can influence brain concentrations. Simultaneous assessment of glucose and lactate in CSF and serum performed at 10, 30, and 60 min after seizures, showed that glucose and lactate levels were higher in CSF and serum samples of the PTZ as compared to control groups (Fig. 2C and D; $P \leq 0.05$). Values of CSF lactate/glucose ratio surpass serum lactate/glucose ratio at all time-points evaluated, except at 10 min, when there was a significant statistical difference between PTZ and control groups (Fig. 2B; * #$P \leq 0.05$).

PTZ-induced seizures could also influence local glucose uptake and glycogen metabolism thereby influencing substrates availability and use in mitochondrial respiration. The 2DG uptake decreased 27% at 10 min after the seizure, returning to control levels at 30 min (Fig. 3A, $P \leq 0.05$). Similarly, there was a decrease of 37% in hippocampal glycogen content at 10 min, which returned to control levels at 30 min after PTZ injection (Fig. 3B, $P \leq 0.05$). Respirometry analysis showed reduced mitochondrial oxygen consumption after 10 min of PTZ-induced seizure in mitochondrial states IV (106.85 ± 30.07 pmol/(s*mg)), III (118.52 ± 30.22 pmol/(s*mg)) and IV_O, (89.08 ± 30.220 pmol/(s*mg)) as compared to both control (268.80 ± 56.06 pmol/(s*mg); 437.01 ± 194.50 pmol/(s*mg); 155.82 ± 67.50 pmol/(s*mg); States IV, III and IV_O respectively) and 24 h groups (413.11 ± 175.16 pmol/(s*mg); 614.48 ± 277.95 pmol/(s*mg); 387.34 ± 155.67 pmol/(s*mg); States IV, III and IV_O respectively). Such decrease is sustained until 60 min in mitochondrial State IV (129.67 ± 66.83 pmol/(s*mg)) (supplementation of substrates) and State III (134.99 ± 78.74 pmol/(s*mg)). At 24 h, State III and State IV respiration is increased relative to 10 and 60 min, whilst in State IV_O (correspondent to leak respiration) the value was significantly increased relative to control (Fig. 4A, * $P < 0.05$, # $P < 0.05$ as compared to controls and 24 h seizure groups respectively).

When analyzing OxPhos Coupling efficiency, an estimation of mitochondrial efficiency we observed decreased values in PTZ compared to Control group (Fig. 4, Control: 0.647 ± 0.104; 10 min: 0.234 ± 0.165; 60 min: 0.300 ± 0.222; 24 h: 0.354 ± 0.0624) (Fig. 4B, *$P < 0.05$ as compared to controls). These mitochondrial abnormalities parallel with a significant decrease in cell viability at 10 and 60 min after seizure (Fig. 5, *$P \leq 0.05$).

4. Discussion

Although limited exceptions exist, the brain houses no energetic reservoirs. The generally accepted view is that its energy requirements, which fluctuate with the level of cerebral activity, are met by variations in local cerebral blood flow, as well as variations in energetic substrate levels supplied directly to the CSF, from which it enters neurons and

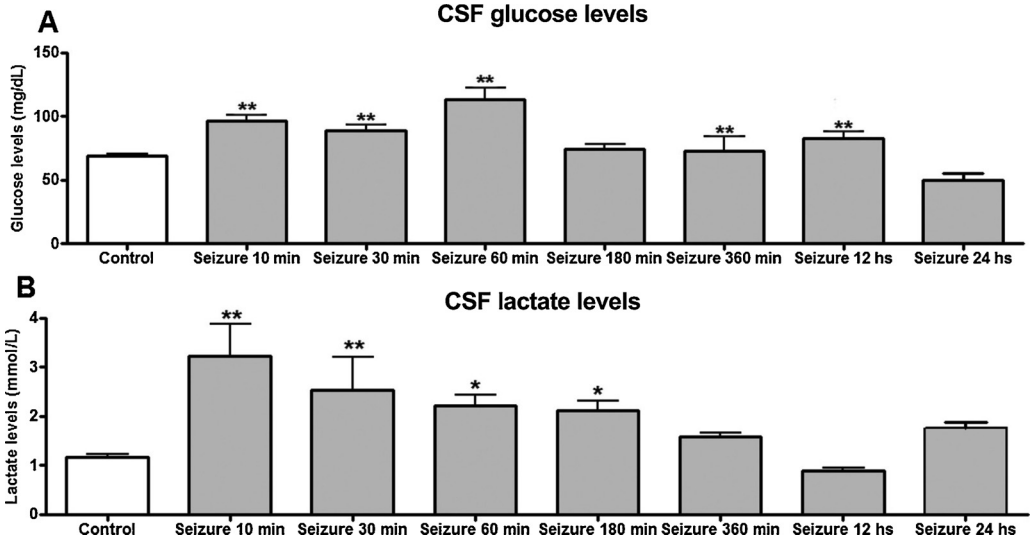

Fig. 1. Cerebrospinal fluid (CSF) profile of glucose (A) and lactate (B). Concentrations measured in control (white bars) and PTZ (black bars) groups at 10, 30, 60, 180, 360 min, 12 h and 24 h after seizures. The data represent mean ± S.D. of at least 8 animals. *$P \leq 0.05$ and **$P \leq 0.01$ when compared with respective controls according to one-way ANOVA.

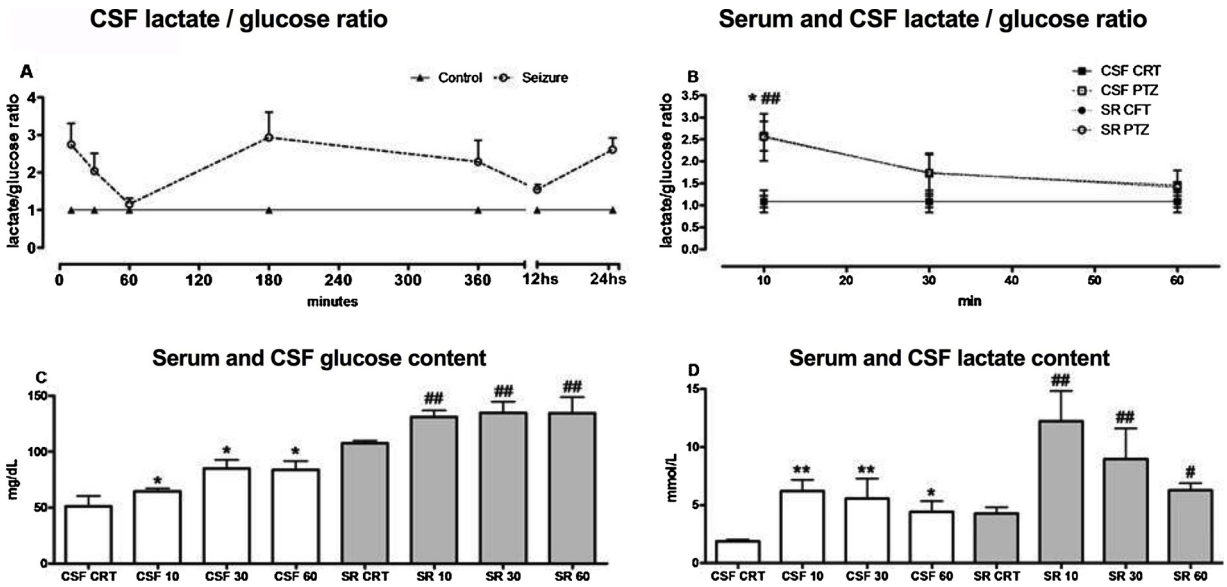

Fig. 2. Cerebrospinal fluid (CSF) and serum lactate/glucose ratio. The ratios in control and PTZ groups were calculated at 10, 30, and 60 min after seizures. The data represent mean ± S.D. of at least 8 animals. $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$ for CSF. $^{\#}P \leq 0.05$ and $^{\#\#}P \leq 0.01$ for serum, when compared to controls according to one-way ANOVA.

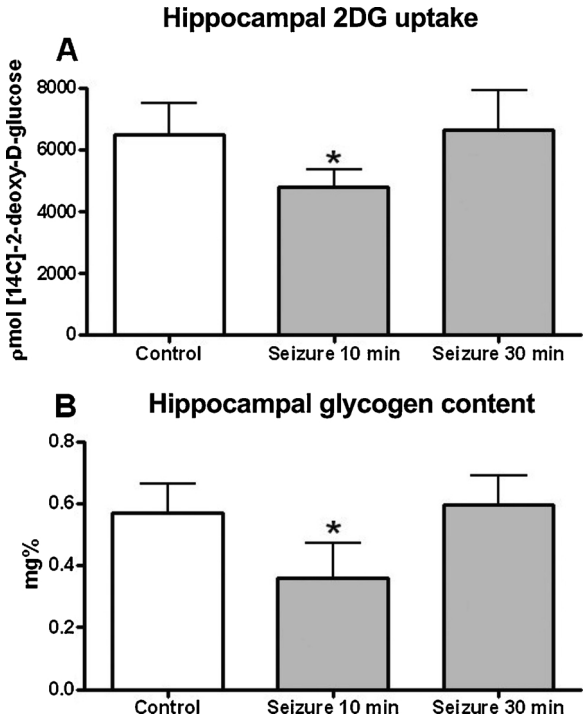

Fig. 3. Hippocampal uptake of [^{14}C]-2-deoxy-D-glucose (A) and glycogen content (B). These parameters associated with glucose availability/mobilization are showed in control (white bars) and PTZ (black bars) groups at 10 and 30 min after seizures. The data represent means ± S.D. of at least 8 animals. $^{*}P \leq 0.05$ and when compared with controls according to one-way ANOVA.

astrocytes by the actions of transporters. In the present study, extracellular glucose and lactate levels promptly increased in the CSF after PTZ-induced seizures, whereas glucose uptake and glycogen level rapidly decreased in hippocampus, which was followed by the concomitant decrease in oxidative energy production and cell viability. Here, we observed that CSF glucose and lactate concentrations peaked similarly at 10 min, but glucose showed more sustained temporal elevation relative to lactate (Fig. 1, A and B).

Seizures are known to increase ATP demands in the brain, putatively increasing the flux of glucose through the glycolytic pathway and TCA cycle in neurons and astrocytes (Carmody and Brennan, 2010). Given this, a sustained support of glucose seems to be necessary to avoid the detrimental consequences of a metabolic crisis (Akman et al., 2010; Bidmon et al., 2008; Darbin et al., 2005). However, it was reported similar increments of glucose and lactate concentrations in striatum microdialysates during the recovery period following electroshock-induced seizure (Darbin et al., 2005) reinforcing our results in the CSF that extracellular glucose/lactate levels are highly available to neural cells and may serve as indicators of hyperglycolisys and mitochondrial failure, which ultimately imply in metabolic desynchronization.

Moreover, alterations in the blood-brain barrier (BBB) permeability in a rodent model and human epileptogenic brain tissue were already demonstrated. Actually, the BBB has long been recognized as crucial for maintenance of the brain microenvironment, and its disruption has been linked with the pathogenesis of epilepsy (Han et al., 2017). Based on this, we tested whether a single convulsive seizure could affect the influx/efflux of lactate and glucose through the CSF. Thus, central and peripheral lactate/glucose ratios showed an overlap (from 10 to 60 min after seizure) suggesting a similar contribution in the substrates availability from peripheral and central compartments (Fig. 2B and C). Although alternative and complex kinetics analysis validated by Duarte and Gruetter (2012) (Duarte and Gruetter, 2012), allows performing a dynamic measurement of brain glucose/plasma *in vivo*, and the determination of glucose uptake and consumption kinetics, paving the way for more accurate mechanistic insights, our approach was even more simplistic but nonetheless goes in the same direction. Actually, as pointed by Korf (2006) (Korf, 2006), CSF lactate level is determined by rates of influx and efflux across the BBB, lactate influx and efflux across the membranes of brain cells, lactate formation from glucose and glycogen, and also blood lactate concentrations. The same factors also determine glucose and oxygen levels, with the obvious differences in transport capacity and diffusion capability. It is known that the extracellular availability of energetic substrates is not tightly associated with consumption. Accordingly, epileptic seizures are proposed to cause glucose hypometabolism, through mechanisms that are currently unclear but likely involve mitochondrial dysfunctions. The use of radiotracers such as [^{18} F] Fluoro-2-deoxyglucose (FDG) or [^{14}C]-2-deoxy-D-glucose (2DG) have allowed the identification of a focal decrease in

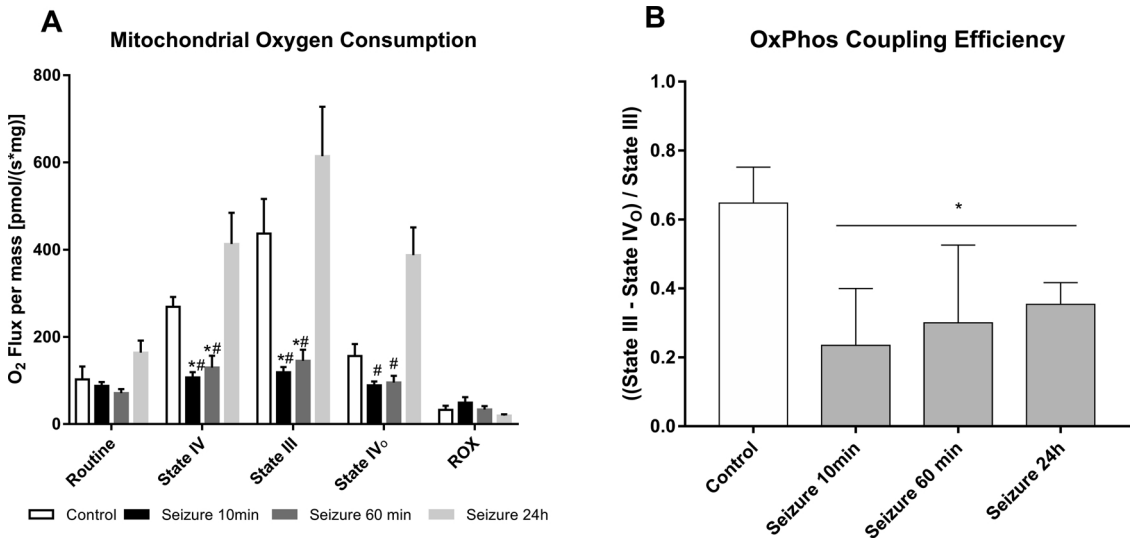

Fig. 4. Mitochondrial parameters linked to energy production. Absolute mitochondrial oxygen consumption decreases in preparations of hippocampus homogenates in States IV; III; IV_O and ROX at 10 min and 60 min after seizures, and trend to normalize by 24 h (A). Seizures trigger increased State IV_O oxygen consumption after 24 h relative to control. The effective capacity of mitochondria in coupling oxygen consumption with ATP synthesis (OxPhos coupling efficiency) was reduced by seizures at 10 and 60 min, and 24 h as compared to controls. State IV (complex I and II substrates pyruvate, malate, glutamate and succinate). State IV (titration with ADP allowing maximum mitochondrial oxygen consumption linked to ATP synthesis). State IV_O (addition of F1Fo ATP-synthase inhibitor Oligomycin; represents oxygen consumption uncoupled with ATP synthesis). Data represent mean ± SD, n = 6 animals per group. *P < 0.05 as compared to controls, #P < 0.05 as compared to 24 h group according to one-way ANOVA.

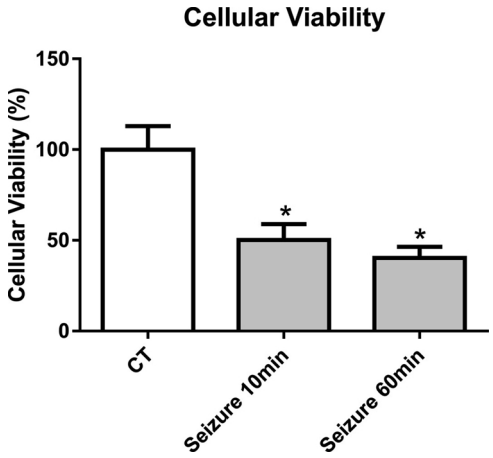

Fig. 5. Hippocampal cells viability. By MTT assay we found reduced cell viability at 10 and 60 min after seizures implying in an acute massive loss of neural cells due to energy deficits. The data represent means ± S.D. of at least 8 animals. *P ≤ 0.05 when compared with controls according to one-way ANOVA.

glucose consumption concordant with the seizure onset zone (Akman et al., 2010). A previous study suggests that whilst strong excitation and strong inhibition would increase brain metabolism, the impaired inhibitory synaptic activity would result in decreasing energy requirements, and therefore, reduced glucose uptake (Akman et al., 2010). Furthermore, increased epilepsy duration in patients correlated with hypometabolism in the hippocampus, parahippocampal gyrus, and ipsilateral thalamus (Akman et al., 2010). Our study showed a short-term decrease in hippocampal glucose uptake after a single seizure (Fig. 3A) but we were not able to replicate prolonged uptake deficits. Also, glycogen pool in astrocytes responds rapidly even to the weakest sensory stimuli whereas the inhibition of glycogen metabolism during periods of intense neuronal activity has dramatic negative effects (Brown et al., 2005; Brown and Ransom, 2007; Walling et al., 2007). Thus, a rapid glycogen mobilization seems to provide additional neuroprotection against metabolic deficits (Brown and Ransom, 2007; Pellerin et al., 2007; Sickmann et al., 2009), even though, here the concomitant reduction in glucose uptake, increase in the glycogen degradation and lactate production parallel with decreased cell viability.

Remarkably, the observed reduction in 2DG uptake and astrocytic glycogen content 10 min posterior to seizure occurs concomitantly with reductions in mitochondrial oxygen consumption up to 60 min after seizures (Fig. 4A); culminating in persistent (24 h) energy deficits (Fig. 4B). Patients with mitochondrial mutations leading to malfunction often manifest seizures as symptom (Hallmann et al., 2014; Molinari et al., 2005; Poduri et al., 2013). Alternatively, previous works have suggested mitochondrial dysfunction not as a cause, but as a consequence to increased neuronal activity during sporadic seizures (Kann et al., 2011; Kann and Kovacs, 2007; Kovacs et al., 2002; Zsurka and Kunz, 2015). Acute reduction of GABAergic inhibition in interneurons leads to increased glutamate release in synapse, and glutamatergic excitotoxicity, which is likely to occur due to PTZ injection (Schroeder et al., 1998). Excitotoxic calcium influx from NMDA receptors activation can drastically affect mitochondrial respiration, and initiate intrinsic apoptotic signalling, as both are tightly related to calcium buffering in mitochondria, further supporting reduction in cerebral cell viability after seizures (Kudin et al., 2009; Meldrum, 1986; Sztriha et al., 1985; Wang et al., 2015), which is observed both 10 and 60 min after seizure when compared to controls (Fig. 5).

Whereas glucose CSF concentrations may reflect the balance between blood supply and neuronal and glial utilization (Fellows et al., 1992), elevated CSF lactate concentration may serve as a proxy biomarker of astrocytic hyperglycolisis and neuronal suffering (Frykholm et al., 2005; Sarrafzadeh et al., 2003; Stefani et al., 2017). However, although the maintenance of high lactate/glucose ratio (> 2:1), accompanied by mitochondrial dysfunction after seizure, demonstrated in this piece of work is a suggestive evidence to support astrocytic hyperglycolisis, further studies are necessary to prove such concept. Also, these set of data showed here is only valid for a single seizure episode in a naïve individual, which must be taken under account for the limitations and translational value.

In summary, after a convulsive seizure glucose and lactate levels immediately rise within the brain, however, considering the acute impact of this metabolic crisis mitochondria are not able to increase

energy production through the FoF1-ATP synthase activity, thereby affecting cell viability. Therefore, in rats injected with PTZ, cerebral substrate availability and mitochondrial bioenergetics are desynchronized, which could account for the hypometabolism after seizure.

Conflict of interest

The authors have no conflict of interest to disclose.

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