

Selective hippocampal cell damage and mossy fiber sprouting induced by chronic intracerebral injections of 2-deoxy-D-glucose

Evgeniia Samokhina, Anton Malkov, Alexander Samokhin and Irina Popova

Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Russia

Abstract. A reduction in glucose consumption has been shown in both patients with acquired epilepsy and in animal epilepsy models. However, the question remains whether the disturbance of glucose metabolism is the driving force of epileptogenesis. We have recently reported that a chronic partial inhibition of brain glycolysis by the non-metabolizable glucose analogue 2-deoxy-D-glucose (2-DG) triggers epileptogenesis in initially healthy rats. In this study, we further investigated whether chronic 2-DG treatment caused a cellular loss in the dorsal hippocampus and mossy fiber sprouting in the dentate gyrus. We found that prolonged (four weeks) treatment with 2-DG induced a neuronal loss in the CA1 field and the dentate hilus. We also found mossy fibers reorganization in the 2-DG group. In addition, we showed that pentylenetetrazole-induced convulsions were considerably strengthened and prolonged in 2-DG-treated rats. Our results demonstrate that the chronically impaired brain glucose metabolism likely leads to a structural remodeling resembling epileptogenesis and has a proconvulsive effect.

Key words: 2-DG — Epilepsy — Hippocampus — Pentylenetetrazole — Epileptogenesis

Abbreviations: 2-DG, 2-deoxy-D-glucose; PET, positron emission tomography; PTZ, pentylenetetrazole; TLE, temporal lobe epilepsy.

Introduction

Epilepsy is a neurological disease characterized by spontaneous recurrent seizures (Fisher et al. 2014). Temporal lobe epilepsy (TLE) is the most common type of a drug-resistant partial epilepsy with seizures originating from or involving mesial temporal structures. Reduced glucose utilization during interictal periods detected by fluorine-18-tagged fluorodeoxyglucose (18 F-FDG) positron emission tomography (PET) technique is a generally recognized characteristic of the patients suffering from TLE (Pan et al. 2008; Cendes et al. 2016). Interestingly, genetically determined disruption of energy supply (mitochondrial disorders, creatine deficits and glucose transport deficiency) is known to be associated with different types of epileptic seizures (Reid et al. 2014). It

has been proposed that the energy metabolism dysfunction (Kudin et al. 2009; Jupp et al. 2012; Lee et al. 2012; Reid et al. 2014), associated with oxidative stress (Azam et al. 2012; Martinc et al. 2012) and neuroinflammation (Wilcox and Vezzani 2014) is a trigger and driving force for acquired epileptogenesis (Zilberter and Zilberter 2017; Patel 2018).

The metabolic rate of glucose consumption increases during a seizure due to intensive neuronal firing (Wasterlain et al. 1993). During the postictal phase, however, glucose utilization is deficient (Guo et al. 2009; Jupp et al. 2012; Lee et al. 2012; Zhang et al. 2015). Moreover, the predictive value for the epilepsy outcome defined by the PET-identified glucose metabolism failure at an early phase of epileptogenesis has been demonstrated in different epilepsy models (Shultz et al. 2013; Bascuñana et al. 2016). Surprisingly, despite glucose hypometabolism is a generally recognised feature of epileptogenesis (Goffin et al. 2008; Pittau et al. 2014; Patel 2018), in the last decade an antiepileptic approach by glycolysis suppression has been suggested by a number of authors (Garriga-Canut et al. 2006; Stafstrom et al. 2009; Koenig et al. 2019; Rho et al. 2019), although a short time

Correspondence to: Evgeniia Samokhina, Laboratory of Systemic Organization of Neurons, Institute of Theoretical and Experimental Biophysics of Russian Academy of Sciences, 3 Institut'skaya Str., Pushchino, Moscow Region, 142290, Russia
E-mail: evgeniia.sam@iteb.ru

2-DG treatment revealed opposing effects (Stafstrom et al. 2008; Gasior et al. 2010; Rho et al. 2019), that is in agreement with *ex vivo* results demonstrating strong 2-DG effects on multiple neuronal parameters underlying excitability, such as resting potential, synaptic transmission, energy metabolism and Cl⁻ homeostasis (Zhao et al. 1997; Samokhina et al. 2017; Nedergaard and Andreassen 2018).

A rationale for such an approach was its apparent similarity to the low-carb, high-fat ketogenic diet (KD), which also leads to the suppression of glycolysis (Rho et al. 2019). However, in KD, glucose is partially substituted by ketone bodies as a mitochondrial fuel (Bentourkia et al. 2009, Prince et al. 2013). Thus, the radical difference of KD and direct glycolysis inhibition is the absence of cellular energy deficiency in KD.

We have shown recently *in vivo* that chronic partial inhibition of brain glycolysis by 2-DG significantly diminished the energy reserve store of glycogen and adenosine phosphates and induced epileptiform activity in initially healthy rats (Samokhina et al. 2017). Furthermore, a bidirectional positive feedback connecting seizures and energy failure has been shown *in vitro* (Malkov et al. 2018).

In this study, using 2-DG as a specific inhibitor of glycolysis (Chandramouli and Carter 1977), we investigated whether chronically reduced cellular glucose supply caused a neuronal loss and synaptic reorganization in the hippocampus. In addition, we treated rats with pentylenetetrazole (PTZ) to determine whether a chronic decrease in glycolysis could have a proconvulsive effect.

Materials and Methods

Animals

Experiments were performed on young adult male Wistar rats (250–300 g) obtained from the Experimental Animal

Center of the Institute of Theoretical and Experimental Biophysics (Pushchino, Russia). Animals were housed in pairs with food and water ad libitum. Animal handling and housing protocols were in accordance with the European Union guidelines (Directive 1986/609/EEC) for the use of experimental animals in scientific research. The protocol was approved by the Committee on the Bioethics of Animal Experiments of the Institute of Theoretical and Experimental Biophysics of the Russian Academy of Sciences.

All rats randomly allocated to three groups: 2-DG ($n = 13$), Saline ($n = 15$), and Control ($n = 4$). Rats in the 2-DG group received 2.5 μ l 2-DG (20 mM) dissolved in sterile normal saline. Administration was daily for 28 days (Fig. 1A). Rats in the Saline group received equivalent volumes of 0.9% saline. Naive rats from the Control group were deprived of any neurosurgical and traumatic manipulations.

Surgery

Experimental animals underwent a neurosurgical operation (Fig. 1A) under general anesthesia (intramuscular injection): mixture (half-and-half Tiletamine hydrochloride and Zolazepam hydrochloride (18 mg/kg) and Xylazine hydrochloride (12 mg/kg). The body temperature was maintained by a heating pad and the cardiopulmonary parameters were monitored during the surgery by an Oxy9Vet Plus pulse oximeter (Bionet, South Korea). Rats were placed in a stereotaxic apparatus and a guide cannula (stainless steel, 21 gauge) was implanted above the left lateral brain ventricle (AP = -0.8; L = 1.5; H = 3.0); according to the rat brain atlas (Paxinos and Watson 2007).

Drugs and drug administration

Drug administrations to animals were started after the post-surgical recovery period (Fig. 1A). 2-DG (purity 98%; Sigma-Aldrich, Saint Louis, Missouri) was dissolved in saline

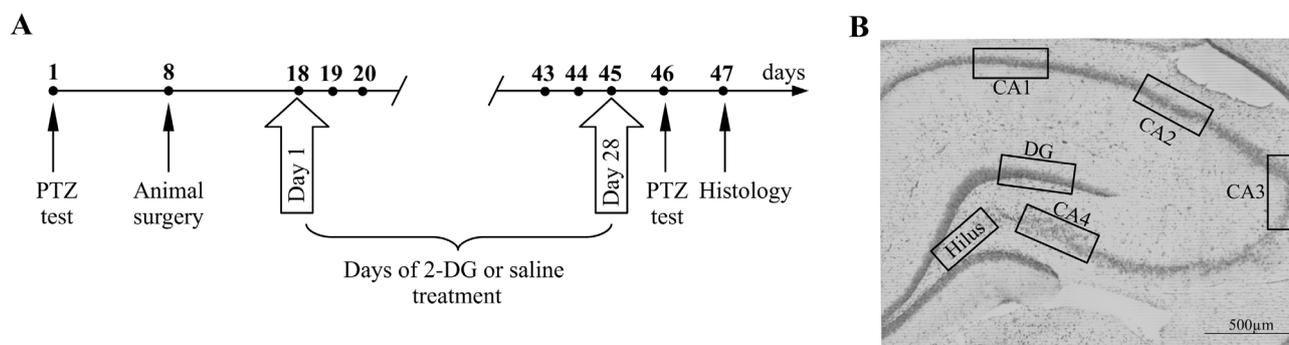


Figure 1. Experimental design. **A.** Timeline diagram showing the experimental protocol, including pentylenetetrazole (PTZ) seizure test, animal surgery, *i.c.v.* injections of 2-DG/saline and histological staining. **B.** Nissl-stained coronal slice of the dorsal hippocampus. Squares indicate the counting frames in the hippocampal fields (CA1, CA2, CA3, CA4), hilus and dentate gyrus (DG).

solution (20 mM). 2-DG (2-DG group) or 0.9% saline solution (Saline group) injected intracerebroventricularly (*i.c.v.*) through the guide cannula to awaken rats using a Hamilton microsyringe type 75N at an infusion rate of 1 μ l/min. For brain adaptation, the injection was usually started 1 min after the microsyringe insertion. The injection needle was slowly pulled out of the brain 2 min after the drug administration to avoid outflow. All substances were injected in equal volumes (2.5 μ l). The dose of 2-DG was chosen based on our preliminary experiments (Samokhina et al. 2017).

PTZ seizure test

Rats from 2-DG and Saline groups were twice injected with pentylenetetrazole (PTZ). The proconvulsive agent PTZ was shown to stimulate seizure activity by blocking γ -aminobutyric acid (GABA)-mediated transmission (Ramzan and Levy 1985). Sub-threshold injections of PTZ (35 mg/kg, intraperitoneal (*i.p.*)) were made: one week before surgery to estimate the pre-treatment seizure susceptibility and on the next day after the end of 2-DG or saline treatment to determine whether a chronic 2-DG treatment enhances susceptibility to PTZ (Fig. 1A). Following each PTZ injection, animals were placed into an acrylic glass transparent box, and behavioral seizures were monitored using a Sony Alpha5000 camera (Japan) for a period of 30 min.

Because the chosen PTZ dose did not induce tonic-clonic convulsions in sufficient number of experimental rodents, two PTZ trails were conducted. In the first PTZ trail, seizure severity was estimated; in the second PTZ trail, latency time and duration of tonic-clonic convulsions were assessed. All results were analyzed in a blind manner.

Histology

On the next day after the final PTZ test, all animals were deeply anesthetized with an overdose of pentobarbital (80 mg/kg, *i.p.*), perfused intracardially with 0.37% sodium sulfide in Timm buffer (0.12 M $\text{NaH}_2\text{PO}_4 \cdot 2 \text{H}_2\text{O}$, 0.10 M NaOH, 0.18 mM CaCl_2 , pH 7.4) for 20 min (15 ml/min) followed by 4% paraformaldehyde solution for 20 min (15 ml/min). Brains were removed from the skull and postfixed overnight in 4% paraformaldehyde at 4°C. After cryoprotection in a gradient of sucrose (10% and 20% sucrose in buffer at 4°C for 24 h each), brains were rapidly frozen in the vapor phase of liquid nitrogen. Coronal sections (20 μ m) were cut from the brain block with a Thermo Shandon Cryotome E (Thermo Scientific, USA) at -19°C and collected onto poly-L-lysine-coated slides for subsequent Nissl and Timm staining. Nissl staining was used to verify cannula placements and to detect the safety of the left lateral ventricle and neuronal damage to the dorsal hippocampus (anterior posterior level between -3.5 and -4.5 according to

the Paxinos and Watson (2007)). Slide-mounted tissue sections were dried at room temperature overnight, submerged in bi-distilled water with acetate buffer for 5 min, and stained in fresh 0.1% cresyl violet for 5–8 min until the desired depth of staining was achieved. To visualize supragranular mossy fiber sprouting in the dentate gyrus, Timm staining of 8–10 slices from each animal (2 slides) was performed at the levels corresponding to AP = 3.5–4.5 as in (Paxinos and Watson 2007). Slide-mounted slices were developed using the following solution: 60 ml of 50% gum arabic, 7.3 ml of citrate buffer (2.55 g sodium citrate, 2.35 g citric acid), 30 ml of 5.9% hydroquinone, and 0.5 ml of 17% silver nitrate. The physical development was performed in the dark at 26°C for 60–90 min. Washing the slides in tap water for 10–15 min terminated the development of stain. The stained slides were dehydrated in graded ethanols, cleared in xylene, and coverslipped with Eukitt (Fluka, Germany) mounting medium.

Bright field images were acquired on a Nikon E200 microscope (Japan) with a Sony Alpha5000 camera (Japan). All tissue sections were photographed under identical conditions. Counts were performed on at least 5 sections. Cell quantifications were carried out manually using the “Cell counter” plugin of the ImageJ software (1.50i, USA) by an investigator who was blind to experimental group.

Statistical analysis

The results are presented as the mean \pm standard deviation. The *n* values represent the number of animals used in the experiments. All statistical tests were performed using GraphPad Prism version 7 (GraphPad Software, Inc., USA). The D’Agostino-Pearson normality test (D’Agostino 1986) was applied in order to assess how far from Gaussian the data are. All data set fails the normality test as the *p* value is ≤ 0.05 , meaning that it deviates significantly from the normal distribution. Then, nonparametric statistical tests were executed. Statistical analysis of cell density in different areas of the hippocampus from the control and drug-treated groups was performed in two steps: statistically significant results by the Kruskal-Wallis test were analysed by the Dunn’s post-hoc test. Changes in the severity of PTZ-induced seizures were tested by the Wilcoxon signed rank test for paired comparisons and Mann-Whitney U test for independent data. $p \leq 0.05$ was considered statistically significant.

Results

PTZ sensitivity test

Seizures induced by PTZ were scored as follows: stage 0, no response; stage 1, ear and facial twitching; stage 2, convulsive waves moving axially through the body; stage 3,

Table 1. The effects of chronic treatment with 2-deoxy-D-glucose (2-DG) and saline on susceptibility to seizure induced by PTZ (35 mg/kg, *i.p.*).

Group	Seizure stage		Latency time (s) to		Duration tonic convulsions (s)
	before treatment	after treatment	2nd seizure stage	4th seizure stage	
Saline	1.3 ± 1.2 (<i>n</i> = 15)	2.6 ± 1.7 (<i>n</i> = 15)	62.00 ± 32.47 (<i>n</i> = 15)	455 ± 155.37 (<i>n</i> = 4)	35.25 ± 12.70 (<i>n</i> = 4)
2-DG	0.8 ± 1.3 (<i>n</i> = 13)	3.6 ± 0.8 [§] (<i>n</i> = 13)	65.83 ± 30.00 (<i>n</i> = 13)	422.33 ± 189.39 (<i>n</i> = 6)	76.5 ± 11.28* (<i>n</i> = 6)

Chronic 2-DG treatment induced a significant difference in seizure stages vs. seizure stages before treatment ([§]*p* = 0.03, Wilcoxon signed paired test) as well as the duration of stage 4/5 seizures as compared to saline-treated rats (**p* = 0.03, Mann-Whitney U test). PTZ, pentylenetetrazole; *n*, the total number of animals.

myoclonic jerks and rearing; stage 4, clonic convulsions with the animal falling on its side; stage 5, repeated severe tonic-clonic convulsions or lethal convulsions. As shown in Table 1, there was a significant difference between the seizure stage after chronic 2-DG treatment and the initial seizure stage (before treatment): 0.8 ± 1.3 before 2-DG treatment (*n* = 13) and 3.6 ± 0.8 after 4 weeks of daily 2-DG injections (*n* = 13), (*p* = 0.03, Wilcoxon signed paired test). In the Saline group, convulsions did not significantly

intensify during four experimental weeks (Table 1). Also we observed a significant increase in the duration of clonic convulsions in the 2-DG group in comparison with the saline-treated group: from 35.25 ± 12.70 (s) in the Saline group (*n* = 4) to 76.5 ± 11.28 (s) in the 2-DG group (*n* = 6), (*p* = 0.03, Mann-Whitney U test). However, our analysis did not reveal statistically significant differences in the latency time for behavioural seizures between these two groups of animals.

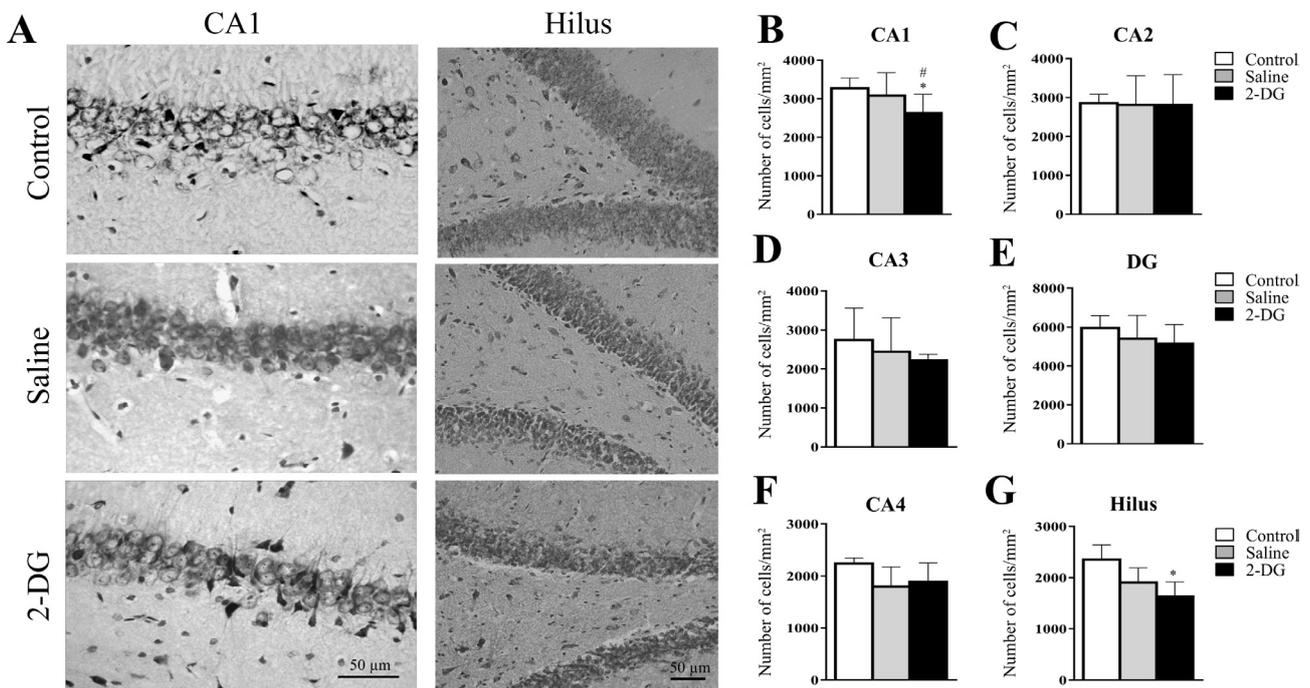


Figure 2. Nissl-stained sections and quantitative analysis of cell density in the dorsal hippocampus. 28 days following 2-DG treatment, neuronal loss was found in the hilus (A, G) (**p* = 0.01 vs. Control) and field CA1 (A, B) of the 2-DG group (**p* = 0.03 and #*p* = 0.04 vs. Control and Saline groups, respectively). The cell densities in other hippocampal fields (CA2, CA3, CA4) and dentate gyrus (D, G) in the 2-DG group did not differ from those in the Control or Saline groups (C, D, E, F). Each bar represents the mean ± SD. No statistically significant difference in the average cell density was revealed in saline-treated animals vs. control animals in all analyzed hippocampal fields. Kruskal-Wallis test followed by Dunn's post-hoc test was performed.

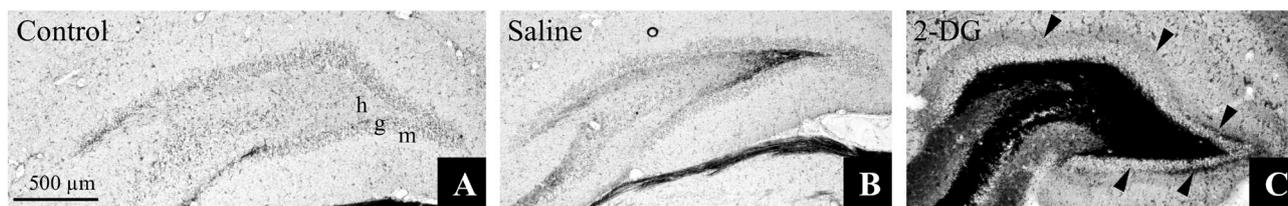


Figure 3. Sections of the dorsal hippocampus stained by the Timm method for identification of mossy fiber sprouting in the dentate gyrus. These sections demonstrate Timm-negative stain from the Control animal group without any injection (A); from the Saline group (B) and Timm-positive stain (arrowheads) in the dentate inner molecular layer of the 2-DG group (C). h, hilus; g, granule cell layer; m, molecular layer.

Neuronal loss in hippocampus

Assessment of neuronal density was performed on the Nissl-stained sections. Neuronal quantification was carried out in the dentate gyrus, hippocampal pyramidal cell layers: fields CA1, CA2, CA3, CA4 (counting frame $7,225 \mu\text{m}^2$) and dentate hilus (counting frame $14,450 \mu\text{m}^2$) (Fig. 1B).

Four weeks after the 2-DG treatment, light microscopy showed that the degradation of the pyramidal layer in CA1 reached of 20% of Control group ($p = 0.03$) and 15% of Saline group ($p = 0.04$): cell density in the Control ($n = 4$) and Saline group ($n = 15$) were 3271 ± 264 and 3082 ± 594 , respectively, as compared to 2631 ± 482 in the 2-DG ($n = 13$) (Fig. 2A, B). In the 2-DG group, the cell density in the hilus of the dentate gyrus was also considerably reduced: the neuron loss was 31% for the 2-DG group relative to the Control (Fig. 2A, G). The cell density was 2352 ± 285 in the Control ($n = 4$) as compared to 1636 ± 279 in the 2-DG group ($n = 7$, $p = 0.01$). Importantly, the cell densities in CA2, CA3, CA4, and dentate gyrus in the 2-DG group did not differ from those in the Control or Saline groups (Fig. 2 C–F). No statistically significant differences in the average densities of neurons were shown in saline-treated animals vs. naive animals in all analyzed hippocampal fields.

Importantly, neuronal densities in CA2, CA3, CA4 and dentate gyrus in the 2-DG group did not differ from those in the Saline group (Fig. 2C–F). No statistically significant differences in the average densities of neurons were shown in saline-treated animals vs. naive animals in all analyzed hippocampal fields except hilus.

Detection of mossy fiber sprouting

Timm staining was performed to measure the mossy fiber sprouting in hippocampus. The degree of mossy fiber sprouting was rated using semi-quantitative analysis (Cavazos et al. 1991) as follows: (1) sparse Timm granules in the supragranular zone; (2) more numerous granules in continuous distribution; (3) prominent granules and patches; (4) a dense laminar band in the supragranular layer; and

(5) a dense laminar band extending to the inner molecular layer. Representative images of Timm staining are shown for three groups: Control ($n = 4$; Fig. 3A), Saline ($n = 9$; Fig. 3B), and 2-DG ($n = 12$; Fig. 3C). The results indicated that aberrant mossy fiber sprouting from the dentate gyrus toward the inner molecular layer was significantly increased in the 2-DG group (2.16 ± 1.11 , $p = 0.02$) (Fig. 4) compared to the Saline group (1.11 ± 0.92) (Fig. 4). In naive control rats ($n = 4$), there was a subtle black Timm staining in the granule cell layer and absence of aberrant mossy fiber sprouting into the inner molecular layer (0.75 ± 0.5). No statistically significant differences were found in Saline vs. Control group.

Discussion

A novel finding of this study indicates that chronically reduced cerebral glucose utilization *via i.c.v.* 2-DG injection leads to the hippocampal neuron loss and synaptic reorganization. Specifically, we demonstrate that chronic 2-DG treatment (daily for 28 days) induces a significant reduction of neuronal density in both the hippocampal

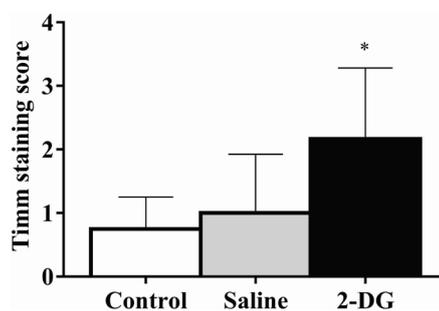


Figure 4. Timm staining scores of rats after 4 weeks of 2-DG/saline treatment. Horizontal bars represent the group means. Each column corresponds to the mean group score \pm SD. The score difference between 2-DG ($n = 12$) and Saline ($n = 9$) groups was statistically significant (* $p = 0.02$). Kruskal-Wallis test followed by Dunn's post-hoc test was performed.

CA1 field and the hilus of dentate gyrus. We also show that the 2-DG treatment initiates sprouting of mossy fibers in the inner third part of dentate gyrus molecular layer. Thus, the chronic energy deficiency state created by 2-DG leads to structural and irreversible changes in nervous tissue that are specific for epileptogenesis.

Hippocampal sclerosis, a selective neuronal loss in the CA1/CA3 region of hippocampus and hilus, is a characteristic feature of TLE patients and animal epilepsy models (Berkovic et al. 1991; Curia et al. 2008; Lévesque and Avoli 2013). Interestingly, in our work, neuronal loss in CA1 and dentate hilus was more dramatic, whereas a reduction of cell density in CA3 was significantly less pronounced. Whether CA3 area is more resistant to glucose metabolism breakdown is unknown. One possibility is that the CA1 region exhibits fewer capillaries than the neighbouring CA3 region (Cavaglia et al. 2001), thus higher stability of CA3 area may be caused by higher vascularization. Previously, it was suggested that CA3 receives two major vascular afferents (dorsal arteries) while CA1 is vascularized by only one arterial branch (ventral artery), that may be the underlying anatomical source of capillary density difference (Marinkovic et al. 1992). Given that hippocampal neurons demonstrate different degree of sensitivity to 2-DG treatment, it will be interesting to explore this phenomenon in future studies.

Most notably, we have shown for the first time that 2-DG treatment induces the development of mossy fiber sprouting in the dentate gyrus of hippocampus. In contrast, morphological alterations were negligible in saline-treated animals. Additionally, in the 2-DG-treated group, we showed a robust decline of threshold for inducing seizures in the PTZ test. This is consistent with literature data showing that acute 2-DG treatment does not protect against pentylenetetrazole-, kainic acid- or maximal electroshock-induced seizures (Lian et al. 2007; Stafstrom et al. 2009; Gasior et al. 2010). While 2-DG induced synaptic reorganization and atrophy in the hippocampal regions, chronic implantation of the intraventricular cannula as well as daily *i.c.v.* injections did not induce neuronal density decline, mossy fiber sprouting or a pro-epileptic effect in the PTZ test. Therefore, the invasive approach we used in experiments did not result itself to posttraumatic epileptogenesis.

An important experimental aspect is the mode of 2-DG administration. In our work, the injection of 2-DG was *i.c.v.*, directly inhibiting glycolysis in brain cells and causing chronic energy deficiency in the brain. In contrast, studies showing the protective role of 2-DG treatment (Shao et al. 2018; Rho et al. 2019) used *i.p.* drug administration, causing a concurrent and significant rise of both blood glucose level and cerebral blood flow, as has been reported for both animals (Combs et al. 1986; Breier et al. 1993; Horinaka et al. 1997; Koenig et al. 2019; Leiter et al. 2019) and humans (Landau et al. 1958; Elman et al. 1999). Importantly, the brain 2-DG

concentration was not estimated in these studies but seems to be insufficient for providing the direct effect on brain cell energy metabolism (Samokhina et al. 2017). Therefore, the 2-DG brain effects in the studies using *i.p.* drug administration were most likely indirect, and referencing in these studies a 2-DG brain glycolysis inhibition is obviously inadequate. Our results show directly the contribution of brain glucose hypometabolism to the development of pathology and confirm the view on acquired epilepsy as a metabolic disease (Zilberter and Zilberter 2017; Patel 2018).

Here we show that chronic 2-DG-induced inhibition of glycolysis results in changes in neuronal density and synaptic reorganization in the hippocampus. We also demonstrate that the chronic 2-DG treatment has a pro-epileptic effect. These results show that the chronic decrease in brain energy metabolism and, in particular, reduction in glucose utilization leads to irreversible structural rearrangements in the nervous tissue, that is important for both the understanding general mechanisms of brain pathogenesis and the development of therapeutic approaches aimed at eliminating the cause, rather than the symptoms, of neurological diseases.

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Conflicts of interest. There are no actual or potential conflicts of interest.

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