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The glycolysis inhibitor 2-deoxy-D-glucose exerts different neuronal effects at circuit and cellular levels, partially reverses behavioral alterations and does not prevent NADPH diaphorase activity reduction in the intrahippocampal kainic acid model of temporal lobe epilepsy

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Abstract

Temporal lobe epilepsy is the most drug-resistant type with the highest incidence among the other focal epilepsies. Metabolic manipulations are of great interest among others, glycolysis inhibitors like 2-deoxy d-glucose (2-DG) being the most promising intervention. Here, we sought to investigate the effects of 2-DG treatment on cellular and circuit level electrophysiological properties using patch-clamp and local field potentials recordings and behavioral alterations such as depression, anxiety-like behaviors, and changes in nitric oxide signaling in the intrahippocampal kainic acid model. We found that epileptic animals were less anxious, more depressed, with more locomotion activity. Interestingly, by masking the effect of increased locomotor activity on the parameters of the zero-maze test, no altered anxiety-like behavior was noted in epileptic animals. However, 2-DG could partially reverse the behavioral changes induced by kainic acid. The findings also showed that 2-DG treatment partially suppresses cellular level alterations while failing to reverse circuit-level changes resulting from kainic acid injection. Analysis of NADPH-diaphorase positive neurons in the CA1 area of the hippocampus revealed that the number of positive neurons was significantly reduced in dorsal CA1 of the epileptic animals and 2-DG treatment did not affect the diminishing effect of kainic acid on NADPH-d^+ neurons in the CA1 area. In the control group receiving 2-DG, however, an augmented NADPH-d^+ cell number was noted. These data suggest that 2-DG cannot suppress epileptiform activity at the circuit-level in this model of epilepsy and therefore, may fail to control the seizures in temporal lobe epilepsy cases.

Introduction

Epilepsy is one of the most complicated neurological diseases that is characterized by neuronal hyperexcitability and sudden, simultaneous discharges which appear as seizures. Almost 1 % of the general population are diagnosed with epilepsy and about 40% of the cases are pharmacoresistant. Temporal lobe epilepsy has the highest incidence among the other types of epilepsy and exhibits considerable resistance to antiepileptic agents [1]. Epilepsy is associated with many behavioral disorders, including anxiety and depression, as reported in epileptic patients and animal models; therefore, introducing novel and potent approaches and compounds to control the seizures is of paramount importance.

Among many novel strategies, metabolic manipulations have widely attracted attention as the ketogenic diet (KD), a low carbohydrate and high-fat diet is shown to have been effective in many drugs resistant cases. KD acts through a great many mechanisms among which, the most important is to cut out glycolysis and subsequent attenuation of lactate shuttle between neurons and astrocytes [1] and, more importantly, decreased cytosolic ATP concentration. Lactate dehydrogenase inhibition which results in direct inhibition of glycolysis is demonstrated to have suppressed paroxysmal discharges in the intrahippocampal kainic acid model of epilepsy, which is one of the most drug-resistant models [2].

2-deoxy D glucose (2-DG) is another glycolysis inhibitor that has been studied over the last few years for its possible therapeutic actions [3–7]. However, interestingly, both its pro-convulsant and anticonvulsant

effects have been observed according to the different methods of epilepsy induction in animals. For instance, in i.v PTZ, i.v kainic acid and electroshock induced seizures, 2-DG decreased seizure threshold while in the 6-Hz seizure test it led to seizure threshold increment [5]. Furthermore, in pilocarpine-induced epilepsy, while elevating seizure latency, 2-DG diminished seizure duration and severity [8]. In in-vitro models like high $[K]_o$, likewise, 2-DG dwindled interictal epileptiform activity [9]. 2-DG is of interest not only for its possible anticonvulsive implication but also for its potency in the inhibition of cancerous cell growth. Indeed, it is already in clinical use for treating SARS-Cov-2 [10] and has had promising results in the suppression of cancerous cell growth in clinical trials [11].

The intrahippocampal kainic acid model, which is deemed as an appropriate simulator of human temporal lobe epilepsy due to hippocampal sclerosis seen in this model [12], has long been used to assess the therapeutic effects of nominated compounds to control the seizures and/or treat epilepsy. Although a previous study has reported that comorbid psychiatric symptoms including, anxiety- and depression-like behaviors are not significantly different between control and epileptic animals in this model [13], severe cell loss in the dorsal hippocampus and general ipsilateral hippocampal deformation plants doubt in the mind whether follow-up tests would probably bring out different results. Previous lesion studies have posited a link between dorsal and ventral hippocampus lesions, which are seen in this model, and diminished anxiety levels [14]. additionally, shriveled hippocampus is associated with depression-like behavior [15].

Considering previous observations, the present study aims to further explore the impact of 2-DG on the electrophysiological consequences of epilepsy-induced by intrahippocampal injection of kainic acid in mice which bears a striking resemblance to human temporal lobe epilepsy. Moreover, how behavioral comorbidities like anxiety and depression, if present, were affected by 2-DG injection. Furthermore, since the nitrergic system is involved in the regulation -of excitatory and inhibitory neurotransmission, and becomes imbalanced during epileptogenesis [17], [18], we also evaluate alterations in the NOergic neurotransmission by using NADPH-diaphorase staining.

Materials & Methods

1. Animals

In this study, 64 male *NMRI* mice (30 to 35g; Pasteur institute, Tehran) were used. They were housed with free access to food and water and were kept under 12:12 hours light: dark cycle. All experimental procedures and animal care were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Biomedical Research Ethics Committee of the National Institute for Medical Research Development (Approval ID: IR.NIMAD.REC.1399.259) and the Ethics Committee of Shahid Beheshti University of Medical Sciences (Authorization code: IR.SBMU.MSP.REC.1400.630)

2. Study design

The present study intended to investigate the behavioral, electrophysiological and histochemical consequences of glycolysis inhibition on the intrahippocampal kainic acid model of temporal lobe epilepsy. Animals were divided into four groups: control group (undergone surgery and received i.p. and intrahippocampal saline, which was used as a solvent for both kainic acid and 2-DG); control+2-DG group (received i.p. injection of 2-DG and intrahippocampal saline), epileptic group (received an intrahippocampal injection of kainic acid), and epileptic+2-DG group (received intrahippocampal kainic acid and i.p 2-DG). Three separate groups of mice were used to assess the effects of glycolysis inhibition by 2-DG on kainic acid-induced hyperexcitability in CA1 pyramidal neurons using Patch-clamp recording, local field potential (LFP) recordings to measure epileptiform activity, and behavioral tests to assess the locomotor activity, anxiety-like and depression-like behaviors. To assess histological alterations, however, the animals were randomly chosen from the animals which had undergone behavioral tests. It should be noted that according to previous studies in our laboratory, saline and surgery do not lead to considerable variation between the groups; hence, the study does not contain intact and sham groups. After surgery (day 0) (intrahippocampal kainic acid or saline injection; also electrode implantation in LFP groups), the animals recovered for 3 or 4 weeks before data acquisition; Data from the control and the epileptic animals were taken on the day 21st whereas the epileptic+2-DG and control+2-DG groups received 300 mg/kg 2-DG for 7 days, once a day, and underwent the experiments on the day 28th. In the LFP groups, epileptic and epileptic+2DG groups consist of the same animals; it is to say, the epileptic animals were recorded on the day 21st, and then, received 2-DG, and recorded once again on the day 28th. Furthermore, to evaluate whether increased epileptiform activity in epileptic+2-DG group was a result of 2-DG injection, or epilepsy progression resulted in this increment, an extra group, epileptic+saline, was added to the experiments. In this additional group, similar to the epileptic+2-DG group, the animals were recorded once on the day 21st, but received saline 300 mg/kg instead of 2-DG for 7 days, once a day, and recorded again on the day 28th. All the injections were performed 90 minutes before the tests, the time in which 2-DG induced ketosis is observed [19]. Because 2-DG had no significant impact on control animals in behavioral tests (see results), the control+2-DG group was omitted from patch-clamp and LFP recording groups.

3. Epilepsy induction

Temporal lobe epilepsy was induced as previously described by Sada et al [2]. Briefly, mice were anesthetized by intraperitoneal injection of ketamine (100mg/kg) and xylazine (10mg/kg) and fixed in the stereotaxic frame. Then, 0.8 nmol kainic acid was dissolved in 40 nL normal saline and directly injected into the dorsal hippocampus (-1.6 mm to the Bregma, 1.6 from the midline, and 1.2 mm deep from the dura mater) according to the atlas of Paxinos and Franklin (2001). Due to the non-convulsive status epilepticus, verification of model induction was endorsed by frequent interictal epileptiform activity (sharp-wave complexes) (see below for details) as well as severe cell loss in the dorsal CA1 pyramidal cell layer (see Nissl Staining) (Fig. 1 C& D). After the experiments, the anesthetized animals were decapitated and the brains were dissected out for injection site verification (Fig. 1 A).

4. Behavioral tests

4.1. Open field test

To measure the locomotor activity, animals were placed in the center of an open field (35×35×35 cm) after half an hour of habituation to the experimental room. In the following ten minutes, moved distance and velocity were analyzed by EthoVision XT 11 software. The arena was cleaned with ethanol 85% between the trials.

4.2. Zero maze test

Anxiety-like behavior was evaluated by using zero maze apparatus. The apparatus (60 cm in diameter, 5cm wide circular corridor, 16 cm high walls and 60 cm high from the floor) was made of wood and painted dark black. After a half an hour habituation to the experiment room, each animal was placed in an open arm-closed arm intersection, facing the closed arm. During the following 5 minutes, the animal was videotaped and analyzed offline afterwards. Five parameters were assessed including the time spent in the open arms, the number of entries to the open arm, the latency of the first open arm entry, head dipping frequency, and body stretching frequency. Between the trials, the apparatus was cleaned using 70% ethanol.

4.3. Sucrose preference test

Rodents are shown to prefer sweet water rather than tap water. Suffering from depression, however, they tend to consume less sweet water in comparison with tap water in normal conditions [20]. To perform the test, the animals were given access to two tap water bottles for 24 hours as habituation in their home cage. During the next 24 hours, both bottles were taken and replaced with two new bottles, one containing 3% sucrose solution while the other filled with tap water. As the diameter of the drinking hole had been noted to have influence on the amount of water consumed by the animal [21], the holes were all equalized in size using a 2mm drill. The proportion of sweet/tap water consumption was calculated afterwards.

5. Local field potentials (LFP) recordings

Mice underwent stereotaxic surgery to record local field potentials. They were anesthetized with an intraperitoneal injection of ketamine (100mg/kg) and xylazine (10mg/kg). The ear bars were placed delicately prior to muzzle fixation. Lidocaine 2% was injected under the scalp skin 5 minutes before making an approximately 2 cm incision in the skin. Following Bregma-Lambda adjustment to a plane level, three holes were made by a fine drill. To prepare electrodes, 2 stainless steel wires (127 µm in diameter, A.M. system Inc., USA) were intertwined to give the electrode suitable strength and flexibility. The electrode, then, was soldered to a connector and placed in the dorsal hippocampus (-2.1mm AP, 1.5mm ML, 1.2mm DV). 6 screws (one as the reference electrode above the cerebellum) were screwed to the scalp. Lastly, dental cement was used to fix the electrodes. The LFP signals were continuously recorded for 13 hours at 1KHz sample rate and low-pass filtered at 250Hz while the animals were freely moving. Interictal epileptiform discharges were defined as sharp-waves, having more than twofold

amplitude compared with baseline, as well as having a frequency between 1 to 20 Hz. The discharges were detected and analyzed by MATLAB 2016 software. At the end of the experiments, brains were removed to verify the proper placement of the electrode (Fig. 1B).

6. Patch-clamp recording

To investigate the possible cellular-level effects of epilepsy induction by kainic acid on the electrophysiological properties of hippocampal CA1 pyramidal neurons, and whether 2-DG can reverse these possible alterations, whole-cell patch-clamp was performed as follows. Briefly, the animals were deeply anaesthetized with ether and then decapitated. The brains were removed immediately and placed in ice-cold artificial CSF (ACSF) containing (in mM): 206 sucrose, 2.8 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 2 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 D-glucose, and saturated with 95% O2 and 5% CO2 (pH 7.3-7.4; 300 mOsm). Transverse slices (300 µm) were cut by using a vibroslicer (7000smz-2, Campden Instruments Ltd, UK). Slices were placed in a holding chamber containing ACSF composed of (in mM) 125 NaCl, 2.5 KCl, 1.5 $CaCl_2$, 1.25 NaH_2PO_4, 25$NaHCO_3$, 10 D-glucose, pH 7.4, 300 mOsm for at least 60 minutes at 32-35˚C. The slices were kept at room temperature (23–25°C) before transferring to the recording chamber. After incubation for at least 1 h, each slice was individually transferred to a submerged recording chamber on the stage of an upright microscope (BX51WI, Olympus); they were continuously superfused with oxygenated ACSF at a rate of 2–3 ml/min at 23-25˚C afterwards. Patch pipettes (borosilicate glass capillary (1.5 mm O.D., 0.86 mm I.D)) were pulled with a PC10 two-stage vertical puller (Narishige, Japan). The pipettes' resistance was 3-6 MΩ when filled with an internal solution containing (in mM): 135 potassium gluconate, 10 KCl, 10 HEPES, 1 MgCl2, 2 Na2ATP and 0.4 Na2GTP. The pH of the internal solution was set to 7.3 by KOH, and the osmolarity was adjusted to 290 mOsm. Whole-cell patch-clamp recordings were performed using Multiclamp 700B amplifier equipped with Digidata 1320 A data acquisition board and pClamp 9 software (Axon, Molecular Devices, CA, USA). All recordings were done from CA1 pyramidal neurons in Current-clamp mode. The recordings were filtered at 5 kHz, sampled at 10 kHz and stored on a personal computer for offline analysis. The data were analyzed offline by using Clampfit version 11.2 (Molecular devices) and MATLAB 2016 software.

The passive electrical properties of the CA1 pyramidal neurons were measured by applying hyperpolarizing current pulses (-50 to -400 pA, 800 ms). The resting membrane potential was recorded after the initial break-in of the cell membrane. To obtain input resistance, current-voltage curve was drawn and its slope was measured as the resistance using first 4 sweeps (0 to -150 pA). Membrane time constant (tau) was evaluated by exponential fitting of capacitive voltage relaxation. Further, membrane capacitance was obtained by dividing the time constant by the input resistance.

Spontaneous activity was recorded and analyzed in a 60 seconds epoch. The firing regularity was quantified by the coefficient of variation (CV) of the ISI (inter-spike interval) which was calculated as the ratio of the standard deviation to the mean of ISI. The amplitude of AHP was measured from the threshold to the peak of the hyperpolarization following the action potential. To investigate the impact of kainic acid and 2-DG injection on rebound APs, a hyperpolarizing ramp current (1000 ms, -300 pA with a

slope of 0.345 pA/ms) followed by a depolarizing current pulse (100 pA for 300 ms) was applied. Burst activity was assessed in 125 seconds epochs. It should be noted that due to severe cell loss in CA1, finding healthy neurons was a daunting task as the remaining neurons were rather fragile. Consequently, sample size is rather small (see below). Even so, for the first time, as far as we know, in the intrahippocampal kainic acid model, we report the passive properties of the cell membrane and action potential (AP) properties of the dorsal CA1 pyramidal neurons, using whole cell Patch-clamp.

7. Histochemical Assessment

7.1. Nissl Staining

To show the brain injury induced by intrahippocampal injection of kainic acid, Nissl staining was performed. Following anesthesia (100 mg/kg ketamine and 10 mg/kg xylazine), transcardial perfusion was performed with saline and 4% paraformaldehyde, 1.33% picric acid in 0.1 M phosphate buffer (pH 7.4). Then, the mice were decapitated and brains were removed and post-fixed in the same fixative. To verify injection and electrode sites, the brains were cryoprotected in 20% sucrose buffer at 4°C overnight. Coronal sections (20 µm) containing the hippocampus were serially cut using a cryostat (Leica CM1850, Germany). However, to evaluate cell loss, the brain blocks were processed and embedded in paraffin and 8 µm sections were obtained using rotary microtome apparatus (Cut5062, Germany) and mounted on gelatin-coated slides. Nissl staining (0.1% Cresyl violet) was performed afterwards. To assess morphological properties of the CA1 pyramidal neurons (diameter of the soma), the long axis length of the soma was measured in the neurons containing visible nucleus, nucleolus, and primary dendritic cone (from the neck of the dendritic cone to the opposite pole of the soma) using a computer-based image analysis system (Olympus BX60, DP12, Olysia Soft Imaging System, Japan).

7.2. NADPH Diaphorase staining

Mice were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine, i.p) on the day 21st (epileptic and control groups) or 28th (epileptic+2-DG group) and were perfused transcardially with a cold fixative containing 4% paraformaldehyde and 1.33% picric acid in 0.1 M phosphate buffer (PB, pH 7.4) following 0.9% saline perfusion. The brains were then dissected out from the skull, post-fixed overnight in the same fixative at 4°C and cryoprotected by being immersed in 20% sucrose until they sank. The brains were freeze-sectioned coronally at 50 µm thickness, between the AP 1.2 mm and 2.4 mm posterior to the Bregma (Paxinos & Franklin, 2001) using a cryostat (Leica CM1850, Germany). NADPH-d staining was performed by incubating free-floating sections in the light-protected 0.1 M PB (pH 7.4) solution, containing 1 mg/ml nicotinamide adenine dinucleotide phosphate diaphorase (β-NADPH-d), 0.1 mg/ml nitroblue tetrazolium (NBT), and 0.3% Triton X-100 (all reagents were obtained from Sigma, St. Louis, MO, USA) at 37°C for 1-2h. The sections, then, were mounted on the gelatin-coated slides and cover-slipped with Entellan. Seven sections from the anterior-posterior axis of hippocampal CA1 area per animal were examined under light microscopy to localize NADPH-d^{+} neurons. The NADPH reactive cells were photomicrographed by the same Olympus microscope as mentioned above and manually counted.

8. Statistical analysis

SPSS 26 (IBM SPSS Statistics. Armonk, NY: IBM Corp) and GraphPad Prism 8 software (GraphPad, La Jolla, CA, USA, respectively) were employed to compare the data between groups and significance levels. One-way ANOVA and student's t-test were used to make a comparison between independent variables while the ANCOVA test was utilized to mask the effect of locomotion on anxiety-like behavior (see results and discussion). Pearson's test (or Spearman's test when a non-parametric test was needed) was employed to assess the correlation between the variables. Numerical data were expressed as mean ± standard error of the mean (SEM) and a value of $P < 0.05$ was considered as statistically significant.

Results

1. Behavioral Tests.

Epileptic animals showed increased locomotor activity, but 2-DG treatment was not able to reverse kainic acid-induced enhancement in locomotor activity in the epileptic group.

The epileptic and epileptic+2DG animals travelled a longer distance than the control group (2749 ± 224, 2276 ± 125.8, 4207 ± 379.7, 4140 ± 426.8 cm for control (N=8), control+2-DG (N=6), epileptic (N=8), and epileptic+2-DG (N=8) groups, respectively for all the behavioral tests unless noted, $P<0.05$ for both; Fig. 2 A). Furthermore, the velocity of the movement had a similar pattern (4.53 ± 0.38, 3.79 ± 0.20, 7.01 ± 0.63, 6.90 ± 0.71 cm/s, $P<0.05$ for both; Fig. 2B) indicating more locomotor activity in epileptic and epileptic+2-DG animals, and 2-DG treatment could not reverse the changes.

Epileptic animals expressed reduced anxiety-like behavior, 2-DG only slightly reversing the changes.

Less anxiety-like behavior in the epileptic animals compared to control group was extrapolated from more time spent in the open arms (43.1 ± 6.34, 23.68 ± 8.93, 115 ± 11.47, 109.8 ± 23.56 (N=7), $P<0.01$; Fig. 2C), more open arm entry (7± 1.52, 2.83 ± 1.01, 18 ± 2.68, 11.43 ± 3.21 (N=7), $P<0.01$; Fig. 2D), smaller latency of first entry to open arm (122.7 ± 31.55, 181.9 ± 39.45, 20.95 ± 5.91, 56.49 ± 20.02, $P<0.01$; Fig. 2E), and more head dipping frequency (23.56 ± 2.10, 20.17 ± 2.96, 40.56 ± 4.41, 49.25 ± 7.02, $P<0.05$; Fig. 2F). Nevertheless, body stretching frequency, which is deemed to reflect anxiety level the most [22] is not significantly different between the groups (14.58 ± 0.84, 16.5 ± 0.67, 18.9 ± 1.69, 20.25 ± 3.16; Fig. 2G). Even though 2-DG seems to have slightly reversed the alterations (number of open arm entry and latency of first open arm entry), the drug group cannot resemble the control group. 2-DG had a similar effect on control+2DG animals as it had on the epileptic animals (Fig. 2).

Increased locomotor activity seems to be the main player in the zero-maze test.

ANCOVA (analysis of covariance) revealed that the locomotion state of the animals affects the zero maze parameters as a covariate. Pearson's test (or Spearman's test) demonstrated a significant correlation between moved distance in the open field and all the zero maze parameters except for body stretching frequency; there was no interaction between the group and locomotor variables hence, a necessary

prerequisite of ANCOVA test was met. Masking locomotion's effect by the ANCOVA test, animals in the control, epileptic, and epileptic+2-DG showed no significant difference in the parameters of anxiety-like behavior but head dipping frequency (Table1).

Table 1

Correlation between locomotion status and zero maze parameters and the effect of masking locomotion's interference in zero maze results.

	Open-arm time	Number of open-arm entry	Latency of first open-arm entry	Head dipping frequency	Body stretching frequency
Correlation	r=0.588**	r=0.511*	r=0.39*	r=0.635**	r=0.224
ANCOVA test p-value	0.16	0.15	0.08	0.04	-
ANOVA test p-value	0.019	0.03	0.033	0.006	-

There was a significant correlation between locomotion status and the zero maze parameters except for body stretching frequency (the first row). ANCOVA test results revealed that there was no significant difference in the zero maze parameters between control, epileptic, and epileptic+2-DG animals after masking locomotion's interference (second row) while ANOVA test results had shown a significant decrease in anxiety status of the animals (last row; and figure 2). Head dipping frequency, however, was significantly different between the groups even after masking locomotion's effect. *P < 0.05, **P < 0.01.

Epileptic animals appeared to be depressed-like, 2-DG partially leading to more sweet water consumption.

The ratio of sweet water to tap water consumption was higher in the epileptic animals, being partially reversed in the epileptic group receiving 2-DG (9.57 ± 0.96, 8.68 ± 2.10, 3.82 ± 1.54, 6.25 ± 1.31, P<0.05; Fig. 2H). It is worth noting that depression-like behavior deduced from the result of this test suggests an all-or-none pattern. It is to say, each epileptic animal was either depressed (tap water consumption was much higher than sweet water consumption) or not-depressed (sweet water consumption was much higher than tap water consumption). 2-DG had no considerable effect in the control+2-DG group (Fig. 2H).

2. Electrophysiology

2-DG failed to suppress interictal sharp-wave complexes.

A preferential enhancement of glycolysis in the activated brain during diseases, like epilepsy, has been reported [4], and based on this, the antiepileptic effect of glycolysis inhibition has been proposed [5, 7, 9, 23]. Therefore, we next examined the electrophysiological consequences of 2-DG treatment, which

inhibits competitively glycolysis and prevents ATP production, in a kainic acid model of temporal lobe epilepsy.

13 hours of continuous LFP recording was conducted from the dorsal hippocampus of the epileptic animals. Sharp-waves were frequently seen in epileptic group while were never observed in control animals (Fig. 3A&B). analysis of these sharp-wave complexes revealed no significant difference in the ratio of total sharp-wave complex time (1.87 ± 0.71, 1.25 ± 0.32 in epileptic+2DG and epileptic+saline groups respectively; Fig. 3C), the ratio of mean sharp wave duration (1.15 ± 0.09, 1.03 ± 0.08; Fig. 3D), and sharp wave frequency (1.02 ± 0.07, 1.01 ± 0.04; Fig. 3E) between the Epileptic-2-DG (N=5) and Epileptic-Saline (N=5) groups; The ratios were obtained by division of the quantities taken in 21^{st} and 28^{th} days in each group ($28^{th}/21^{st}$). Although the parameters are reported for the whole 13 hours, the sharp-wave complexes were scrutinized in one-hour epochs during the whole 13 hours and no notable suppression was noted (data not shown). LFP results suggest that 2-DG is not able to suppress epileptiform activity at circuit-level.

Glycolysis inhibition by 2-DG cannot completely reverse epilepsy-induced cellular electrophysiological changes in the CA1 pyramidal neurons.

By applying hyperpolarizing currents (Fig. 4A) Patch-clamp Findings showed that resting membrane potential remained unchanged either following induction of epilepsy (-60.43 ± 2.424 in control (N=8); -59.12 ± 3.527, in the epileptic group (N=6)) or inhibition of glycolysis in the epileptic group (-60.37 ± 1.4 mV, epileptic+2-DG group (N=7); Fig. 4B). The membrane resistance was also not affected by intrahippocampal kainic acid injection (122.3 ± 18.59 MΩ in control vs epileptic group 119.2 ± 21.11 MΩ) and 2-DG treatment (105.1 ± 22.90 MΩ; Fig. 4C). However, the time constant was shorter in both epileptic and epileptic+2-DG groups (5.76± 1.26, $P<0.01$ and 5.12±0.80 ms, $P<0.001$, respectively; Fig. 4D) compared to control group (13.11 ± 1.47ms). Furthermore, the membrane capacitance was decreased in these groups (0.05±0.01 pF, $P<0.01$ and 0.06±0.01 pF, $P<0.05$, respectively; Fig. 4E) when compared to the control neurons (0.17±0.03 pF). Changes in membrane capacitance were associated with a significantly smaller cell size of survived pyramidal neurons (Figs. 4F, G&H) both in epileptic ((control group: 13.89 ± 1.24 µm (N=4) vs epileptic group: 8.45 ± 0.09 µm(N=4), $P<0.05$) and in the epileptic plus 2-DG group (8.7 ± 0.15 µm, $P<0.05$; Fig. 4I). As it can be seen in Nissl-stained sections, induction of epilepsy also led to a remarkable cell loss in the epileptic group and inhibition of glycolysis did not stop the loss of neurons in the epileptic group receiving 2-DG (Fig. 1D).

Whole-cell current-clamp recordings revealed that neurons from epileptic mice showed enhanced neuronal excitability (Fig. 5A) and exhibited a significant higher firing frequency (1.28 ± 0.42 Hz (N=5) in control neurons vs 4.93 ± 0.63 Hz (N=3), $P<0.05$; Fig. 5C), but treatment with 2-DG following induction of epilepsy reduced the neuronal excitability and firing frequency (2.18 ± 0.74 Hz (N=5), Figs. 5A&C).

Furthermore, although the amplitude of action potential was not affected by epilepsy induction when compared with the control group (80.74 ± 4.81 mV vs 85.16 ± 7.52 mV) inhibition of glycolysis in the

epileptic mice who received 2-DG resulted in a significant increase in the AP amplitude (105.4 ± 7.06 mV (N=4), P= 0.033; Fig. 5D). Induction of epilepsy following intrahippocampal injection of kainic acid led to a slightly slower depolarization phase of action potential (Control:1.09 ± 0.25 ms vs epileptic:1.28 ± 0.07 ms), as evidenced by an increased rising tau compared to control cells, but 2-DG treatment resulted in a significantly faster time constant of the rising phase of the action potential, as compared both to the control and epileptic groups (0.41 ± 0.05 ms, P<0.05; Fig. 5E). We also found that either induction of epilepsy or 2-DG treatment had no significant effect on the AP half-width (Fig. 5F). Nevertheless, due to afterdepolarization (ADP), which is only noted in the epileptic+2-DG group (amplitude=49.891±8.312 mV, decay tau=2.584±0.672 ms, area under curve=143.6±50.87 ms. mV; Fig. 5b), the duration of AP (measured at threshold voltage) was significantly increased both in epileptic and epileptic+2-DG groups compared to control group (4.19 ± 0.55, 9.81 ± 1.63, 16.15 ± 1.40 ms (N=4), in control, epileptic and epileptic+2-Dg groups, respectively, P<0.05; Fig. 5G). The amplitude of AHP did not differ significantly between the groups (Fig. 5H). Inhibition of glycolysis, but not induction of epilepsy alone, was accompanied by a significant increase in the coefficient of variation of interspike interval (ISI) when compared to either control (0.64 ± 0.04 vs 0.97 ± 0.11, P<0.05) or epileptic (0.60 ± 0.12, P<0.05; Fig. 5I) groups. This may indicate the irregularity of firing pattern of the CA1 pyramidal neurons in epileptic mice that received 2-DG.

The latency of the first post-inhibitory rebound spike, following a hyperpolarizing ramp current, was significantly shorter in the epileptic neurons than in control ones (34.19 ± 7.85, 5.36 ± 0.75 ms, P<0.01); likewise, it was significantly lower in epileptic+2-DG group compared to epileptic group (7.15 ± 0.99 ms, P<0.05, Fig. 5J&K).

To further assess the impact of 2-DG treatment on the firing pattern, we analyzed quantitative burst activity. Burst activity in hippocampal pyramidal neurons has already been demonstrated in epileptic cells [24, 25]. Here, neurons obtained from all the epileptic animals showed severe burst activity (Fig. 6A); in the epileptic+2-DG group, however, neurons from 2 of the 5 animals showed no burst activity; the other three animals showed attenuated burst activity compared with the epileptic animals (Fig. 6B), Even though the difference between the parameters did not reach to significance level; the number of bursts decreased by 61% (19 ± 3.60, 11.67 ± 2.33 for epileptic and epileptic+2-DG groups respectively; Fig. 6C), the mean duration of each burst diminished by 207% (4.46 ± 1.27, 1.45 ± 0.62; Fig. 6D), the mean AP number in each burst decreased by 71% (11.78 ± 4.98, 6.88 ± 2.13; Fig. 6E), and the mean pause between the bursts dwindled by 41% (1.99 ± 0.88, 2.812 ± 0.45; Fig. 6F). Patch-clamp results, hence, suggest that 2-DG could notably reverse the alterations that ensued from epilepsy induction at the cellular level. Moreover, it led to alterations in AP properties that did not assemble the control animals.

3. Histology

Kainic acid led to severe pyramidal and NADPH-d positive cell loss in the dorsal CA1 pyramidal layer.

Nissl staining showed that the integrity of the dorsal CA1 pyramidal cell layer was disrupted and remarkable cell loss, especially in CA1, was evident following intrahippocampal kainic acid

administration (Figs. 1C&D). In parallel, *NADPH histochemical staining* for nitrergic neurons revealed that the number of NADPH-d$^+$ cells in the pyramidal layer of dorsal CA1was significantly decreased in the intrahippocampal kainic acid injected animals when compared with the control group (11.27 ± 0.76, 4.93 ± 0.84 in control (N=4 mice, 28 sections) and epileptic (N=3 mice, 21 sections) groups respectively; P<0.05. Figs. 7A, a, B, b, C, c, D, d & E).

2-DG treatment increased NADPH-d$^+$ neurons in the dorsal hippocampus of control animals while failing to alter their number in the kainic acid-treated animals

In control+2-DG group, elevated NADPH-d$^+$ was observed compared with control group (20.71 ± 1.01 in the control+2DG group (N=4 mice, 28 sections); p-value < 0.001; Fig. 7E). In the epileptic group receiving 2-DG, however, no significant alteration in the number of NADPH-d-stained neurons (6.61 ± 1.50 (N=4 mice, 28 sections); p-value = 0.73; Fig. 7E) was noted compared with the epileptic group. In contrast, in the contralateral dorsal hippocampus of epileptic and the epileptic+2-DG group, the NADPH-d$^+$ cell number was approximately 3-fold more in comparison with the ipsilateral side (15 ± 1.375 in the epileptic group, P<0.05, and 13.07 ± 1.64 in the epileptic+2-DG group, P<0.05; Figs. 7F&G); Note that the number of NADPH-d$^+$ neurons in the contralateral hippocampus of these groups was even mildly more compared to control group. There was no notable difference between ipsilateral and contralateral hippocampus in control group (data not shown).

Discussion

Our behavioral findings showed decreased anxiety-like behavior as well as increased locomotor activity and depression-like behavior. It has been elucidated that there is a link between hippocampal sclerosis and depression-like behavior in epileptic models [15, 26]. In our model of temporal lobe epilepsy, hippocampal sclerosis is a well-known remark, as evidenced by a significant cell loss in the CA1 area of the hippocampus. This is consistent with the finding reported by [27] and [28]. Although a previous study showed no depression-like behavior in the mice model of intrahippocampal kainic acid using tail suspension and forced swimming tests [13], here, we report a significant depression-like behavior in the NMRI mice model of epilepsy induced by intra-hippocampal injection of kainic acid. Moreover, hippocampal lesions reduce anxiety-like behavior in rodents. Even so, a considerable controversy is faced when it comes to differentiating behavioral disorders, including anxiety, between the dorsal and/or ventral hippocampus of lesioned animals [29], [30]. Locomotor activity, however, is affected by such lesions especially dorsal hippocampus lesions [31]. Increased locomotor activity has already been reported in the intra-hippocampal kainic acid model of temporal lobe epilepsy [13]; the same study also reported no altered anxiety-like behavior in kainic acid-treated mice in the elevated plus-maze test.

Nonetheless, here, we explicitly show that both locomotor activity and anxiety-like behavior are altered in the epileptic group, as evidenced by increased locomotor activity and decreased anxiety-like behavior. Although cell loss is noted in the ventral hippocampus besides the dorsal part (kainic acid injection site), sclerosis is mostly seen in the dorsal hippocampus [32]. This fact raises a hypothesis about whether

increased locomotion due to dorsal hippocampal sclerosis has affected the zero-maze parameters. To test this hypothesis, we first attempted to see if there was a correlation between the locomotion state and each parameter of the zero-maze test. Interestingly, all the parameters except for body stretching frequency were significantly correlated to the locomotion state of the animals. Hence, using the ANCOVA test, we masked locomotion's effect on the parameters and the results were staggering; except for head dipping frequency, the other three parameters (open-arm time, number of open-arm entry, and latency of first open arm entry) did not reach to significance level between the three groups while ANOVA test results were indicating quite the opposite. All in all, at least to some extent, significantly reduced anxiety- like behavior in the kainic acid-treated animals compared with the control animals, was a result of augmented locomotor activity. Consistently, body stretching is deemed to be the most emotionally-driven posture of the animal in the zero-maze apparatus [22]; here we indicated that body stretching frequency is not affected by locomotion state and is not significantly different between the three groups.

In the present study, we show that 2-DG at the cellular level almost suppresses the electrophysiological alterations induced by intra-hippocampal injection of kainic acid, which produces one of the most drug-resistant epilepsy models with a striking resemblance to human epilepsy because of sclerosis seen in the hippocampus of the treated animals. However, at the neuronal circuit level, 2-DG seems to fail to suppress the sharp-wave complexes. This incident begs the question whether neuronal electrophysiological alterations induced by kainic acid injection are responsible for this incompetency of 2-DG. In this regard, Forte et al. [33] illuminated that 2-DG exerts its anticonvulsant effects through different mechanisms at the cellular and circuit levels; at the cellular level, K_{ATP} channels seem to play the major role while at the circuit level, suppression of epileptiform activity depends on GABA-A receptor activation. This activation is mediated by increased pentose phosphate pathway (ppp) as glycolysis enzyme phospho-fructo kinase is inhibited by 2-DG, and subsequently, upstream substrates are shifted to ppp. As a result of increased NADPH, neurosteroid production is augmented, leading to a GABA spillover outside the synaptic cleft, and subsequent tonic inward Cl^- current which exerts 2-DG's circuit-level anticonvulsive effects. Interestingly, the neurosteroids are produced in excitatory neurons only and inhibition of their production is shown to have abated the GABA-A mediated current. In the intrahippocampal model of temporal lobe epilepsy; however, a remarkable cell loss, particularly in dorsal hippocampus (kainic acid injection site), is noted. Hence, it could be inferred that severe cell loss mediated decrease in neurosteroids, and consequent disappearance of GABA-A induced current, leads to unsuppressed epileptiform activity following 2-DG injection; however, this needs to be further investigated in future studies.

Even though inhibited IPSPs are postulated to be the primary mechanism of hyperexcitability in surviving CA1 pyramidal neurons [34, 35], analysis of passive properties of CA1 neurons showed decreased membrane capacitance which leads to membrane tau decrement and hyperexcitability. The latency of rebound AP following a hyperpolarizing current indicates the neurons' excitability [36]; Consistently, rebound AP latency was shorter in epileptic animals in our groups. This capacitance decrease is likely to occur due to diminished cell size seen in the intra-hippocampal kainic acid-treated animals as we

demonstrated here. However, consistent with the previous reports, 2-DG did not alter the passive membrane properties of CA1 pyramidal neurons [7]. There posited to be a link between firing rate alteration following 2-DG injection and subsequent K_{ATP} channel activation due to ATP deprivation [37]. Here, we revealed that 2-DG can reduce firing rate in epileptic animals substantially. One probable explanation for the reduction in neuronal excitability induced by 2-DG treatment could be the prolonged AP duration and subsequent relative refractory period increment, which is likely to be a key player in burst activity suppression and decreased AP frequency throughout the recording.

Moreover, our findings showed a decrease in the AP rising tau as well as an increase in the AP amplitude and duration in the 2-DG- treated epileptic mice; With glycolysis attenuated by 2-DG, ATP level of the neurons will dwindle. Voltage-gated sodium and potassium channels which are responsible for AP production most, have been shown to be highly regulated by phosphorylation (e.g. protein kinase A and C). Phosphorylated KCNC4 channels are responsible for fast repolarization in neurons; Phosphorylated channels are more likely to be open at more negative potentials [38]. Therefore, with a reduction in the neuronal ATP content and the protein kinase activity, we might explain the prolongation of the action potential duration in the 2-DG received epileptic mice.

Next, we assessed whether induction of epileptic activity and glycolysis inhibition modify the NADPH-diaphorase activity, as a histochemical marker of the nitric oxide synthase (NOS), since there are several reports are confirming the role of nitric oxide system in the pathophysiology of mood disorders, including depression [39, 40], and epilepsy [41–43]. Reduced number of NADPH-d^+ cells in the dorsal hippocampus of i.p and i.c.v kainic-acid treated animals has already been reported [18], [44]. Here we show that NADPH-d^+ cells are smaller in number in the ipsilateral dorsal hippocampus CA1 in the intrahippocampal kainic acid model of epilepsy. Consistent with previous reports [45] however, in the contralateral dorsal CA1 of epileptic animals (and also epileptic + 2-DG group), NADPH-d^+ cells were slightly more frequent than the control animals. This could be a compensatory mechanism attempting to augment NO signaling in the contralateral hippocampus after decreased NADPH-d^+ cell number in the kainic acid-treated side. Although 2-DG was unable to increase NADPH-d^+ cell number in the drug animals, it led to an increase in number of NADPH-d^+ in control-2-DG received rats. It could be speculated that increased NADPH concentration in the interneurons owing to pentose phosphate pathway (PPP) potentiation following glycolysis inhibition by 2-DG leads to such augmentation. To explain more, increased NADPH will bring about increased reduction of nitro blue tetrazolium to diformazan (the visible dye) inside the neurons containing NADPH diaphorase which were not NADPH-d^+ when PPP and, subsequently NADPH concentration was low. Interestingly, NADPH-d^+ neurons in the hippocampus are demonstrated to release GABA too, indicating that NO acts as a paracrine/ retrograde co-transmitter [46, 44]. if so, loss of these GABAergic neurons could be a cause of disappeared IPSPs following kainic acid injection, that mentioned above. Additionally, it is recently argued that NADPH diaphorase activity in aldehyde-fixed tissue is not enzymatic rather, it is mediated by NO-containing factors which promote the reduction of nitro blue tetrazolium to diformazan [47].

In conclusion, while, at the cellular level, 2-DG treatment significantly reverses the electrophysiological alterations following epilepsy induction by intra-hippocampal kainic acid injection, it seems to be incompetent in suppressing circuit-level changes (as shown by interictal epileptiform activity). In behavioral part of our study, on the other hand, only partial improvement was noted which could be a direct impression of hypometabolism induced by 2-DG. Moreover, while glycolysis inhibition by 2-DG was associated with an increase in the number of NADPH-d^+ cells in control group, its application was unable to alter the NADPH-diaphorase activity, as did not change the number of NADPH-d^+ neurons in the epileptic animals, which may imply a severe NADPH-d^+ cell loss.

Declarations

Conflicts of interest

All authors have been substantially involved in the preparation of the present manuscript and no undisclosed groups or persons have had a primary role in the study. All authors have seen and approved the submitted version of the paper and accept responsibility for its content. The authors declare no conflict of interest and declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors Contributions

Khatibi and Rahdar: carried out the experiments, analyzed the data and wrote the draft of the article.

Rezaei, Davoudi, Nazari, Mohammadi: analyzed the data, wrote the Matlab codes.

Raoufy, Mirnajafizadeh, Behzadi and Hosseinmardi: helped supervise the project and critically reviewed the article.

Janahmadi: conceived, designed the experiments, supervised the research, wrote the paper with input from all authors.

Data availability

Data are available from the corresponding author on reasonable request.

Ethics approval

All experimental procedures and animal care were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Biomedical Research Ethics Committee of the National Institute for Medical Research Development (Approval ID: IR.NIMAD.REC.1399.259) and the Ethics Committee of Shahid Beheshti University of Medical Sciences (Authorization code: IR.SBMU.MSP.REC.1400.630).

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Figures

Figure 1

Verification of injection site, electrode site, and cell loss following kainic acid injection.

(A) the injection site of kainic acid or saline. (B) electrode location in LFP group animals. (C) normal dorsal hippocampus (control); (D) severe cell loss in CA1 and, to less extent, in CA3 as well as swollen dentate gyrus in kainic acid-treated hippocampus compared to the control hippocampus. Scale bar: 300 μm.

Figure 2

Decreased anxiety-like behavior as well as increased depression-like behavior in epileptic animals.

(A) and (B) indicates increased travelled distance and velocity of locomotion in epileptic (N=8) and epileptic+2-DG (N=8) groups respectively compared to the control (N=8) animals. Note that 2-DG was unable to reverse altered locomotion induced by kainic acid injection. (C) increased spent time in the open arms in epileptic and epileptic+2-DG groups compared to control group. (D) A significant increase in the number of open arm entries in epileptic animals; 2-DG partially reversed the alterations. (E) latency of first open arm entry was substantially lower in epileptic animals compared to the control group. The epileptic+2-DG group showed lower latency as well but did not reach the significance level. (F) head dipping frequency was higher in epileptic and epileptic+2-DG animals in comparison with control groups. (G) body stretching frequency was not significantly different between the groups. (H) Sucrose preference is shown to be diminished in epileptic animals, 2-DG partially reversing it. Notably, 2-DG led to a slight alteration in the test results in control+2-DG (N=6) group compared to the control animals. The bars represent the mean ± SEM. *P < 0.05, **P < 0.01.

Figure 3

Interictal epileptiform activity and 2-DG's circuit-level effect.

(A) and (B) 10 minutes epochs from control and epileptic animals respectively, indicating interictal epileptiform discharges (sharp-wave complexes) (five complexes are seen in this epoch). These complexes were never seen in control animals. The ratios of total sharp-wave complex time (C), sharp wave frequency (frequency of the discharges within a complex) (D), and sharp-wave duration (mean duration of each complex) (E) were not significantly different between epileptic+2-DG and epileptic+saline groups during 13 hours of recording. Epileptic animals (N=5) were recorded on the day 21^{th} (3 weeks after epilepsy induction), then received 2-DG for a week and recorded on the day 28^{th} again (epileptic+2-DG group). Epileptic-saline group (N=5) was added to figure out whether the increment in epileptiform activity following 2-DG injection was a result of 2-DG or progression of the pathology. The

ratios were obtained by dividing the data acquired on day 28th by the data on day 21th. Data are shown as mean ± SEM.

Figure 4

The electrophysiological consequences of Kainic acid and 2-DG treatment on the Passive properties of dorsal CA1 pyramidal cell membranes.

(A) hyperpolarizing currents to measure membrane resistance, capacitance and tau in control (upper) (N=8), epileptic (middle) (N=6) and epileptic+2DG (N=7) groups. resting membrane potential (B) as well as membrane resistance (C), were similar between the three groups. Membrane tau (D) and capacitance (E), however, were significantly lower in epileptic and epileptic+2-DG groups compared to control animals. The pyramidal layers of CA1 are shown in control (F), Epileptic (G), and epileptic+2-DG (H) groups. Note that only a small proportion of CA1 pyramidal neurons have survived following kainic acid injection (also see Fig. 1). (I) demonstrates the diminished size of the survived cells in epileptic and epileptic+2-DG groups. Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. Scale bar: 50 μm.

Figure 5

Spontaneous activity and action potential properties of dorsal CA1 pyramidal neurons.

(A) 15 seconds epochs from the spontaneous activity of control (upper) (N=5), epileptic (middle) (N=3), and epileptic+2-DG (N=5) groups. Note the high frequency of action potential firing in the epileptic group, being highly reversed by 2-DG. (B) superimposed APs from the three groups clearly indicating the alterations (see below). Note the afterdepolarization (ADP) only in epileptic+2-DG group (b). Dramatically increased frequency of AP firing (C) in a 60 seconds epoch in epileptic animals compared to the control group; 2-DG, however, reversed the changes substantially. (D) AP amplitude augmented significantly compared to both epileptic and control groups. AP rising tau was significantly lower in epileptic+2-DG group compared to control animals. Although not reaching to significance level, it was lower in epileptic group compared to epileptic+2-DG group as well (E). Even though AP half-width was much similar in the three groups (F), AP duration was significantly higher in 2-DG treated epileptic animals (G) owing to ADP noted only in the epileptic+2-DG group. (H) Afterhyperpolariziation, although being lower in the epileptic and epileptic+2-DG groups, did not reach to significance level between the three groups. (I) 2-DG led to increased irregularity compared to the epileptic and control groups. (J) latency of rebound APs is significantly smaller in epileptic and epileptic+2-DG groups compared to control animals. (K)

superimposed rebound APs following a hyperpolarizing ramp current. APs occurred after injection of +100 pA current following the ramp current (dashed line); the APs are shown with a different time scale to show the latency of APs following the injections. The bars represent the mean ± SEM. *P < 0.05, **P < 0.01.

Figure 6

Burst activity suppression by 2-DG.

(A) and (B) 15 seconds epochs from epileptic and epileptic+2-DG animals. In 2 out of 5 epileptic+2-DG animals burst activity was never seen. Mean burst number, mean AP number in each burst, mean burst time, and mean pause between two bursts did not reach to the significance level between the two groups in 125- second epochs (C, D, E, F). Nevertheless, the percentages of alterations were substantial (-61%, -207%, -71%, +41% respectively) indicating remarkable burst activity suppression by 2-DG (N= 3 in epileptic and epileptic+2-DG groups).

Figure 7

Distribution of NADPH diaphorase positive neurons in pyramidal cell layer of dorsal CA1 in control (A, a), control+2-DG (B, b), epileptic (C, c), and epileptic+2-DG (D, d) groups.

2-DG led to a significant increase in the number of NADPH-d^+ neurons in the control+2-DG group (N=4) compared to the control (N=4) group while failed to reverse severe NADPH-d^+ decrement induced by epilepsy induction (N=3 and 4 in the epileptic and epileptic+2-DG groups, respectively) (E). In the contralateral dorsal hippocampus, however, NADPH-d^+ cells were significantly higher compared to the ipsilateral dorsal hippocampus both in epileptic (F) and epileptic+2-DG (G) groups. Note that the number of NADPH-d^+ neurons is slightly higher in the contralateral CA1 of epileptic and epileptic+2DG animals compared to the control animals. The NADPH-d^+ cells are shown by the arrows. Data are shown as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. Scale bars: A 300 μm, B 100 μm.