Metabolic alterations predispose to seizure development in high fat diet-treated mice:

the role of Metformin.

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Abstract

The link between epilepsy and type 2 diabetes (T2DM) and/or metabolic syndrome (MetS) has been poorly investigated. Therefore, we tested whether a high fat diet (HFD), inducing insulin-resistant diabetes and obesity in mice, would increase susceptibility to develop generalized seizures induced by pentylentetrazole (PTZ) kindling. Furthermore, molecular mechanisms linked to glucose brain transport and the effects of the T2DM antidiabetic drug metformin were also studied along with neuropsychiatric comorbidities. To this aim, two sets of experiments were performed in CD1 mice, in which we firstly evaluated the HFD effects on some metabolic and behavioral parameters in order to have a baseline reference for kindling experiments assessed in the second section of our protocol. We detected that HFD predisposes towards seizure development in the PTZ-kindling model and this was linked to a reduction in glucose transporter-1 (GLUT-1) expression as observed in GLUT-1 deficiency syndrome in humans but accompanied by a compensatory increase in expression of GLUT-3. While we confirmed that HFD induced neuropsychiatric alterations in the treated mice, it did not change the development of kindling comorbidities. Furthermore, we propose that the beneficial effects of metformin we observed towards seizure development, are related to a normalization of both GLUT-1 and GLUT-3 expression levels. Overall, our results support the hypothesis that an altered glycometabolic profile could play a pro-epileptic role in human patients. We therefore recommend that MetS or T2DM should be constantly monitored and possibly avoided in patients with epilepsy, since they could further aggravate this latter condition.

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Introduction

A European cohort study has demonstrated that patients with epilepsy (PWE) are at higher risk of comorbid illnesses in comparison to the general population. Predominantly, this risk is increased in patients with uncontrolled epilepsy [1]. Recently, studies documented that patients with type 1 diabetes mellitus have a higher risk of developing epilepsy than the general population, although the underlying mechanisms remain unknown [2, 3]. In contrast, little is known about the risk of epilepsy in patients with type 2 diabetes (T2DM) and/or metabolic syndrome (MetS) [4]. Interestingly, some clinical and preclinical studies reported that hyperglycemia and/or obesity, which represent risk factors for T2DM, can precipitate seizures, particularly those of focal onset [5–9]. Furthermore, it has been well-established that several diseases associated with MetS and/or T2DM such as hypertension and stroke are also predisposing factors to epilepsy [10–12], whereas others such as cognitive impairment, anxiety and depression are well-documented comorbidities of both conditions [13–15].

On the other hand, some antiseizure medications (ASMs) can alter the metabolic milieu of the body; for example, weight gain and hyperinsulinemia are well known side effects of valproic acid, carbamazepine, gabapentin and other ASMs, although not all ASMs may have an impact on weight or may, as with topiramate, even reduce it [16–18]. Furthermore, metabolic disorders typically featured by insulin resistance, such as obesity, dyslipidemia, altered leptin/adiponectin ratio, total antioxidant status and others, are often associated with epilepsy [17, 19, 20]. Interestingly, antidiabetic drugs, with different mechanisms of action, have demonstrated beneficial effects in several animal models of epilepsy [21–24].

Glucose transporters (GLUT) are a family of transport proteins jointly acting to supply glucose in cells through a facilitated passive passage of glucose down its concentration gradient [25]. Specifically, GLUT-1 is present in several human cell types and it is the prominent glucose transporter at both blood brain barrier (BBB) level and astrocytes [26], while GLUT-3 is mostly

expressed in neurons [27, 28]. Astrocytes are contributors to brain glucose uptake and represent the dominant route for uptake during periods of intense synaptic activity [29]. In fact, as previously demonstrated, the expression level of these glucose transporters is regulated in concert with metabolic demand and regional rates of neuronal glucose consumption [30, 31]. An altered glucose transport across the BBB is linked to epilepsy, such as in the case of GLUT-1 deficiency syndrome (GLUT1-DS), due to a mutation of the SLCA1 gene that promotes refractory generalized seizures [32]. Metformin, an insulin sensitizing biguanide derivate, represents the first line oral hypoglycemic drug for the treatment of T2DM [33, 34]. Metformin exerts pleiotropic effects partly through of 5'-AMP-activated protein kinase (AMPK) and insulin receptor substrate-1 (IRS-1) related pathways [35, 36]. Interestingly, metformin has shown anticonvulsant effects in some animal models of epilepsy and has been considered a candidate for drug repurposing [23, 37–39].

Altogether, it seems reasonable to believe that insulin resistance, MetS and T2DM may have an influence on neuronal excitability and therefore on seizure development [4]. Therefore, we hypothesized that MetS linked to T2DM and obesity could increase seizure propensity and that metformin would be effective in reverting this phenomenon. Accordingly, we tested whether a high fat diet (HFD), inducing insulin-resistant diabetes and obesity in mice [31], would increase susceptibility to develop generalized seizures induced by pentylentetrazole (PTZ) kindling, a well-validated model of epileptogenesis and epilepsy, where hypertension is also a predisposing factor [40]. Furthermore, molecular mechanisms linked to glucose brain transport and metformin effects were studied along with neuropsychiatric comorbidities.

Materials and Methods

Animals

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Four-week old male CD1 mice ($n = 188$ were purchased from Charles River Laboratories s.r.l. (Calco, Lecco, Italia) and housed 3/4 per cage and kept under stable conditions of humidity ($60 \pm 5\%$) and temperature (22 ± 2 °C) with a 12/12-h reversed light/dark cycle (lights on at 20:00). Tap water and food pellets were provided *ad libitum* until the time of the experiments. In detail, mice were fed either a normocaloric diet (NCD; standard laboratory chow, Teklad Global Rodent Diets, ENVIGO RMS S.r.l, Italy) or a high fat diet (HFD) containing 59% kcal fat, 15% kcal protein and 25% kcal carbohydrate (Laboratory Dott. Piccioni, Milan, Italy). Procedures involving animals and their care were conducted in conformity with the international and national law and policies (EU Directive 2010/63/EU for animal experiments, ARRIVE guidelines and the Basel declaration including the 3R concept) and approved by the Animal Care Committee (Authorization n° 177/2019-PR) of the University of Catanzaro, Italy. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Experimental Summary

We planned two different sets of experiments: in *experiment #1* we aimed to determine HFD effects on some metabolic and behavioral parameters in the mice, in order to have a baseline reference for kindling experiments in the second section of our protocol. Male CD1 mice were randomly divided into two groups, fed with NCD ($n = 14$) and HFD ($n = 14$) for 8 weeks, respectively (Fig.S1). During this period, mice were weighed weekly and, at the same time, food and drink intake were measured for all animals until the end of the experimental protocol. At the end of the 8th week of dietary manipulation, both groups of mice were subjected to quantitative magnetic resonance (QMR). Subsequently, NCD and HFD mouse groups were randomly divided into two subgroups for type of fed diet ($n = 7$; Fig.S1) before being experimentally evaluated. In detail, the first subgroup, for both types of diet, was subjected to an intraperitoneal glucose tolerance test (IPGTT), whereas the second subgroup, for both types

of diet, was subjected to an insulin tolerance test (ITT). Furthermore, one week after IPGTT and ITT, the first subgroup of mice, for both types of diet, was used to perform the Morris water maze (MWM), whereas the second subgroups were used to carry out the forced swimming test (FST), open field test (OFT) and passive avoidance (PA) test (see Section Behavioral tests). As described above for *experiment#1*, to test seizure susceptibility and its related neuropsychiatric comorbidities in a mouse model of T2DM, as well as the effectiveness of metformin in reverting this phenomenon, CD1 mice (*experiment #2*), after being randomly divided into 2 groups, were respectively fed with NCD ($n = 80$) and HFD ($n = 80$) until the end of the experimental protocol (Fig.1). During the entire experimental period, mice were weighed weekly and, at the same time, food and drink intake were also measured for all animals. At the 7th week of dietary manipulation, mice were randomly divided, for both types of diet, into the following four groups ($n = 20$ in each group; Fig.1):

1) Control group (NCD Vehicle);

2) PTZ-kindling control group (NCD KINDL);

3) Control group treated with metformin (NCD+MET);

4) PTZ-kindling control group treated with metformin (NCD+MET KINDL)

5) High fat diet group (HFD Vehicle)

6) PTZ-kindling HFD group (HFD KINDL)

7) High fat diet group treated with metformin (HFD+MET)

8) PTZ-kindling HFD group treated with metformin (HFD+MET KINDL)

At this time point, the metformin-treated groups started oral drug treatment, up to the end of the experimental protocol, whereas the other untreated mice received vehicle. Metformin was administered orally at a dosage of 300 mg/Kg/day [41], by dissolving 300 mg in 100 ml of drinking water. This dosage was chosen based on the knowledge that mice drink, on average, 10 ml/100 g/day [42]. Subsequently, in order to evaluate the susceptibility to seizures, all

animals, in every group were intraperitoneally (i.p.) injected with either saline or PTZ every other day starting from the end of the 8th week of dietary manipulation (established T2DM in HFD fed mice) up to kindling development [42]. As previously reported, for every single experimental group, testing started 24h after the last PTZ-administration necessary to achieve the kindling criterion within the group [24]. Similarly, testing, for non-kindled groups, commenced contemporarily with their respective kindled control groups. In detail, when an experimental group was defined as kindled, after being weighed, was subjected to QMR (n=20/group). At the end of this procedure, in order to study glucose transporter protein levels and to perform several metabolic and behavioral tests, every experimental group was randomly divided into three subgroups (Fig.1). The first subgroup (n = 6 for both types of diet) was only used to collect brain samples in order to study glucose transporters (GLUT-1 and -3), whereas the second and the third subgroups (n = 7/group), for both types of diet, were subjected to an intraperitoneal glucose tolerance test (IPGTT) and to an insulin tolerance test (ITT), respectively. Furthermore, one week after IPGTT and ITT, the second subgroup of mice was used to perform the MWM, whereas the third subgroup was used to carry out the FST, open field test and passive avoidance test (see Section Behavioral tests).

Intraperitoneal glucose tolerance test (IPGTT) and insulin tolerance test (ITT)

Blood glucose levels were verified by intraperitoneal glucose tolerance test (IPGTT) and insulin tolerance test (ITT), as previously described [41]. Briefly, the IPGTT was performed in mice fasted for 6 hours, administering intraperitoneally (i.p.) glucose at 1 g/Kg body weight. Subsequently, blood samples were collected from the tail vein at 0, 30, 60, 90 and 120 min after glucose injection. Fasting blood glucose (FBG) levels were measured with an automatic glucometer (Glucomen LX, Menarini Diagnostics, Firenze). Likewise, the ITT was carried out after i.p. injection of insulin (1Units/kg body weight Regular®, Novorapid, Novonordisk,

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Rome, Italy), as previously described [43]. Briefly, blood samples were collected from the tail vein at 0, 30, 60, 90 and 120 min and FBG levels were assessed as reported above.

Development of pentylentetrazole kindling

Chemical-kindling was induced by a subconvulsive dose of PTZ (30 mg/kg/i.p.) administered every other day (between 9:00 and 11:00), as previously described [42, 44]. After PTZ injection, mice were placed in a single cage (100 cm wide x 100 cm x 50 cm high) and monitored for 30 min and seizures were scored according to the following scale: 0 = no change in behavior; 0.5 = abnormal behavior (sniffing, extensive washing, orientation); 1 = isolate myoclonic jerks; 2 = atypical (unilateral or incomplete) clonic seizures; 3 = fully developed bilateral forelimb clonus; 3.5 = forelimb clonus with a tonic component and twist of body; 4 = tonic-clonic seizure with suppressed tonic phase; only clonus of all limbs; 5 = fully developed tonic-clonic seizures. The maximum response was recorded for each mouse. Mice were considered fully kindled when exhibiting 3 consecutive stage 5 seizures [42, 44].

Behavioral tests

In order to reduce the number of animals used and avoid the effect played by different tasks in the same rodent, mice were divided as reported above (see Fig.1 and S1). Furthermore, when two tests were carried out on the same mouse, at least 1 day (range 1–3 days) was allowed as previously reported by [45]. All behavioral tests were conducted under controlled environmental conditions, including temperature, humidity, and light intensity (dim illumination), and with the support of video-tracking software (EthoVision XT8; Noldus Information Technology, Wageningen, the Netherlands) [21]. Experiments were always performed between 09:00 and 11:00 in order to avoid possible circadian alterations.

Furthermore, regarding treatment and dietary manipulation, when the same group of mice was subjected to multiple behavioral tests and/or repeated tasks of the same test, drug and diet were continued up to the end of the experimental protocol.

Forced swimming test (FST)

Despite some limitations, the FST is widely used to study depressive-like behavior in rodents [21]. We used a FST protocol previously validated in our and other laboratories [21]. Briefly, mice were placed individually (for 6 min) and forced to swim in a glass cylinder (height: 26.5 cm, diameter: 16.5 cm) containing 15 cm of water, maintained at 22-23°C [46]. The immobility time (IT), directly related with depressive-like behavior, including passive swimming, was assessed during the last 4 min of the test. The condition for passive swimming was floating vertically in the water while making only those movements necessary to keep the head off the surface of the water. After the test, mice were dried and then normally housed. Mean swimming velocity was also evaluated and analyzed.

Open-Field (OFT)

Locomotor performance was investigated for 10 min in an open-field (OFT) maze formed from a white 50 × 50-cm Plexiglas box with its floor divided into 9 squares. The central square was defined as the center of the box in which the mouse was placed at the beginning of the test, while the 8 squares along the walls were considered as the periphery. After each trial, the arena was systematically cleaned (70% ethanol) to remove olfactory cues. The OFT was performed as previously reported [47]. The parameters measured were the time spent in the center, the number of entries in the center, the total distance moved and the mean velocity during 10 min

of test. Diminished exploratory (locomotor) activity in the OFT is commonly considered as a measure of augmented levels of anxiety/emotionality and *vice versa* [47].

Passive Avoidance (PA)

Passive avoidance (PA) is used to study learning and memory in rodents [48]. Mice were tested in a step-through apparatus (Ugo Basile, Varese, Italy) measuring 47x18x25 cm, composed of a cage divided into two compartments (light and dark) by a sliding door. The PA test was performed over two consecutive days, as previously described [49]. Briefly, on day 1, mice were individually placed in the light compartment and allowed to freely explore the entire apparatus for 5 min (habituation trial). Subsequently, 15 min after the habituation, the conditioning or learning trial was started. In detail, mice were individually placed in the light compartment with the sliding door closed. Following 30 sec of delay, the sliding door that separates the two compartments was automatically opened. When the mouse entered into the dark chamber, an electrical foot shock (0.5 mA for 3 sec) was administered by the floor grid. The latency to enter (s) in the dark compartment was recorded and analyzed. After each trial, the apparatus was systematically cleaned to remove olfactory cues (70% ethanol). The retention trial was performed 24h later (day 2) by re-introducing the mouse into the light compartment. The mouse's memory was evaluated by recording the latency (s) to enter into the dark compartment; however, no foot shock was administered. The cut-off time for this session was 300 s. Retention memory is directly related to time taken to enter the dark chamber [49].

Morris Water Maze test (MWM)

The Morris water maze (MWM) test is widely used to investigate spatial learning and memory in rodents [50]. MWM was performed in a circular pool (diameter 93 cm , height =45 cm),

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divided into four quadrant and filled with water to a depth of 24 cm, as previously described [21]. An escape platform (8 cm in diameter) was submerged in the target quadrant, 1 cm below the water surface. On the walls, surrounding the pool, several visual cues were located. Mice were trained to find a hidden platform for 4 consecutive days with 4 sessions on each day as previously described [21]. During each session, mice were placed facing the pool wall in a chosen quadrant and each mouse was given 60 s to find the hidden platform; if a mouse failed to find it, it was guided gently to the platform and allowed to stay on it for 20 s. On the 5th day, after final acquisition, the platform was removed from the pool and a probe test was carried out for 60 s in order to test the mouse's retention memory. The time spent in the target quadrant and in all quadrants were recorded. Mean swimming velocity was also evaluated and analyzed for each group.

EchoMRI - quantitative magnetic resonance

Changes in fat mass composition in response to dietary manipulation were analyzed by quantitative magnetic resonance (QMR) (Echo MRI 700, Echo Medical Systems, Houston, USA) as previously described [41, 51]. Briefly, all mice groups, in each experimental protocol, between 9 and 11 a.m., were subjected to QMR. Total body fat, lean mass, free and total body water were quickly evaluated in a noninvasive manner. Each mouse was tested in duplicate, and readings were accepted if they differed by less than 10% [41]. After each session, the tube was systematically cleaned (70% ethanol) to remove olfactory cues.

Western Blot Analysis

Mice were decapitated, after 6h of fasting, and their brains were quickly removed and submerged in ice-cold artificial cerebrospinal fluid. Subsequently, about 350 mg of brain tissue

was homogenized in Buffer A (Hepes pH 7.9 10 mM, KCl 10 mM, EDTA 0.1 mM, EGTA 0.1 mM, DTT 1mM, PMSF 0.5 mM). Nuclear and cytoplasmic extracts were prepared as previously described [52]. For western blot analysis, cytoplasmic proteins (50 μg) were resolved on 10% SDS-PAGE, transferred to nitrocellulose membrane (0.2 μm Bio-rad cat.1620112). Membranes were first immersed for 2 h in blocking solution (5% non-fat dry milk), then probed with antibodies raised against GLUT-1 (Novus Bio, Centennials, CO) and GLUT-3 (Santa Cruz Biotechnology, Santa Cruz, CA). After overnight incubation at 4°C, goat-anti rabbit IgG horseradish (Dako) and anti-mouse IgG horseradish (Dako) conjugates were added for 1 h at room temperature, and immune complexes were visualized by enhanced chemiluminescence (ECL, Amersham). Western blots were normalized to beta-actin (GLUT-1) or tubulin (GLUT-3), as loading control proteins, and densitometric analysis was performed using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, Inc., La Jolla, CA 92037, USA). The two experimental protocols were separately analyzed and not compared. Data obtained from experiment #1 were analyzed and compared by *Student's t*-test. We used two-way-ANOVA followed by Tukey's post hoc test to analyze and compare data obtained from experiment #2. In detail, we have separately investigated data obtained from dietary manipulation in mice subjected to PTZ-kindling, with diet (two levels) and kindling (two levels) as factors. Two-way-ANOVA was also used to separately analyze and compare data obtained from metformin treatment in mice subjected to dietary manipulation, with diet (two levels) and treatment (two levels) as factors. The effects of metformin treatment in kindled mice were also separately assessed, for type of diet, using a two-way ANOVA with kindling

and treatment as factors. Data were expressed as means ± S.E.M. All tests used were to side and $p < 0.05$ was considered significant.

Results

Effects of dietary manipulation, kindling, treatment and their combinations on body composition

Not surprisingly, in both experimental protocols, HFD-fed mice had a significant weight gain, as a consequence of an increased percentage of body fat ($p < 0.01$; Fig. S2), compared to their respective NCD control group. At odds, no significant difference ($p > 0.05$) was noted on lean mass, or free and total body water (data not shown). Moreover, in both experimental protocols, there was no significant ($p > 0.05$) difference in food intake between NCD and HFD-fed mice groups; however, the amount of drinking water was significantly increased in HFD-fed mice groups in comparison to their respective NCD control group ($p = 0.03$; data not shown). Subsequently, in the experimental protocol#2, PTZ-kindling did not significantly ($p > 0.05$; Fig. S2) alter body weight and the percentage of body fat both in NCD and HFD fed mice groups. Likewise, PTZ-kindling did not alter drink and food intake in these experimental groups (data not shown). However, metformin was able to significantly ($p < 0.01$; Fig. S1 a, c) reduce body weight and the percentage of fat both in kindled HFD-fed mice and in non-kindled HFD-fed mice groups in comparison to their respective HFD untreated controls. Likewise, metformin was able to normalize drink and food intake in all HFD-fed mice groups (kindled and non-kindled mice). In contrast, no significant metformin effect on these parameters was noticed in all NCD mice groups (data not shown).

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Effects of dietary manipulation, kindling, treatment and their combinations on blood glucose levels

IPGTT and ITT analysis, performed after dietary manipulation in both experimental protocols, showed a significant ($p < 0.01$; Fig. S3) increase in FBG levels in HFD-fed mice groups in comparison to NCD-fed mice groups. Moreover, in the experimental protocol #2, kindled NCD and HFD-fed mice had significantly ($p < 0.001$; Fig. S3) higher FBG levels than their respective control groups. Metformin significantly ($p < 0.01$; Fig. S3b, d) reduced FBG levels both in kindled-HFD and in non-kindled HFD-fed mice groups in comparison to their respective untreated control groups. Likewise, it significantly ($p < 0.01$) ameliorated glucose tolerance, and insulin sensitivity, as assessed by IPGTT and ITT, respectively, in kindled NCD mice groups, with FBG levels being maintained similar to NCD-fed control mice. No significant difference in FBG levels was however, detected in NCD-fed mice treated with metformin ($p > 0.05$; Fig. S3b, d).

Development of pentylenetetrazol kindling

Kindled seizures were directly proportional and cumulative with repeated exposure to PTZ injections in all mice. None of the mice convulsed on the first injection, although HFD-fed mice kindled before NCD-fed mice ($p < 0.001$); metformin-treatment was able to significantly ($p < 0.01$; Fig.2) influence the kindling process in both NCD and HFD-mice groups. In particular, the 3rd stage 5 seizure was reached after 16 PTZ injections in the HFD group, after 19 PTZ injections in the HFD+MET group, after 21 PTZ injections in the NCD+MET group and after 22 PTZ injections in the NCD group (Fig. 2). No mortality was observed during the testing period for every group.

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Effects of dietary manipulation, kindling, treatment and their combinations on depressive and anxiety-like behavior: forced swimming test (FST) and open field test (OFT)

Post-hoc analysis revealed that there was no significant difference ($p > 0.05$) in the IT among experimental mice groups, in both experimental protocols, after dietary manipulation (Fig. S4b, c). Regarding the OFT, in the experimental protocol #1, no significant difference was observed between the NCD and HFD-fed mice groups (Fig. S4a). At odds, in the experimental protocols #2, HFD-fed mice had a significant reduction in the time spent in the center ($p < 0.0001$; Fig.3a) and in the number of entries in the center ($p = 0.0003$; Fig. 3b) in comparison to NCD-fed mice, showing an anxious-like behavior. Moreover, PTZ-kindling did not significantly ($p > 0.05$) modify these parameters in HFD-mice, whereas it was able to significantly reduce the time spent in the center and the number of entries ($p = 0.0001$ and $p = 0.0008$, respectively) in NCD-fed mice. Metformin treatment was significantly able to increase these two parameters both in kindled ($p = 0.0001$ and $p = 0.042$, respectively) and non-kindled ($p = 0.0001$ and $p = 0.047$, respectively) HFD-fed mice groups in comparison to their respective untreated control groups, reducing anxiety-like behavior. Likewise, metformin increased significantly these parameters in kindled ($p = 0.0001$ and $p = 0.004$, respectively) NCD-fed mice, with anxiety-like behavior being maintained similar to NCD-fed control mice. No significant ($p > 0.05$) difference was observed among metformin-treated NCD-fed mice groups and metformin-treated HFD-fed mice groups. Mean velocity and total distance moved did not significantly differ ($p > 0.05$) among groups in the OFT, supporting the absence of motor impairment.

Effects of dietary manipulation, kindling, treatment and their combinations on learning and memory performance: passive avoidance (PA)

Learning and memory decline were tested in the PA. After 8 weeks of dietary manipulation (Fig. S5a) no significant difference in cognitive performance was detected between mice

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groups. In contrast, in the experimental protocol #2 (Fig. 4a), the HFD-fed mice group had a significantly ($p = 0.006$) shorter latency (s) to enter in the dark compartment in comparison to the NCD-fed control group, suggesting a cognitive impairment (Fig. 4a). PTZ-kindling did not significantly ($p > 0.05$) modify the latency to enter in the dark compartment neither in NCD-fed mice nor in HFD-fed mice in comparison to their respective control mice groups. At odds, metformin was significantly able to increase the latency to enter into the dark compartment in both treated (kindled and non-kindled) HFD-fed mice groups in comparison to their respective control untreated groups (HFD vs HFD+MET, $p = 0.017$; HFD KINDL vs HFD+MET KINDL, $p = 0.0049$; Fig. 4a). No significant ($p > 0.05$) difference was observed among NCD-fed mice groups.

Effects of dietary manipulation, kindling, treatment and their combinations on learning and memory performance: Morris water maze (MWM)

Post-hoc analysis revealed that after 8 weeks of dietary manipulation, there was no significant difference, neither in the latency to find the platform nor in the probe trial between NCD and HFD-fed mice groups, indicating no deterioration of spatial learning and memory (Fig. S5b, c). At odds, in the experimental protocol #2, both kindled and non-kindled HFD fed mice groups showed a significant ($p < 0.0001$; Fig. 4b) increase in the latency to find the platform and they spent significantly ($p < 0.0001$; Fig. 4c) less time in the target quadrant (s) in comparison to their respective NCD control groups. Moreover, PTZ-kindling did not significantly alter cognitive performance neither in NCD nor in HFD-fed mice groups, with these parameters being maintained similar to their respective control levels. It is noteworthy, that metformin treatment significantly ($p < 0.0001$; Fig. 4b) decreased the latency to find the platform both in kindled and non-kindled HFD-fed mice groups, preventing cognitive decline. Likewise, metformin significantly ($p < 0.0001$; Fig. 4c) increased the time spent in target quadrant in both

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mice groups. No significant difference ($p > 0.05$) was observed for other groups. The mean swimming velocity did not change ($p > 0.05$) among experimental groups, supporting the absence of motor impairment.

Effects of dietary manipulation, kindling, treatment and their combinations on GLUT-1 and -3 expression

The expression of the glucose brain transporters GLUT-1 and GLUT-3 in whole brain, were quantified, only in the experimental protocol #2, by Western blot analysis. In detail, HFD-fed mice showed a significant ($p < 0.0001$; Fig. 5a) reduction of GLUT-1 brain expression in comparison to NCD-fed mice. PTZ-kindling was able to significantly ($p = 0.002$; Fig. 5a) reduce GLUT-1 expression levels in kindled NCD-fed mice in comparison to NCD-fed control mice. Conversely, GLUT-1 expression was significantly ($p < 0.0001$; Fig. 5a) increased in kindled HFD-fed mice in comparison to both non-kindled HFD-fed mice and kindled NCD-fed mice. Metformin treatment was able to normalize GLUT-1 expression in HFD-fed mice groups. In detail, metformin treatment significantly reduced GLUT-1 protein levels in kindled HFD mice whereas it was able to significantly increase GLUT-1 protein in HFD fed mice in comparison to their respective control untreated groups ($p < 0.0001$; Fig. 5a). Surprisingly, metformin-treated NCD-fed mice had a significantly lower GLUT-1 level than untreated NCD-fed mice. Moreover, a slight, not significant ($p > 0.05$; Fig. 5a) increase of GLUT-1 protein levels was observed in the kindled NCD mice group after metformin treatment.

The GLUT-3 expression level was significantly ($p < 0.0001$; Fig. 5b) increased in the HFD-fed mice group in comparison to the NCD-fed mice. PTZ-kindling was also able to significantly increase GLUT-3 expression both in NCD and HFD-fed mice groups ($p < 0.0001$; Fig. 5b) in comparison to their respective non-kindled control mice. Metformin treatment significantly reduced ($p < 0.0001$; Fig. 5b) GLUT-3 expression in the kindled HFD-fed mice group, with the

protein level being maintained similar to non-kindled HFD-fed mice. At odds, metformin did not change GLUT-3 expression levels in non-kindled HFD-fed mice. Furthermore, metformin significantly $(p = 0.022;$ Fig. 5b) reduced GLUT-3 expression in the kindled NCD-fed mice group in comparison to untreated kindled NCD-fed mice, whereas it significantly ($p = 0.015;$ Fig. 5b) increased protein levels in NCD-fed mice. No significant difference on protein levels was observed after metformin treatment in the HFD-fed mice group.

Discussion

In accordance with our initial hypothesis, we have confirmed that HFD, by inducing changes linked to metabolic syndrome such as diabetes and obesity, predisposes towards seizure development in the PTZ-kindling model in mice and this is apparently linked to a reduction in GLUT-1 expression, as observed in GLUT1-DS in humans [53] and accompanied by an increased expression of GLUT-3. Furthermore, while we have confirmed that HFD also induces neuropsychiatric alterations in the mice (*e.g.* cognitive impairment, anxiety), it did not modify the development of kindling comorbidities [54, 55]. Finally, confirming the known anti-seizure efficacy of metformin in animal models of epilepsy [39], we observed that its effects were apparently linked to a standardized normalization of both GLUT-1 and -3 expression, which is observed in either NCD or HFD groups.

It is known that a fat-enriched diet gives rise to obesity, T2DM and/or MetS in several rodent strains, reproducing pathophysiological similarity to human medical conditions [56]; however, the metabolic responses may vary according to the specific rodent strain and duration of the high-fat diet exposure [57, 58]. In our experiments, we observed that, after 8 consecutive weeks of dietary manipulation, HFD-fed mice had both a significant weight gain (severe obesity) and worsening of their glycometabolic profile accompanied by an increased percentage of total body fat and an increased blood glucose level linked to insulin resistance. To note, we did not

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measure blood pressure and serum lipids to respectively evaluate hypertension and dyslipidemia in the mice (which was beyond the main aims of the present experiments) and therefore, we cannot meet the clinical criteria of MetS definition. However, this is debated in preclinical studies and these are considered valid models of MetS [57, 58]. Notably, in our study, after 8 consecutive weeks of fat-enriched diet manipulation in mice, we did not notice any significant difference in all our behavioral tests in contrast to previous observations [13, 31, 41]; however, this is likely due to the duration of HFD. In fact, as previously described for dementia in an experimental model of T2DM [59], we can suppose that a decline of metabolic brain homeostasis precedes and does not accompany neuropsychiatric complications [60]. In agreement, we found that HFD-fed mice showed neuropsychiatric-like comorbidities only after a long-term maintenance of fat-enriched diet (second part of experimental protocol). Finally, we confirmed that HFD-fed mice had a reduced expression of GLUT-1 and an increased expression of GLUT-3, in comparison to NCD controls [61, 62]. Interestingly, alterations of GLUT-1 expression levels have often been reported during cognitive impairment and mood disorders [63–66], therefore we can also hypothesize that any neuropsychiatric modifications we observed may be linked to a deficit of GLUT-1 [62]. Furthermore, null heterozygous GLUT-3 mice show several behavioral features suggesting autism spectrum disorders as well as electrographic discharges, but no clinical generalized seizures; however, GLUT-3 mutations would not seem to be related to generalized epilepsy in humans [67]. On the other hand, an impaired glucose transport through the BBB results in subjects with GLUT1-DS, a genetic neurometabolic disorder linked to a mutation in the GLUT1 (encoded by the SLC2A1 gene), characterized by severe infantile seizures, developmental delay, cognitive impairments and microcephaly [53, 67].

Based on this background, we believe that the observed higher susceptibility to develop PTZ-kindling of HFD-fed mice may be linked to GLUT1-DS, while the observed modifications in

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GLUT-3 expression may be a compensatory mechanism to maintain cellular glucose intake. This is in agreement with previous observations indicating that cerebral glucose demand and its consumption are higher in epileptic brain, due to the increased neuronal excitability, than other brain activities [68, 69]. This higher propensity to develop chronic seizures by PTZ-kindling are in keeping with both clinical [5, 6] and preclinical evidence [7, 9, 70, 71], reporting that T2DM and its main drivers, such as hyperglycemia and/or obesity, can often precipitate seizures [4]. Despite methodological differences, these findings are partially consistent with previous findings demonstrating that the increase in brain metabolic energy demands during seizures experimentally induced by kainate and PTZ administration, lead to an increased GLUT-1 and GLUT-3 protein abundance in several brain regions [72]. Notably, the accompanying behavioral modifications in PTZ-kindling are not different between NCD and HFD-fed mice, indicating that this latter treatment does not apparently have an impact on comorbidity development.

In addition, we have also confirmed that metformin may ameliorate both the metabolic profile and several behavioral deficits linked to diabetes [41, 73, 74] as well as significantly decreasing seizure susceptibility in the PTZ-kindling model, thus showing *antiepileptogenic* effects [24, 75]. Furthermore, we observed that metformin treatment led to a renormalization of GLUT-1 and GLUT-3 expression levels and this could contribute to it antiepileptic properties. However, other mechanisms possessed by this drug may also have contributed to its effects [39]. For example, these beneficial effects could be due to metformin pleiotropic properties (*e.g.* antidiabetic, anti-inflammatory, antioxidative and neuroprotective effects), which have led to the suggestion that this drug can also be useful as add-on therapy, to manage several neurological diseases including epilepsy [76, 77].

Conclusions

Overall, our results confirm the hypothesis that an altered glycometabolic profile could play an epileptogenic role. In agreement with our data, it is interesting to hypothesize that metformin could be a potential and promising drug particularly for patients who have concurrent conditions of T2DM and epilepsy. Interestingly, other antidiabetic drugs, with different mechanisms of action, have demonstrated beneficial effects in several animal models of epilepsy and epileptogenesis [21, 22, 78], suggesting a link between these two disorders. Despite this, to date, the relationship between epilepsy and T2DM is poorly understood and suffers from a lack of epidemiological evidence and continued biomedical research efforts [4]. Moreover, based on the multifactorial etiology of T2DM and epilepsy [79, 80], the causes behind this relationship might be many and various, warranting for further preclinical and clinical studies. In particular, these studies should also clarify what diabetic drivers (*e.g.* obesity, hypertension, hyperglycemia) are more likely predisposing towards seizure onset. To notice, recent studies have highlighted the contribute of gut inflammation and the consequent dysbiosis in both these pathologies [81, 82], therefore future studies should also focus on the potential role of peripheral inflammation and microbiome in these conditions [83]. Furthermore, another question that deserves to be addressed is whether epilepsy represents a cause and/or a consequence of diabetes. Interestingly, it has been reported that common mechanisms such as mitochondrial dysfunction and dysregulation of energy demand predispose people towards seizure development, obesity and diabetes [4, 31]. In ours model, the increased seizure susceptibility and neuropsychiatric comorbidities could arise independently and separately from the same brain network abnormalities linked to diabetes. Finally, our data indicate that MetS or T2DM should be constantly monitored and possibly avoided in patients with epilepsy, since they may further aggravate this latter condition.

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Acknowledgments

Author Contributions

Conceived and designed the experiments: ER, AL, AB. Performed the experiments: VN, AL, MT, BA. Analyzed the data and wrote the paper: AL, ER, VN, AC. Commented on the paper and provided feedback on the discussion: GDS, RC. Corrected and modified the manuscript: all authors.

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Compliance with Ethical Standards

Ethical Approval

The experimental protocols and the procedures reported here were approved (Authorization n° 177/2019-PR) by the Animal Care Committee of the University of Catanzaro, Italy.

Conflict of interest

The authors declare that there are no conflicts of interest to be disclosed.

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Figure Captions

Fig. 1 Scheme of the experimental protocol (# 2) used in this study. HFD = High fat Diet; NCD = normocaloric Diet; PTZ = pentylentetrazole; QMR = quantitative magnetic resonance; IPGTT = intraperitoneal glucose tolerance test; ITT = insulin tolerance test; FST = forced swimming test; OFT = open field test; PA = passive avoidance; MWM = Morris water maze

Fig. 2 Pentylentetrazole kindling development. Progression of kindling in four groups (metformin-treated and untreated NCD and HFD-fed mice) showing a faster development in HFD-fed mice in comparison to NCD- fed mice ($p < 0.001$). Metformin treatment in both groups of mice reduced seizure propensity. Data marked with * and # are significantly different ($p < 0.01$ and $p < 0.001$, respectively) from control mice

Fig. 3 Open field test performed, at the end of PTZ-kindling procedure (experimental protocol #2), in mice. Bars indicate a) the time spent in the center expressed in seconds (s), b) number of center entries. Data marked with * are significantly ($p < 0.01$) different from NCD-fed control mice, ° significantly ($p < 0.01$) different from NCD KINDL, and §, # are significantly ($p < 0.01$) different from HFD and HFD KINDL mice. Values are means ±S.E.M. NCD = normocaloric diet group; NCD KINDL = kindled normocaloric diet group; NCD+MET = metformin-normocaloric diet group; NCD+MET KINDL = metformin kindled-normocaloric diet group; HFD = high fat diet group; HFD KINDL = kindled high fat diet group; HFD+MET = metformin- high fat diet group; HFD+MET KINDL = metformin kindled-high fat diet-treated group

Fig. 4 Passive avoidance and Morris water maze tests, performed, at the end of PTZ-kindling procedure (experimental protocol #2), in mice. Passive avoidance: a) Bars indicate the latency to enter into the dark chamber expressed in seconds. Morris water maze test: b) Learning curve (latency to platform) over 4 consecutive days; c) performance in the probe trial on the 5th day. Data marked with *,° significantly ($p < 0.01$) different from controls and §,# significantly ($p < 0.01$) different from HFD and HFD KINDL. Values are means ±S.E.M. NCD = normocaloric diet group; NCD KINDL = kindled normocaloric diet group; NCD+MET = metformin-normocaloric diet group; NCD+MET KINDL = metformin kindled-normocaloric diet group; HFD = high fat diet group; HFD KINDL = kindled high fat diet group; HFD+MET = metformin- high fat diet group; HFD+MET KINDL = metformin kindled-high fat diet-treated group

Fig. 5 Western Blotting experiment panel on GLUT-1 and GLUT-3 expressions. a) Columns represent mean relative protein levels of GLUT-1 normalized versus actin. b) Columns represent mean relative GLUT-3 protein levels expression normalized versus tubulin. Data marked with * are significantly ($p < 0.01$) different from NCD, ** are significantly different from HFD ($p < 0.01$) and §, # significantly ($p < 0.05$) different from NCD+MET and HFD+MET. Values are means ±S.E.M of n = 6 experimental replicates. NCD = normocaloric diet group; NCD KINDL = kindled normocaloric diet group; NCD+MET = metformin-normocaloric diet group; NCD+MET KINDL = metformin kindled-normocaloric diet group; HFD = high fat diet group; HFD KINDL = kindled high fat diet group; HFD+MET = metformin-high fat diet group; HFD+MET KINDL = metformin kindled-high fat diet treated group