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Research report

Ketogenic diet prevents neuronal firing increase within the substantia nigra during pentylenetetrazole-induced seizure in rats

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ABSTRACT

The mechanism responsible for the anti-seizure effect of ketogenic diets is poorly understood. Because the substantia nigra pars reticulata (SNr) is a "gate" center for seizures, the aim of the present experiment was to evaluate if a ketogenic diet modifies the neuronal response of this nucleus when a seizure-inducing drug is administered in rats. Two groups of rats were given a standard diet (group 1) or a ketogenic diet (group 2) for four weeks, then the threshold for seizure induction and the firing rate of putative GABAergic neurons within the SNr were evaluated with progressive infusion of pentylenetetrazole under general anesthesia. The results demonstrated that the ketogenic diet abolished the correlation between the firing rate response of SNr-neurons and the seizure-threshold. This result suggests that the anti-seizure effect of ketogenic diets can be due to a decrease in reactivity of GABAergic SNr-neurons.

1. Introduction

Ketogenic diets (KD) are acknowledged for their anti-epileptic efficacy. Many different hypotheses have been advanced, but its mechanism of action is still poorly understood (Masino and Rho, 2012; Lutas and Yellen, 2013; Rho, 2015). Because ketone bodies are elevated during KD, many studies have been focused on a potential anti-seizure effect of ketone bodies. In previous studies it has been tried to use synthetic-ketones to replicate the effects of a KD (D'Agostino et al., 2013; Viggiano et al., 2015). On the other hand, it has been demonstrated that calorie restriction is another relevant factor to obtain anti-seizure effects (Bough, 2008; Viggiano et al., 2016). Besides the identification of the anti-seizure molecules contained in KD or metabolically induced by KD, it is of great interest to identify the anatomical structure or the neuronal population within the central nervous system that is affected by KD in such a way that give rise to an anti-seizure effect. In a previous experiment it has been demonstrated that ketone bodies affect in vitro the spontaneous firing rate of neurons within the substantia nigra pars reticulata (SNr; Ma et al., 2007), which is considered a "gate" center for seizures (Iadarola and Gale, 1982; McNamara et al., 1984; Gale, 1986; Depaulis et al., 1994). The aim of the present work was to evaluate the role of the SNr in the anti-seizure effect of KD in a whole animal model, as previously described (Viggiano et al., 2015). The model consisted of a continuous infusion of the seizure-inducing drug pentylenetetrazole (PTZ) in anesthetized rats, until seizure discharges appear in the cortical-electroencephalogram (cEEG). A treatment is thus considered to have an anti-seizure effect if a higher amount of PTZ is necessary to produce a seizure discharge. In the present experiment, the unit activity of neurons within the SNr was also recorded; in fact, there is typically an increase in the firing rate of SNr-neurons during a PTZ infusion. The aim was to verify if the KD treatment modified such PTZ-induced changes in the firing rate of the SNr-neurons.

2. Experimental procedures

The experimental design consisted of a simple two groups design which differed only for the diet; the control group was fed with standard a diet while the treated group was fed with a ketogenic diet. At the end of the treatments all rats were evaluated

Abbreviations: KD, ketogenic diet; PTZ, pentylenetetrazole; βHB, β-hydroxybutyrate; cEEG, cortical-electroencephalogram; SNr, substantia nigra pars reticulata.

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for the PTZ-threshold for seizure induction and for the changes in SNr-neurons activity due to PTZ.

2.1. Animals

Ten male Wistar rats (Harlan Laboratories, Udine, Italy), age P30 and weighting 105 ± 16 g (mean ± S.D.), were randomly divided into two groups. Rats were housed in standard temperature and humidity conditions with a 12 h light–dark cycle (lights on from 07:00 a.m. to 07:00 p.m.). Animals belonging to group 1 were given free access to standard food and tap water. Animals belonging to group 2 were, instead, given a calorie-restricted KD as described elsewhere (Bough and Eagles, 1999); briefly, they were given each day approximately 90% of the calculated daily calorie-requirement by a food mix composed of more than 78% fat (Bio-Serv cF3666, Frenchtown, NJ, USA). Rats were fed with the above diets for four weeks, then they undergone the surgical procedure. All procedures fulfilled the requirements of the European Communities Council Directive of 22 September 2010 (2010/63EU). Animals were kept under general anesthesia with urethane during all surgical procedures and seizure induction; the research was approved by the local ethical committee of the University of Salerno and by the Ministry of Health of the Italian Government.

2.2. Surgery

The day before the experiment, at 19:00, food was removed from the cages of all the animals; this assured to have all the animals in the same acute fasting conditions and permitted to ascribe the results to the effects of the prolonged KD-treatment and not to those of an acute calorie restriction of the last day.

On the day of the experiment, the rats were anesthetized with 1.5 g/kg b.w. of urethane (Sigma-Aldrich, Milano, Italy). From this time on, body temperature was maintained at 36.5 ± 0.5 °C with a thermostated heating pad. The femoral vein was then accessed by inguinal incision and insertion of a polyethylene catheter (outer diameter 0.6 mm) to a depth of 6 cm. Placement of electrodes to record cEEG was achieved by percutaneous incision of the scalp and osteotomy of the calvarium. Two holes (1.4 mm in diameter) were drilled on the skull at the following coordinates: first hole at 2 mm lateral from midline and 2 mm anterior from bregma; second hole at 2 mm lateral from midline and 2 mm posterior from bregma. Two stainless steel screws (1.6 mm in diameter; Novara Metalli, Novara, Italy) were placed into these holes to a depth of 2 mm from the skull surface and served as recording electrodes. A safety pin, the reference electrode, was placed subcutaneously in the posterior of the neck. A third hole was drilled at the following coordinates: 5.2 mm posterior from bregma, 2.6 mm lateral from midline. A custom made tetrode attached to a microdrive was then lowered through the third hole to a depth of 7.0 mm from the skull, and served to reach and record a single neuronal spontaneous firing activity within the SNr (expected within 7.8 mm and 8.8 mm in dept; Paxinos and Watson, 1997). The tetrode consisted of four twisted, insulated nichrome-wires (0.001 in. in diameter, AM-Systems, Sequim, WA, USA). After the stereotaxic placement, the microdrive was secured to the other screws with dental cement.

2.3. cEEG and single neuron activity recording

The recording and reference electrodes were connected to a custom made digital amplifier (Viggiano and Coppola, 2013) with minor modifications; the amplifier provided a gain of 5000 V/V, a band pass filter from 1.6 Hz to 100 Hz, and an analog-to-digital conversion with a resolution of 14 bits and a sampling frequency of 9000 samples/s (NI_USB-6009 OEM, National Instruments, Austin, Texas, USA). The digital readings were sent to a PC through a standard USB port; a custom software written with LabView (National Instruments, Austin, Texas, USA) provided real time visualization and recording of the cEEG and the tetrode signals for subsequent analysis and allowed manual marking of the events of interest (e.g. perfusion start and perfusion stop).

After placement of all the electrodes, as described in the previous section, the tetrode was slowly advanced vertically by turning a miniature screw on the microdrive. By this way, the tetrode was lowered in steps of 10 μm, checking at each step for the presence of an appreciable single-unit activity. The lowering was stopped when a single neuron signal was found having a spontaneous firing rate of 10–40 spikes/s, a biphasic shape and a duration of 0.6–1.5 ms. Such kind of signal is supposed to pertain to GABAergic neurons (Gernert et al., 1999). The electrophysiological recording was started in all cases at 60 min after the initiation of anesthesia. After a basal recording of 10 min, blood glucose (GlucoMen LX plus, Menarini Diagnostics, Firenze, Italy) and blood β-hydroxybutyrate (βHB; Freestyle Optium, Abbott, Rome, Italy) were determined by tail prick; the femoral catheter was then connected to a syringe-pump prefilled with 100 mg/ml of PTZ (Sigma-Aldrich, Milano, Italy), and PTZ infusion was started at a rate of 0.25 ml/min. When a sustained high frequency- and high amplitude-seizure discharge was evident (greater than 0.5 mV from the zero level and lasting more than 5 s), the infusion was interrupted and recording was maintained for an additional 30 min.

For each cEEG recording, the onset-time of the seizure discharge was measured, defined as the time from the infusion start to the first of the above-threshold peaks; above-threshold peaks were defined by having an amplitude greater than 0.5 mV, with inter-peak latency shorter than 1 s (Fig. 1). For each rat, the PTZ-threshold for seizure induction was defined as the injected dose of PTZ at the onset-time of the seizure discharge (mg/Kg b.w.).

For each recording the mean firing rate was calculated: (1) during the basal period and (2) during the period from PTZ infusion start until the onset-time of the seizure discharge, but not during the seizure discharge; for each rat the ratio between these two values was considered an index of the response of SNr neurons to PTZ.

2.4. Histological controls

At the end of the seizure recording procedures, rats were sacrificed by anesthetic overdose. The brains were immediately removed and stored in 4% formaldehyde/phosphate buffer solution. Brains were then processed by routine histological procedures to verify the placement of the tetrode tip.

2.5. Statistical analysis

Data are expressed as means ± S.E. Statistically significant differences were evaluated by the Student's *t*-test.

3. Results

3.1. Body weight

As expected, KD produced a significant impairment of growth. The mean body weights at the time of the PTZ-seizure test were 120 ± 11 g for the KD-treated group, and 164 ± 6 g for the control group; the Student's *t*-test demonstrated a significant difference between the KD-treated group and the control group ($p < 0.01$).

3.2. Blood βHB and glucose

The effects on blood βHB and glucose were comparable with those find in a previous work (Viggiano et al., 2015). As expected,