Epilepsy Research (2011) **96**, 166—171

journal homepage: www.elsevier.com/locate/epilepsyres

SHORT COMMUNICATION

Ketogenic diet does not change NKCC1 and KCC2 expression in rat hippocampus

Gisela Gómez-Lira, Julieta Griselda Mendoza-Torreblanca, Leticia Granados-Rojas*

Laboratorio de Neuromorfometría, Instituto Nacional de Pediatría, Insurgentes Sur 3700-C, Col. Insurgentes Cuicuilco, Del. Coyoacán, México D.F. 04530, México

Received 24 November 2010; received in revised form 16 May 2011; accepted 22 May 2011

KEYWORDS
Ketogenic diet;
NKCC1;
KCC2;
Optical fractionator;
Hippocampus;
Rat

Summary In control rats, we examined the effects of ketogenic diet on NKCC1 and KCC2 expression levels in hippocampus. Neither the number of NKCC1 immunoreactive cells nor the intensity of labeling of KCC2 was found to modify in hippocampus of the rats after ketogenic diet treatment. These results indicate that ketogenic diet by itself does not modify the expression of these cation chloride cotransporters.

Introduction

Ketogenic diet (KD), a high fat, low carbohydrate diet, is a non-pharmacological treatment employed in refractory epilepsy. Bough et al. (2002) and Noh et al. (2003) reported the anticonvulsive effect of KD. This was in accordance with earlier report by Muller-Schwarze et al. (1999) where KD was shown to have anti-epileptogenic properties. Despite these findings, the mechanism of action of KD is still unknown. Melø et al., 2006 reported the induction of ketosis by KD, and the use of ketone bodies as alternative metabolites by the brain. Several reports have suggested that KD, and specifically, ketone bodies, could interact with the cells, thereby increasing neuronal inhibition. Rheims et al. (2009) showed that β-hydroxybutyrate (β-HB) reduced the depolarizing GABA action by inducing E_m and E_{GABA} hyperpolarization in neocortical neurons during postnatal development *in vitro*. This suggests the mediation of β-HB due to cation chloride cotransporter Na^+—K^+—$2Cl^-$ (NKCC1) and bicarbonate-chloride exchangers activity regulation. In contrast, Kirmse et al. (2010) and Tyzio et al. (2011), showed that β-HB does not change E_m and E_{GABA} in cortical neurons and suggested that ketone bodies does not modified the co-transporters activity.

The intracellular chloride concentration determines the strength and polarity of gamma-aminobutyric acid (GABA)-mediated neurotransmission. The cation chloride cotransporters (NKCC1 and K^+—Cl^- (KCC2)), which transport chloride within and outside the cell, respectively (Delpire, 2000), are responsible for the intracellular chloride concentration. In resected tissues from patients with refractory epilepsy and in rat kindling model, NKCC1 expression is up-regulated while KCC2 expression is down-regulated

* Corresponding author. Tel.: +52 55 1084 0900x1425; fax: +52 55 1084 3883.
E-mail addresses: ggomez@fisio.cinvestav.mx (G. Gómez-Lira), julietamt14@hotmail.com.mx (J.G. Mendoza-Torreblanca), lgranados_2000@yahoo.com.mx (L. Granados-Rojas).

0920-1211/$ — see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.eplepsyres.2011.05.017

in hippocampus and cortex (Rivera et al., 2002; Okabe et al., 2003; Palma et al., 2006; Aronica et al., 2007; Shimizu-Okabe et al., 2007). In addition, GABA is potentially depolarizing in certain neurons of the epileptic cortical network (Cepeda et al., 2007), and in an experimental model, the intracellular concentration of chloride increased, while calcium influxes were induced by GABA (Sugimoto et al., 2003). These data suggest that the depolarizing action of GABA is due in part to alteration in NKCC1 and KCC2 expression levels.

For the fact that NKCC1 and KCC2 have been implicated in the regulation of chloride homeostasis in brain, and that β-HB (a ketone body) probably modifies NKCC1 activity *in vitro*, the objective of this study was to evaluate the effects of KD on NKCC1 and KCC2 expression levels in hippocampus of control rats *in vivo*.

In the study, we estimated the total number of NKCC1 immunoreactive (NKCC1-IR) cells by stereology and determined KCC2 labeling intensity by densitometry, in five regions of hippocampus of normal diet (ND) or ketogenic diet (KD) fed rats.

Materials and methods

Animals and diet administration

Male Sprague-Dawley rats were bred and maintained in controlled conditions of temperature (22–24 °C) and light:dark cycle (12:12 h). At postnatal day 21 (P21), the rats were subjected to a one-day fasting and were later fed with either ND (PMI Nutrition, Lab Rodent Diet 5001) or KD (Harland Tekland TD 96355) for 4 weeks. At the end of treatment (P51), the body weight and β-hydroxybutyrate (β-HB) blood levels of the animals were measured. To measure β-HB levels, an Optium Xceed system and test strips (Abbott Labs) were used. This research was approved by our Institutional Committee and conducted in accordance with the ethical principles and regulations as specified by the internal guidelines of the Institution (NOM-062-ZOO-1999) and general principles of NIH (publication 85-5).

Tissue sample collection and immunocytochemical staining

After treatment, rats were sacrificed and transcardially perfused with PFA (4%). Serial coronal sections (50 μm) of all rat hippocampus (−1.72 to −7.08 mm posterior to Bregma) were cut and collected. To select the sections from the serial cuts per animal, we used a systematic random procedure consisting of choosing one of every eight sections that resulted in 10–12 sections (anterior, middle and posterior hippocampus). The sections from ND and KD rats were processed in parallel and incubated with NKCC1 antibody (1:1500; Millipore) or KCC2 antibody (1:2000; Millipore). Both antibodies recognize the total protein. After, the sections were incubated with anti-rabbit biotinylated IgG (1:500; Vector Labs.), washed and incubated with avidin peroxidase complex (ABC kit: Vectastain; Vector Labs.). Finally, to reveal peroxidase activity, we used 3,3′-diaminobenzidine (DAB; Vector Labs.). The sections processed with NKCC1 antibody were counterstained with methyl green (Sigma).

Stereology

A systematic random procedure using an optical fractionator (West et al., 1991) was employed in counting the number of NKCC1 stained cells in hippocampus of ND and KD fed rats. The hippocampus was divided in five regions: molecular layer (ml), granule layer (gl), hilus (h), CA3 and CA1 areas. NKCC1-IR cell number was counted in each region. The counting frame size was 70×45 μm, height dissector 13 μm, and guard zones were defined at 1.5 μm from the upper and lower borders of the counting frame. The estimated total number of NKCC1-IR cells was calculated using the Stereo investigator 9 program in a semi-automatic stereological system (MicroBrightField Inc.). The counting was made at 100× and the coefficient of error (Gundersen, $m = 1$) was <0.1. The cell density was the total number of cells per unit volume (volume was obtained using Cavalieri method, grid size was 200×200 μm, except the granular layer which was 100×100 μm, and the coefficient of error Gundersen <0.01).

Intensity of labeling

KCC2 staining images in hippocampus of ND and KD fed rats were obtained using Olympus BX51 microscope connected to a digital video camera (mbf Bioscience), and analyzed using NIH ImageJ v1.43s program. The images were randomly obtained in the five regions of hippocampus using an objective lens of 20×. A mean of 30 images were analyzed in every region, and the values were expressed as optical density (OD) units. Each value of OD was corrected using background subtraction.

Statistics

All data were expressed as mean ± se. *t*-test or Mann–Whitney *U*-test was performed using a SigmaStat v3.5 program (Systat Software, Chicago, IL.).

Results

Body weight and ketonemia

The body weights of ND ($n = 9$) and KD ($n = 9$) rats were measured at P51, the body weight of KD rats decreased significantly in comparison with ND rats (KD = 164.4 ± 8.1 g, ND = 245.6 ± 9.1 g; $p < 0.001$, Mann–Whitney *U*-test). With regard to β-HB blood level after diet treatment, the parameter showed a significant increase in KD rats (4.33 ± 0.65 mmol/l) when compared with ND rats (0.31 ± 0.04 mmol/l) with $p < 0.001$ (Mann–Whitney *U*-test).

NKCC1 and KCC2 immunoreactivity

The analysis of NKCC1 staining pattern revealed presence of clearly stained cells, and processes in all regions of hippocampus (Fig. 1), which was similar in both groups of

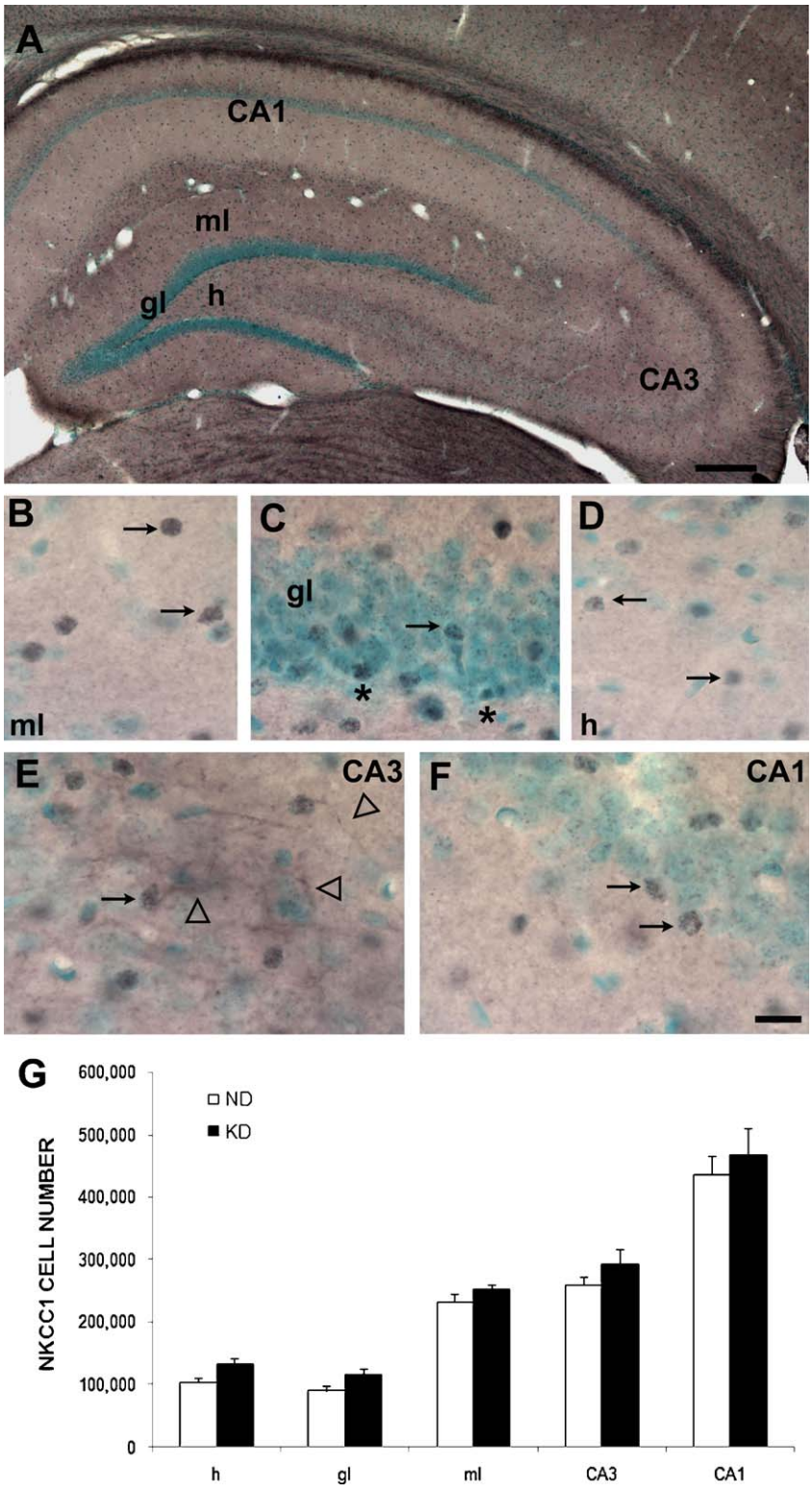

Figure 1 NKCC1 stained section of the hippocampus of rats fed with normal diet. A shows clearly stained NKCC1-IR cells in the whole hippocampus. B, D and F show magnified stained cells (arrows) in the molecular layer (ml), hilus (h) and CA1 region respectively. C shows NKCC1-IR cells in the granular layer (arrow), and in the border between the granule cell layer and the hilar region (asterisk). E shows NKCC1-IR cells (arrow) and processes (arrow head) in CA3 region, while in G, the bar graph shows NKCC1 cell number estimated by stereology in the five regions of entire hippocampus of rats fed with normal diet (ND) or ketogenic diet (KD). The estimate of cells number showed that ketogenic diet (KD) does not significantly change the cell number when compared with ND rats. The photomicrographs were taken at 4× (A) and 100× (B, C, D, E, and F), and the calibration bar was 250 μm and 20 μm respectively.

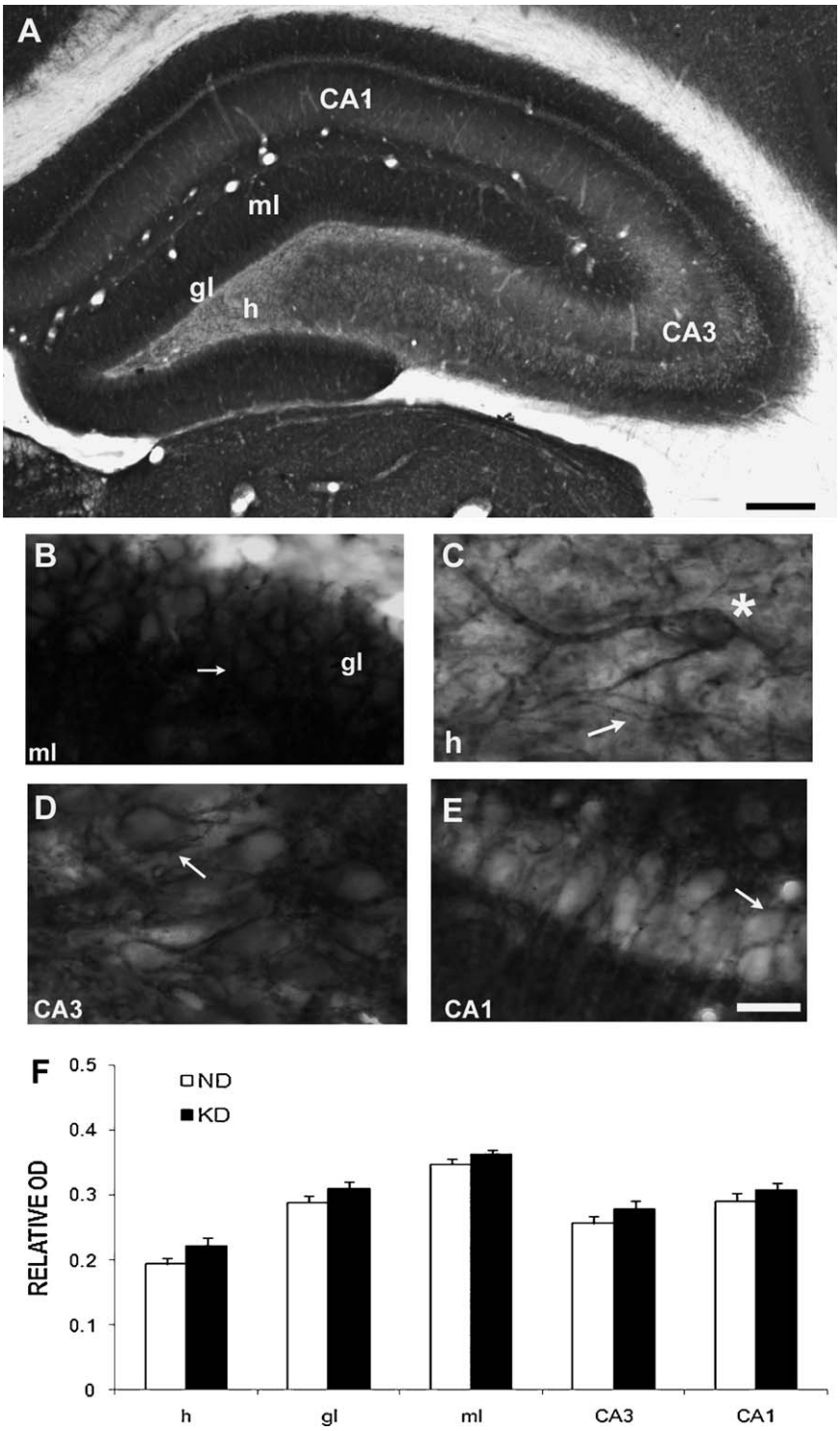

Figure 2 KCC2 stained section of the hippocampus of rat fed with normal diet. The pattern of KCC2 staining showed a diffuse staining in all regions of hippocampus (A). In the granule layer (gl), the neurons showed a strong staining around the cell bodies (arrow) corresponding to the nerve terminals of basket cells (B). In the hilus (h), a discrete staining of dendrites (arrow) and somas of some neurons (asterisk) is shown (C). In CA3 region, strong staining is observed around the cell bodies (arrows, D), while in CA1 region, a weak staining around the cells is shown (arrow, E). In F, the bar graph shows the relative OD of KCC2 staining in the five regions of the entire hippocampus of rats fed with normal diet (ND) or with ketogenic diet (KD). The slight increase induced by KD was not statistically significant. The photomicrographs were taken at 4× (A) and 100× (B, C, D, and E), and the calibration bar was 250 μm and 25 μm respectively.

animals studied. Moreover, stereological count of NKCC1-IR cells did not show any significant difference between ND and KD rats even when there was a slight increase in each region of the entire hippocampus of KD rats (Fig. 1G). In addition, we analyzed the NKCC1-IR cells in anterior and posterior hippocampus, and no differences were found neither in the estimated total number nor density cells between ND and KD rats when anterior and posterior regions of hippocampus were separately analyzed (data not shown).

KCC2 staining pattern showed diffuse stains in all regions (Fig. 2) with a strong and discrete stain in dendrites and somas of some neurons (Fig. 2C, arrow and asterisk, respectively). The analysis of labeling intensity in the five regions of hippocampus showed that KD does not change KCC2 expression in the entire rat hippocampus, although a slight increase in OD relative values after the treatment with KD was observed, these differences were not significant (Fig. 2F). In this case, we also analyzed the intensity of labeling of KCC2 in the anterior and posterior hippocampus; the KD does not change the KCC2 expression (data not shown).

Discussion

For the fact that NKCC1 and KCC2 regulate the polarity of GABAergic transmission, and that ketone bodies probably modify NKCC1 activity in immature neurons *in vitro,* it was expected that KD could change these cotransporters expression levels *in vivo*. However, neither the number of NKCC1-IR cells nor the intensity of KCC2 labeling was modified by KD in any of the regions of the entire or anterior-posterior hippocampus. These findings led us to conclude that KD by itself does not modify the expression of these cation chloride cotransporters in hippocampus.

Stafstrom et al. (1999) found that KD does not modify several aspects of synaptic transmission such as EPSP slopes, population spike amplitude, input/output curves and response to evoked stimulation in hippocampus of adult rats. In similar form, Thio et al. (2000) did not find any evidence that KD products (the ketone bodies) modified the currents mediated by postsynaptic receptors of GABA or glutamate in control conditions.

In a subsequent study by Bough et al. (2003), it was shown that ketogenic calorie-restricted diet enhances GABAergic inhibition *in vivo*, suggesting that these modifications are dependent on $GABA_A$ receptor activation. These findings are in accordance with the results of this work, where it was found that KD does not change NKCC1 and KCC2 expression levels in rats. All these suggest that the polarity of GABAergic transmission, as reflected in the electrophysiological responses, is not modified by KD under control conditions.

Rheims et al. (2009), in their recent work showed that β-HB, a physiological concentration (2 mM), is able to induced E_m and E_{GABA} hyperpolarization in immature neurons, which goes to suggest that the presence of β-HB could modify NKCC1 activity. However, even when we reached higher levels of β-HB in the blood of ketogenic rats (4.33 ± 0.65 mmol/l), and observed a slight tendency of increase in NKCC1 or KCC2 expression levels, the changes were not found to be significant. This could be explained on the basis that contrary to what occurs in immature neurons, GABA signaling is inhibitory and not excitatory in rats at P51.

Another possible explanation is that the β-HB does not alter the depolarizing action of GABA, as evidenced by Kirmse et al. (2010) and Tyzio et al. (2011). These authors showed that in immature neurons, β-HB does not induce E_m and E_{GABA} shift and that the activation of $GABA_A$ receptors rose the intracellular calcium concentration and depolarize the membrane potential, suggesting that the antiepileptic actions of ketone bodies are not mediated by GABA signaling or by modification of the co-transporters activity.

In spite of the results obtained in this study, the exclusion of NKCC1 or KCC2 as anticonvulsive target of KD cannot be concluded. It is possible that the modifications in the levels of expression and/or activity occurred during or after the establishment of epilepsy, as Kwon et al. (2008) observed on neurogenesis in hippocampus. Kaila and colleagues have shown that the expression of KCC2 is regulated by the activity in immature and mature neurons, and that BDNF plays an important role in this event (Rivera et al., 2004; Khirug et al., 2010). Also it has been demonstrated that the activity of the cotransporters depends on phosphorylation/dephosphorylation mechanism (Kahle et al., 2005), which suggests that the expression of these proteins does not necessarily reflect if the cotransporters have biological activity. We therefore suggest that further studies be carried out to determine KD effect on the expression levels of these cotransporters after induced epilepsy.

Acknowledgements

This work was supported by a grant from Consejo Nacional de Ciencia y Tecnología (86784), México to L. Granados-Rojas. We thank Sara Navarrete and Verónica Cordóva for their technical assistance and Dr. Cyril Ndidi N., for his help in the preparation and correction of the manuscript.

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