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Original article

Immunoreactivity for calretinin in interneurons of the hippocampal CA1 field and dentate gyrus in adult rats after administration of habanero peppers *(Capsicum chinense Jacq.)*

J. Jaworska-Adamu[1], A. Krawczyk[1], K. Rycerz[1], M. Gołyński[2], A. Wawrzyniak[1], K. Lutnicki[2], I. Balicki[3]

[1] Department of Animal Anatomy and Histology, Faculty of Veterinary Medicine, University of Life Sciences, Akademicka 12, 20-033, Lublin, Poland
[2] Subdepartment of Internal Diseases of Farm Animals and Horses, Faculty of Veterinary Medicine, University of Life Sciences, Głęboka 30, 20-612 Lublin, Poland
[3] Department and Clinic of Animal Surgery, Faculty of Veterinary Medicine, University of Life Sciences, Głęboka 30, 20-612 Lublin, Poland

Abstract

Calretinin (CR), a calcium-binding protein from EF-hand family, is localised in non-pyramidal GABA-ergic interneurons of the hippocampus. CR takes part in maintaining calcium binding homeostasis, which suggests its neuroprotective role. Hippocampal neurons contain membrane transient receptor potential vanilloid 1 (TRPV1) which binds to capsaicin (CAP) contained in habanero pepper fruits. Few in vivo studies have revealed the effect of CAP on interneurons containing CR. The aim of the present study was to investigate the CR immunoreactivity in interneurons of the hippocampal CA1 field and dentate gyrus (DG) in adult rats after intragastric administration of the habanero pepper fruits. Wistar rats received a peanut oil – control group (C), and oil suspension of habanero pepper fruits at doses of 0.025 g dm/kg b.w. – group I and 0.08 g dm/kg b.w. – group II for 28 days. After euthanasia, the brains were collected and embedded in paraffin blocks using a routine histological technique. Frontal hippocampal sections were immunohistochemically stained for CR by using a peroxidase-antiperoxidase method. CR immunoreactive (CR-IR) interneurons were morphologically and morphometrically analyzed under a light microscope. The results showed similar shapes and distribution of cells in both areas of the brain in group C and I of animals. However, CR-IR interneurons in the hippocampal CA1 field and in DG were occasionally observed in the group II of rats.

The results of morphometric studies did not reveal statistically significant differences in the surface area and shape index of cells between examined brain regions from groups I and II compared to group C.

Only in group II of rats, an increase in the digital immunostaining intensity of CR-IR interneurons was found in DG. Low number of CR-IR interneurons in the hippocampal CA1 field and in the DG, under the influence of a large dose of habanero pepper fruits containing CAP, may be caused by the activation of TRPV1 receptors and the increase in $Ca2^{+}$ ions in these cells. This phenomenon may ultimately lead to neuronal death and may disturb neuronal conduction.

Key words: capsaicin, interneurons, calretinin, hippocampus, rats

Correspondence to: J. Jaworska-Adamu, jadwiga.jaworska@up.lublin.pl

Introduction

Calretinin (CR) belongs to the EF-hand family of calcium-binding proteins (CaBPs). It was first detected in 1987 in the retina of the eye (Rogers 1987). CR is a buffer protein that regulates homeostasis of Ca^{2+} ions in neurons. It also participates in intracellular processes as a sensory protein (Billing-Marczak and Kuźnicki 1999).

The hippocampus plays an important role in learning and memory processes and controls many mechanisms affecting the behaviour of animals. In humans and other mammals, CR in the hippocampus is localised mainly in non-pyramidal GABAergic interneurons. In the rat, this area contains about 13% of GABAergic cells that participate in passive Ca^{2+} buffering (Jacobowitz and Winsky 1991, Miettinen et al. 1992, Nitsch and Ohm 1995). CR-positive interneurons are specialized in inhibition of other interneurons in the local circuit of neuronal networks and they also support the principal cells (Gulyás et al.1996). The neuroprotective role of CR is suggested, but it is not clear and remains to be determined. Interneurons expressing this protein are "resistant" to various pathological processes (epilepsy, depression, Alzheimer disease) (Barinka and Druga 2010). However, recent investigations have shown that hippocampal interneurons with CR are vulnerable to temporal lobe epilepsy (Tóth and Maglóczky 2014). In the hippocampus, changes in the expression of CR in interneurons of the circling mouse were observed. Ca^{2+} homeostasis disorders can cause damage to hippocampal processes (Maskey et al. 2012).

Habanero pepper (*Capsium chinensis Jacq.)* produces active alkaloid capsaicin (CAP) and is used as a spice in dietary supplementation. This substance is characterised by an exceptionally sharp, burning taste. The most important CAP features are: the irritating and analgesic effect as well as the influence on thermoregulation and fat tissue metabolism. In addition, it has antioxidant, hypertensive, antibacterial properties and lowers body weight (Olszewska 2010). After oral administration, the alkaloid is absorbed and hydrolysed in the gastrointestinal tract. Less than 5% of unchanged CAP enters the brain by blood (O'Neill el al. 2012). CAP is neurotoxic at high doses (Olszewska 2010). Moreover, it induces neuronal death in cell cultures derived from rat hippocampus (Drebot et al. 2008). This substance binds to the neuronal membrane transient potential vanilloid 1 receptor (TRPV1) (Olszewska 2010). In the CA1 field of the hippocampus this receptor affects the plasticity of GABAergic synapses. TRPV1 agonists affect long-term potentiation (LTP) by GABAergic interneurons (Tóth et al. 2005, Benion et al. 2011, Ho et al. 2012).

The effects of intragastric administration of habanero pepper fruits and thus CAP on hippocampal interneurons have not been investigated. Hence, the purpose of this study was to perform the morphological and morphometric analyses of CR-immunoreactive neurons in the hippocampal CA1 field and dentate gyrus in adult rats after intragastric application of different doses of the habanero pepper fruits.

Materials and Methods

The experiments were approved by the Second Local Ethics Committee in Lublin (No. 21/2013). The study was conducted on 15 adult male Wistar rats (120-125 g) from the laboratory animal farm. The animals were kept in an air-conditioned room with a relative humidity of 45-47% at 22-23°C in a 12 h light / 12 h dark cycle. The rats were fed commercial fodder for laboratory animals (LSM, Agropol Motycz Poland) and they had permanent access to water. The acclimation period was 16 days before the experiment. The rats were divided into 2 experimental groups (I, n = 5 and II, n = 5) and control group (C, n = 5). Dried fruits of the habanero pepper with HPCR - specific content of capsaicin 7.64mg / dm (capsaicin and dihydrocapsaicin) were suspended in peanut oil. The suspension was administered intragastrically (*i.g*) using special automatic plastic rod-feeding tubes (Instech Laboratories, Inc., Plymouth Meeting, PA USA). Group I received habanero peppers at a dose of 0.025 g dm/kg b.w. for 28 days. Group II received 0.08 g dm/kg b.w. of these fruits which were also applied for 28 days. Daily doses were divided into 2 equal parts and administered every 12 h. Each animal received 0,5 ml of the suspension at the appropriate concentration. Group C received 0.5 ml of pure peanut oil using the same method. After the rats were euthanized, the brains were removed immediately, fixed in fresh buffered 10% formalin (pH-7.0) for 12 h at 4°C and embedded in paraffin blocks using a routine histological technique. Six-μm-thick frontal sections which contained hippocampus and a dentate gyrus (DG) were obtained from each animal using a microtome.

Immunohistochemistry

To demonstrate CR-immunoreactive (CR-IR) interneurons in hippocampal CA1 field and in DG in all of the rats (C, I and II), an immunohistochemical, indirect peroxidase-antioxidase method (PAP) was performed. To eliminate the endogenous peroxidase activity, sections were treated with 3% H_2O_2 for 30 min at room temperature (rt). In order to remove background staining, sections were treated with 10% normal goat

serum (Sigma-Aldrich, St. Louis, Missouri, USA, G9023, 1:10) at rt for 20 min. The antibodies and reagents (Sigma-Aldrich, St. Louis, Missouri, USA) were diluted in 0.5M TBS (Tris-buffered saline) pH-7.6 according to the manufacturer's recommendations. The sections were incubated with an anti-calretinin antibody produced in rabbits (C7479, 1:1000) at 4°C for 48 h. Next, secondary goat antibody against rabbit IgG conjugated with the peroxidase complex (A9169, 1:400) was applied for 1h at rt. 3,3'diaminobenzidine tetrahydrochloride (DAB) was used as a chromogen for 30 min at rt. Additionally, sections were washed in distilled H_2O and counterstained with Mayer's hematoxylin, and mounted in DPX (Fluka, Buchs, Switzerland). Control of the reaction specificity was performed by omitting the primary antibody and replacing it with normal goat serum. CR-IR interneurons in hippocampal CA1 field and DG were analysed and photographed in the Olympus BX51 light microscope (Olympus, Tokyo, Japan) with a digital camera (Olympus Colour View III). The microphotographs were archived and morphologically and morphometrically analysed.

Morphological analyses

Distribution and shapes of CR-IR interneurons in CA1 layers (SO- *stratum oriens*, SP-*stratum pyramidale*, SR- *stratum radiatum*, SLM-*stratum lacunosum moleculare*) and in DG (SM-*stratum moleculare*, SG-*stratum granulosum*, H-*hilus*) were analysed.

Morphometric and statistical analysis

Because of the rare occurrence of CR-IR interneurons, in both brain regions in all rat groups (C, I, II), their density was not quantified. The surface areas of the CR-IR interneurons, shape index and digital immunostaining intensity was analysed in CA1 and in DG in C, I and II groups of animals. The area of randomly selected 50 interneurons was measured by manual outlining of their bodies using a suitable tool. The results obtained were compared and presented as means with standard deviation (Fig. 2).

In order to demonstrate changes in CR-IR interneurons, their shape index was calculated as the quotient of 4π and the ratio of the cell surface area to the square power of its body perimeter. Fifty randomly selected CR-IR cells in all the animals were analysed. The results are shown as mean values along with the standard deviation (Gittins and Harrison 2004) (Fig. 3).

In addition, immunostaining intensity was measured in 50 randomly selected cells in a square of 1 μm^2 of their neuroplasm. The results were inverted so that the higher values represent a darker colour. The results were then standardized to remove differences in light exposure. The data are presented in the form of average digital immunostaining intensity along with standard deviation in optical units/μm^2 (ou/μm^2) (Matos et al. 2006, Nguyen et al. 2013) (Fig. 4).

By means of one-way analysis of variance (ANOVA) and post-hoc Tukey HSD test, the results obtained in the form of means with standard deviation were statistically compared. Normal distribution of data was assessed using the Shapiro-Wilk test. Data which did not meet the condition of the normal distribution were compared using the non-parametric Kruskall-Wallis test. The significance factor for all tests was $\alpha = 0.05$. Statistical analyses were carried out using R 3.3.1 (Free Software Foundation's GNU General Public License, http://www.r-project.org/).

Results

Immunohistochemical studies

Group C of rats

In all layers of the CA1 of rats from C group, singular interneurons of different shapes were observed. In the SO oval, fusiform and round cells were rarely seen. In the SP, among densely stacked pyramidal principal neurons arranged in several layers without reaction for CR, there were dark brown, oval, round, fusiform and triangular CR-IR interneurons. These cells sometimes had prominent nerve processes. In the SR, mainly oval and triangular CR-IR interneurons were rarely observed. At the boundary of the SR and SLM fusiform, triangular cells sometimes with prominent processes were localised. Multiform CR-IR interneurons were observed in the SLM.

In the SM of DG, single, round CR-IR interneurons were rarely found. In the vicinity of H, in SG, round, oval and fusiform CR-IR cells were seldom encountered. On the other hand, granular neurons showed no reaction. In the H, various (triangular, oval, circular) interneurons with visible nerve processes were distributed (Fig. 1A).

Group I of rats

In group I of rats, no differences in the distribution, shapes of CR-IR interneurons were found both in CA1 and DG compared to group C (Figs. 1A and 1B).

Group II of rats

In group II of rats, CR-IR interneurons were very rare both in CA1 and in the DG. In SO, single, oval and round cells were distributed near SP. Among pyramidal cells in SP, there were triangular, oval and round CR-IR

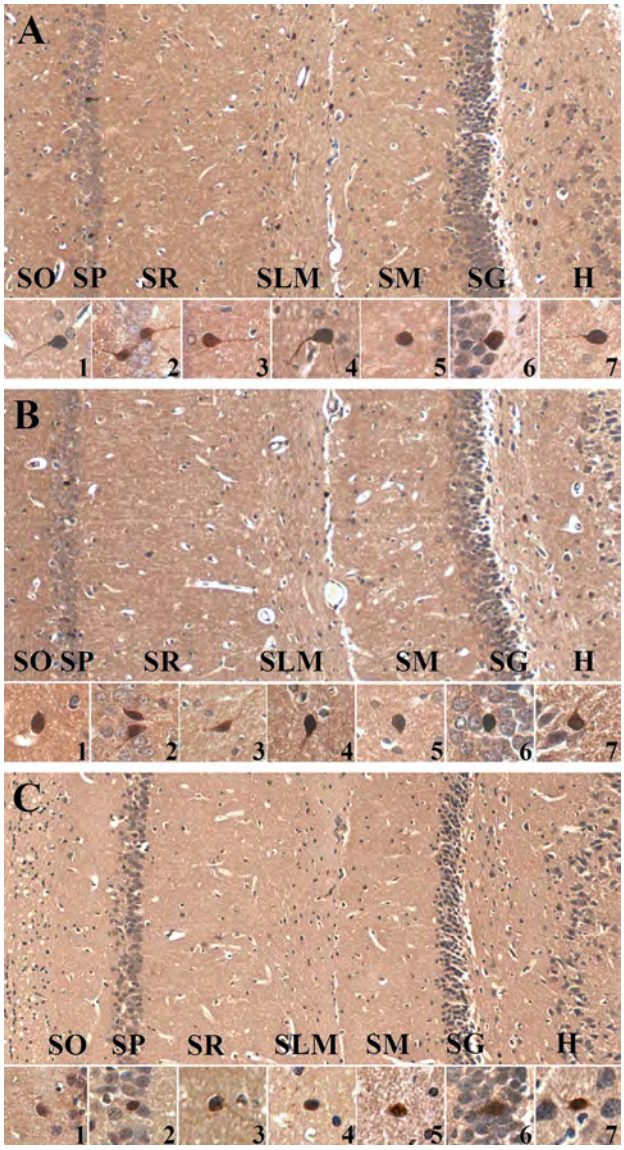

Fig. 1. CR-IR interneurons in the hippocampal CA1 field (SO-*stratum oriens*, SP-*stratum pyramidale*, SR-*stratum radiatum*, SLM-*stratum moleculare lacunosum*) and dentate gyrus (DG) (SM-*stratum moleculare*, SG-*stratum granulosum*) with hilus (H) in rats from the control group (A), group I (B), group II (C); x10 in (A, B, C), x60 in (1-7).

interneurons. Similarly, in SR, round and oval CR-IR cells were observed. Sporadically they were located on the SR/SLM boundary. In SM, closer to SG, oval CR-IR interneurons were observed. In the deeper part of SG, among granular neurons, there were rare triangular interneurons. In H, just beneath SG, oval or triangular CR-IR cells, and multiform neurons which were located deeper were found (Fig. 1C).

Morphometric and statistical analysis

The results of morphometric analyses concerning the surface area and shape index of CR-IR interneurons in CA1 and in DG of rats from groups I and II did

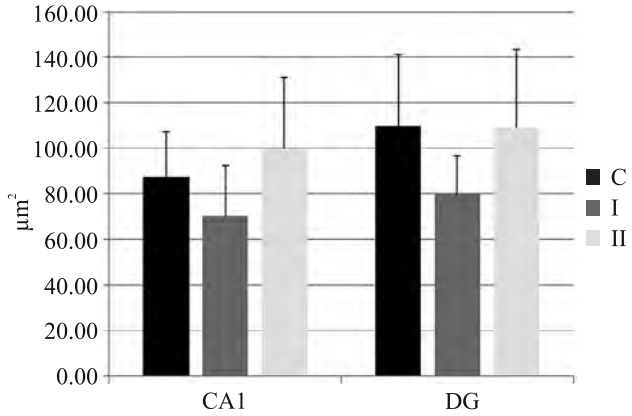

Fig. 2. The average surface area with standard deviation bars of CR-IR interneurons in the hippocampal CA1 field and DG in rats (groups C, I and II).

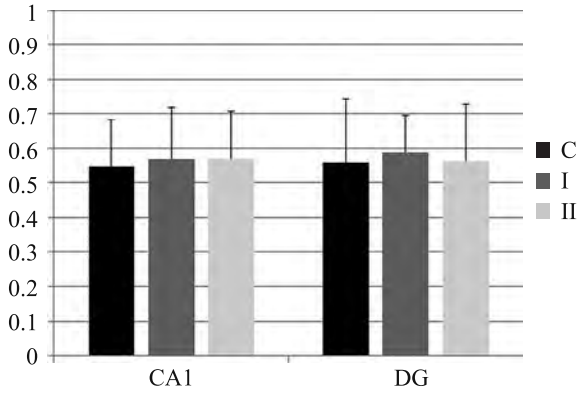

Fig. 3. The average shape index with standard deviation bars of CR-IR interneurons in hippocampal CA1 field and DG in rats (groups C, I and II).

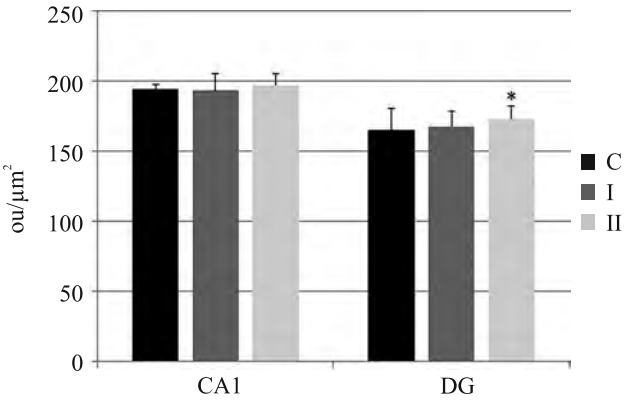

Fig. 4. The average digital immunostaining intensity of CR-IR interneurons in the hippocampal CA1 field and DG in rats (groups C, I and II). * statistically significant difference (ANOVA $p<0.05$).

not differ significantly in comparison to group C. Changes between the results were within the standard deviation range (Fig. 2, 3). The average mean of digital immunostaining intensity of CR-IR interneurons was similar in both studied areas in groups C, I and II of the animals. Only in group II in DG, the increase in

this parameter was statistically significant for CR-IR interneurons compared to group C (ANOVA $p<0.05$) (Fig. 4).

Discussion

The results obtained provide information on the effect of intragastric administration of pepper fruits containing CAP, administered intragastrically for 28 days to rats, on CR-IR interneurons from CA1 and DG. The smaller dose (0.025 g dm/kg b.w.) of these fruits (group I) did not change CaBP immunoreactivity in the cells in both brain areas examined. The higher dose (0.08 g dm/kg b.w.) caused a less frequent distribution of CR-IR interneurons in the layers of CA1 and DG. Analyses of the surface area and shape index of CR-IR interneurons did not reveal statistically significant differences in CA1 and DG between groups I and II compared to group C. These results indicate that CR-IR interneurons did not change their shape and did not undergo swelling or shrinkage. The digital analyses of immunostaining intensity for CR interneurons showed a statistically significant increase in only DG between group II and group C rats.

In the hippocampus, CR interneurons are specialized to control other interneurons and principal neurons. Their unique connections may play a critical role in generating rhythmic synchronization activity of the hippocampus by controlling terminations of other interneurons on dendrites and principal neuronal cell bodies (Gulyás et al. 1996).

CR which belongs to the calmodulin superfamily occurs in certain subpopulations of interneurons. Under neuropathological conditions, CR is more "resistant" to lesions than other CaBPs. It has been shown that CR expression is not affected by schizophrenia, deep depression, Alzheimer's disease, and multiple sclerosis (Rogers 1987, Fonseca and Rosiano1995, Beasley et al. 2002, Clements et al. 2008, Barinka and Druga 2010). However, in the rat depression model, the number of CR-immunopositive cells was increased in SO of the hippocampal CA1 field and SM of DG (Nowak et al. 2012).

In our study, the increase in the immunostaining intensity of CR in the interneurons of DG in the group II rats may indicate an increased intracellular concentration of Ca^{2+} ions. When these ions are attached to CR, the conformation of this CaBP changes, leading to an increase in CR immunoreactivity (Kuźnicki and Filipek 1997). CAP as an agonist of neuronal TRPV1 receptors induces the opening of calcium channels. Excessive influx of calcium ions into cells can lead to neuronal death mainly through apoptosis pathways (Pecze et al. 2013). This may explain the less frequent occurrence of CR-IR interneurons in CA1 and DG in group II rats. In the CA1, TRPV1 receptors are involved in the modulation of synapses plasticity of pyramidal neurons, which may influence long-term potentiation (LTP) in learning and memory. Inhibitory CR-containing cells in the hippocampus form connections with dendrites of nearly all calbindin-containing interneurons. In this way, CR-IR interneurons affect the "inhibition" of the main neurons (Nowak et al. 2012). Loss of CR-IR interneurons can disturb the GABAergic system by altering LTP in the hippocampus, which can affect the proper course of memory processes.

In conclusion, we have demonstrated that CR-IR interneurons in the hippocampal CA1 field and in DG were insensitive to the lower concentration of habanero pepper fruits containing CAP administrated intragastrically to rats for 28 days. The higher concentration of habanero pepper fruits containing CAP, however, resulted in a decrease in the number of CR-IR interneurons in hippocampal CA1 and DG, most likely as a result of apoptosis induced by excess of calcium ions. These results suggest that CAP can cause changes in interneuronal networks, which can eventually lead to neuronal conduction abnormalities.

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